UNIVERSITAT DE BARCELONA

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Digestive physiology and nutrition during the early life stages of the Amazonian catfish *Pseudoplatystoma punctifer* (Castelnau, 1855) in culture

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TESIS DOCTORAL

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DIANA CASTRO RUIZ

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Tesis Doctoral

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Memoria presentada por
DIANA CASTRO RUIZ
para optar al grado de
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Tesis realizada bajo la dirección de
Dr. Maria José Darias Cáceres del IRD, MARBEC (Francia) y
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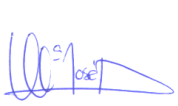
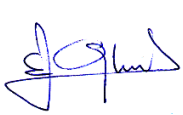

Dr. María J. Darias	Dr. Enric Gisbert	Dr. Isabel Navarro	Diana Castro Ruiz
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DEDICATORIA

A Graciela y Luis Baltazar,

A Werner

A Diana Valentina

Amores eternos,
vivirán siempre...
En mi corazón

¡Amar y luchar es la vida del hombre hasta el final!

Lucano

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RESUMEN

El pescado es una de las principales fuentes de proteínas para la población de la cuenca amazónica, la cual posee la mayor biodiversidad de peces del mundo. La acuicultura se ha desarrollado de manera constante en la región durante las últimas décadas para contribuir a las necesidades alimentarias de una población en rápido crecimiento, y varias especies de peces nativos han sido identificadas como de interés desde hace tiempo. Entre ellas, el pez gato *Pseudoplatystoma punctifer* (Castelnau, 1855) es muy apreciado por la calidad de su carne y la ausencia de espinas intramusculares, aunque su cultivo comercial aún no se ha desarrollado en el Perú debido a las altas tasas de mortalidad durante las primeras etapas de vida, principalmente causadas por una alta incidencia de canibalismo y la dificultad para adaptarse a los alimentos formulados. Para superar estos obstáculos, el objetivo de esta tesis fue estudiar la ontogenia del sistema digestivo de *P. punctifer* y sus necesidades nutricionales durante las primeras etapas de vida. Los resultados revelaron que el sistema digestivo estaba completamente desarrollado y funcionalmente maduro entre 10 y 13 días post fertilización (dpf, *ca.* 11-14 mm TL), lo que indicó la transición del modo de digestión larvario al adulto y el momento apropiado para el destete. Los estudios nutricionales revelaron que una dieta con 45% de proteína cruda (harina de pescado) y 15% de lípidos (lecitina marina y de soja) permitió adelantar el destete, promovió un mejor y más rápido desarrollo de la función digestiva y un mejor crecimiento (6 veces mayor) y redujo la incidencia de canibalismo (a la mitad) al final del primer mes de vida en comparación con protocolos anteriores. Además, los resultados revelaron que la nutrición durante la etapa larvaria afectó la nutrición y el comportamiento de los juveniles tempranos. Específicamente, el DHA dietético (4% TFA) proporcionado durante la fase larvaria influyó en la expresión génica de las enzimas digestivas y contribuyó a reducir el comportamiento caníbal en la etapa juvenil temprana. En conclusión, esta tesis aporta valiosos conocimientos sobre la nutrición temprana de esta especie, sentando así las bases para la mejora de los protocolos de alimentación, adaptados a sus capacidades digestivas y necesidades nutricionales durante las primeras etapas de vida, y representa un paso importante hacia su cultivo a nivel comercial.

SUMMARY

Fish is one of the main sources of protein for the population of the Amazon Basin, which has the world's highest fish biodiversity. Aquaculture has developed steadily in the region over the past decades to contribute to the food needs of a rapidly growing population, and several indigenous fish species have long been identified as of interest. Among them, the catfish *Pseudoplatystoma punctifer* (Castelnau, 1855) is highly appreciated for the quality of its meat and the absence of intramuscular spines, although its commercial farming has not been yet developed in Peru due to high mortality rates during the early life stages, mainly caused by a high incidence of cannibalism and the difficulty to adapt to formulated feeds. In order to overcome these obstacles, the objective of this thesis was to study the ontogeny of the digestive system of *P. punctifer* and its nutritional needs during the early life stages. Results revealed that the digestive system was completely developed and functionally mature between 10 and 13 days post fertilization (dpf, *ca.* 11-14 mm TL), indicating the transition from the larval to the adult mode of digestion and an appropriate weaning time. Nutritional studies indicated that a diet containing 45% of crude protein (fishmeal) and 15% lipids (marine and soybean lecithin), allowed to advance the weaning time, promoted a faster and better development of the digestive function and an increased growth (by 6-fold), and reduced the incidence of cannibalism (by half) at the end of the first month of life compared to previous protocols. In addition, results revealed that the nutritional history during the larval stage affected the nutrition and behavior of early juveniles. Specifically, the dietary DHA (4% TFA) provided during the larval phase influenced the gene expression of digestive enzymes and contributed to reducing the cannibalistic behavior at the early juvenile stage. In conclusion, this thesis provides valuable knowledge on the early nutrition of this species, which sets the basis for the improvement of feeding protocols for the early life stages adapted to its digestive capacities and nutritional needs, and represents an important step towards its farming at commercial scale.

TABLA DE CONTENIDOS

INTRODUCCIÓN

La acuicultura en el mundo y en Perú

Los organismos acuáticos desempeñan un papel importante en la alimentación de la población mundial y contribuyen fuertemente a la seguridad alimentaria en muchas regiones del planeta. Éstos son, además, una fuente de nutrientes fundamentales, tales como proteínas de alta calidad y aminoácidos esenciales, ácidos grasos poliinsaturados de cadena larga omega-3 y minerales y vitaminas esenciales (Golden et al., 2021). La creciente demanda de productos acuáticos ha generado una alta presión de pesca a nivel mundial, poniendo en riesgo las poblaciones naturales de estos organismos y la sostenibilidad de la industria pesquera. Dado que la pesca de captura mundial se ha mantenido relativamente estable desde finales de la década de 1980 (87 a 96 millones de toneladas, Mt), el desarrollo de la acuicultura ha sido esencial para satisfacer la demanda de alimentos de origen acuático por parte de una población mundial creciente (FAO, 2020). La producción acuícola mundial ha aumentado en apenas tres décadas de 15 a 82 Mt (principalmente peces, moluscos y crustáceos; las cifras excluyen la producción de algas; FAO, 2020; Figura 1). La producción mundial de animales acuáticos

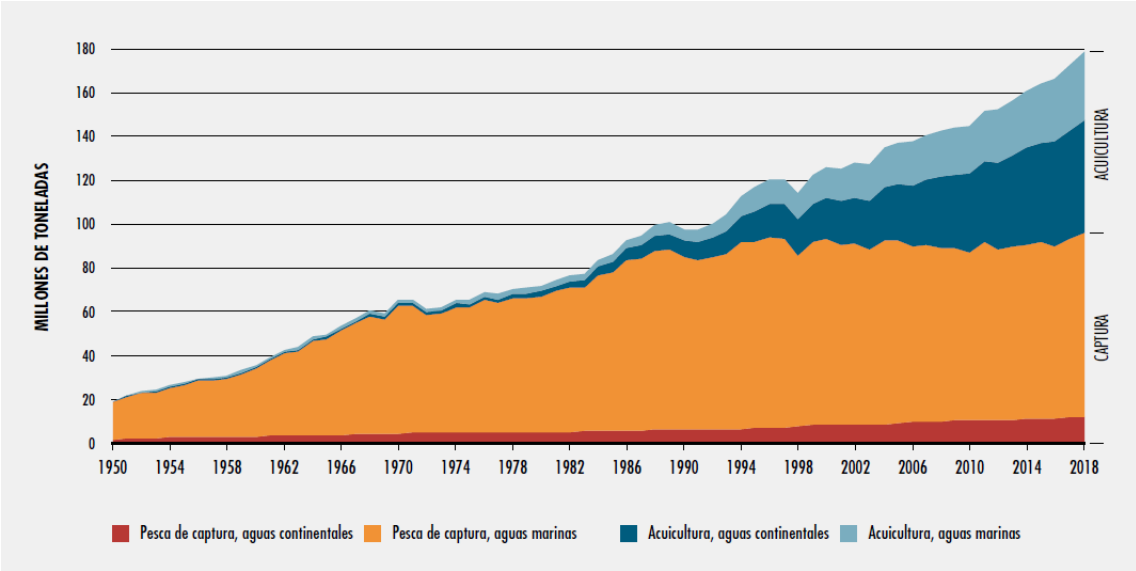

Figura 1. Producción mundial de pesca de captura y de acuicultura (FAO, 2020).

alcanzó 178.5 Mt en 2018, de las cuales 156 Mt fueron destinados al consumo humano, lo que equivale a un suministro anual de 20,5 kg per cápita (FAO, 2020). En 2018, la producción acuícola representó el 46% de la producción total (Figura 2) y el 52% del pescado disponible para el consumo humano. Para 2030, se estima que el 59% del pescado disponible para el consumo humano provendrá de la acuicultura (FAO, 2020).

La acuicultura continental, realizada principalmente en agua dulce, representa la mayor parte de la producción acuícola mundial, siendo los peces los organismos más cultivados globalmente, si bien los moluscos son los más cultivados en ambientes marinos y costeros (Tabla 1). Las especies de peces

más cultivadas son las carpas (*Ctenopharyngodon idellus*, *Hypophthalmichthys molitrix*, *Cyprinus carpio*, entre otras) y la tilapia del Nilo, *Oreochromis niloticus* (FAO, 2020).

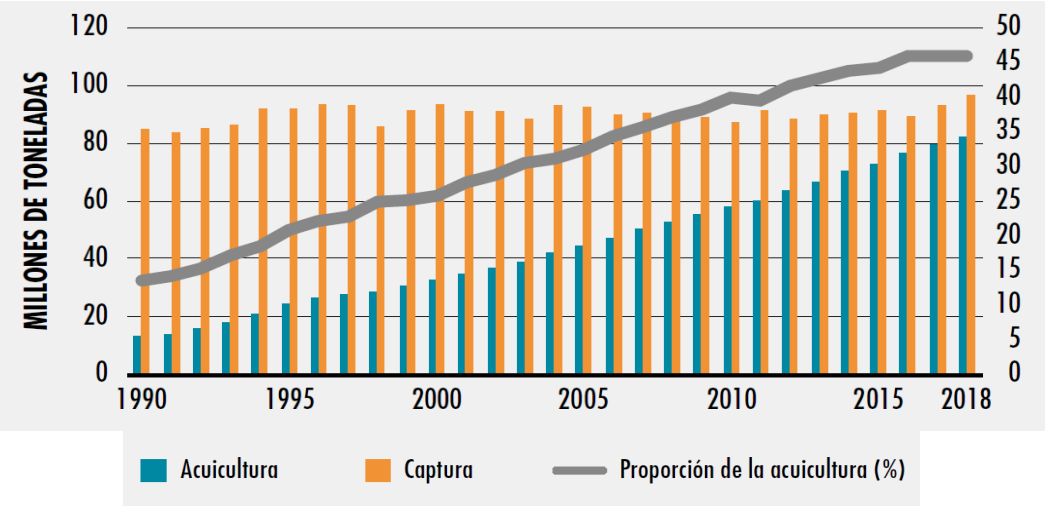

Figura 2. Contribución de la acuicultura a la producción total de organismos acuáticos (FAO, 2020).

Tabla 1. Producción acuícola de 2018 por continentes y por principales grupos de especies (excluyendo las algas) (FAO, 2020)

	África	Américas	Asia (– Chipre)	Europa (+ Chipre)	Oceanía	Mundo
	(en miles de toneladas, peso vivo)					
Acuicultura continental						
1. Peces de aleta	1 893	1 139	43 406	508	5	46 951
2. Crustáceos	0	73	3 579	0	0	3 653
3. Moluscos	...	...	207	...	...	207
4. Otros animales acuáticos	...	1	528	0	...	528
Total parcial	***1 893***	***1 213***	***47 719***	***508***	***6***	***51 339***
Acuicultura marina y costera						
1. Peces de aleta	291	1 059	3 995	1 892	92	7 328
2. Crustáceos	6	888	4 834	0	6	5 734
3. Moluscos	6	640	15 876	680	102	17 304
4. Otros animales acuáticos	0	...	387	3	0	390
Total parcial	***302***	***2 587***	***25 093***	***2 575***	***200***	***30 756***
Toda la acuicultura						
1. Peces de aleta	2 184	2 197	47 400	2 399	97	54 279
2. Crustáceos	6	961	8 414	0	6	9 387
3. Moluscos	6	640	16 083	680	102	17 511
4. Otros animales acuáticos	0	1	915	3	0	919
Total	**2 196**	**3 799**	**72 812**	**3 083**	**205**	**82 095**

NOTA: 0 = cantidad de producción inferior a 500 toneladas; … = sin producción o datos de producción no disponibles.
FUENTE: FAO.

Asia es, con diferencia, el mayor productor acuícola mundial (73 Mt), seguido por el continente americano (3,8 Mt), que representa el 4,63% de la producción mundial (Tabla 1). Chile es el principal productor de la región (1,3 Mt). En comparación, 1.9 y 0.6 Mt son producidas en el resto de América Latina y el Caribe y en América del Norte, respectivamente (FAO, 2020). La acuicultura en América Latina juega un papel importante en la producción de alimentos y en la economía local. Las principales

especies producidas son el camarón blanco *Penaeus vannamei*, el salmón del Atlántico *Salmo salar* y *O niloticus*, siendo Ecuador, Chile y Brasil, respectivamente, los principales productores de estas especies en la región (FAO, 2021).

En Perú, la acuicultura ha crecido de unas 5.000 toneladas de producción anual en 1990 a más de 150.000 toneladas en 2019 (FAO, 2021) (Figura 3) y se proyecta que supere las 200.000 toneladas en 2025 (Berger, 2020). En la actualidad, Perú es el sexto mayor productor acuícola de Latinoamérica

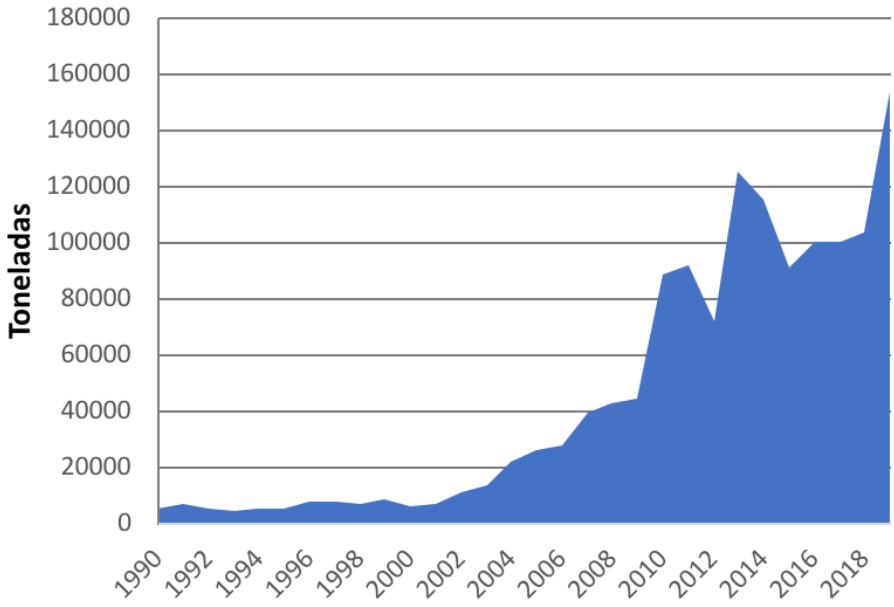

Figura 3. Producción acuícola peruana de 2000 a 2019 (FAO, 2021).

y produce una variedad de especies, tanto en aguas continentales como marinas y salobres. La producción acuícola está dominada por la concha de abanico *Argopecten purpuratus*, la trucha arcoíris *Oncorhynchus mykiss*, *Penaeus vannamei* y *O. niloticus*, seguido por especies de peces nativos de la región amazónica como el paco *Piaractus brachypomus*, la gamitana *Colossoma macropomum*, el sábalo cola roja *Brycon amazonicus*, el paiche *Arapaima gigas* y el boquichico *Prochilodus nigricans* (Figura 4) (FAO, 2021).

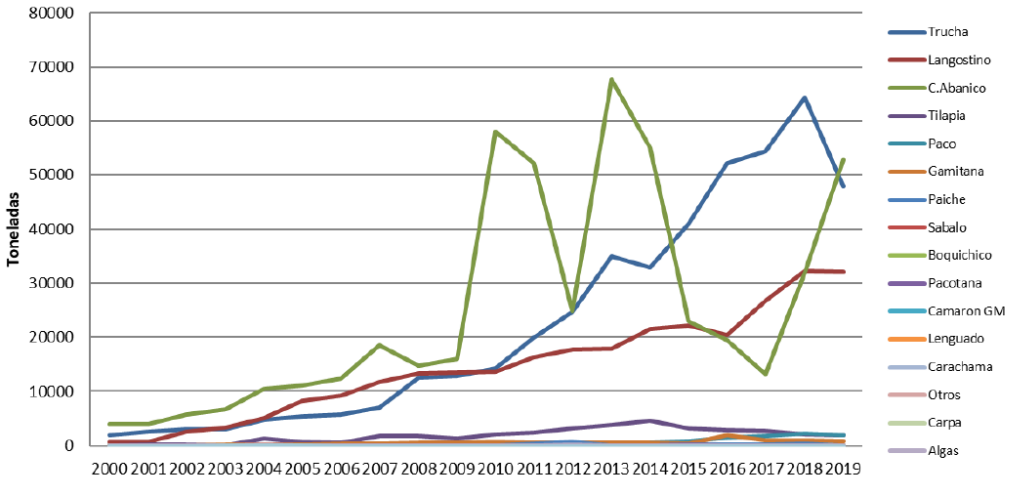

Figura 4. Producción acuícola peruana de 2000 a 2019 por especies (PRODUCE, 2020).

La Amazonía peruana, que representa más del 60% del territorio nacional (García Dávila et al., 2018), reúne unas condiciones favorables para el desarrollo de la acuicultura continental, tales como abundante agua y terreno, temperaturas tropicales y una gran variedad de especies de peces de interés. No en vano, con sus más de 6.000.000 km^2, la cuenca amazónica alberga la mayor diversidad de peces del mundo con 2.406 especies, de las cuales 1.402 son endémicas (Jézéquel et al., 2020). El pescado es la principal fuente de proteínas, ácidos grasos esenciales y micronutrientes para la población local, especialmente para las familias de bajos ingresos, y el consumo de pescado per cápita es uno de los más altos del mundo (Isaac y de Almeida, 2011). Sin embargo, las poblaciones naturales de peces se enfrentan cada vez más a numerosas amenazas como la contaminación, la deforestación, las represas hidroeléctricas, las especies invasoras y la sobrepesca (Carolsfeld et al., 2003; Winemiller et al., 2016). Para contrarrestar estos efectos, la acuicultura se ha desarrollado de manera constante durante las últimas décadas para contribuir a las necesidades alimentarias de una población en rápido crecimiento (FAO, 2020) y varias especies nativas han sido identificadas desde hace tiempo como especies de interés para la acuicultura amazónica (Saint-Paul, 1986). Entre las distintas especies de peces nativos, el pez gato o bagre amazónico *Pseudoplatystoma punctifer* (Castelnau, 1855) es muy apreciado por la calidad de su carne y la ausencia de espinas intramusculares. Reúne una serie de características como una alta fecundidad, la posibilidad de manipular el desove en cautiverio y una conversión alimenticia eficiente, por las que esta especie, así como otras del género *Pseudoplatystoma* Bleeker, 1862, ha sido considerada de gran interés para la diversificación acuícola en la región desde hace ya varias décadas (Kossowski, 1996).

Taxonomía, distribución geográfica, morfología externa y biología de *Pseudoplatystoma punctifer*

Pseudoplatystoma punctifer (Castelnau, 1855), denominada comúnmente doncella (Perú), pintadillo o cachara (Colombia), surubí (Bolivia) o sorubim (Brasil), es miembro de la familia Pimelodidae y del orden Siluriformes (García Dávila et al., 2018). Es una de las ocho especies del género *Pseudoplatystoma* propuestas en la última revisión taxonómica realizada por Buitrago–Suárez y Burr (2007), quienes ampliaron las tres especies reconocidas previamente y redefinieron su distribución geográfica (Tabla 2). Sin embargo, una serie de inconsistencias han sido observadas entre la taxonomía del género propuesta por Buitrago–Suárez y Burr (2007) y posteriores estudios moleculares y morfológicos (Carvalho-Costa et al., 2011; Estivals et al., 2015; García-Dávila et al., 2013; Torrico et al., 2009), lo que pone de manifiesto la necesidad de reexaminar la clasificación de las especies de este género.

En la Amazonía peruana, *P. punctifer* tiene una amplia distribución y ha sido registrado en numerosos ríos de las regiones de Loreto, Ucayali y Madre de Dios (Figura 5). Esta especie de bagre alcanza una longitud total máxima de hasta 1,3 m y un peso de hasta 20 kg (García Dávila et al., 2018). Tiene un cuerpo alargado con una coloración gris en el dorso y blanco en el vientre y con barras

Tabla 2. Especies del género *Pseudoplatystoma* antes y después de la revisión taxonómica de Buitrago-Suárez y Burr (2007) y su distribución geográfica (Gisbert et al., 2022)

Especies		**Distribución geográfica**	
Antes	**Después**	**Cuenca**	**Países**
P. fasciatum	*P. punctifer*	Amazonas	Bolivia, Brasil, Colombia, Ecuador, Perú, Venezuela
	P. reticulatum	Amazonas central, Paraná	Argentina, Bolivia, Brasil, Paraguay, Uruguay
	P. orinocoense	Orinoco	Venezuela
	P. fasciatum	Guayanas	Guyana, Surinam, Guayana Francesa
	P. magdaleniatum	Magdalena, Cauca	Colombia
P. tigrinum	*P. tigrinum*	Amazonas	Brasil, Colombia, Ecuador, Perú, Venezuela
	P. metaense	Orinoco	Colombia, Venezuela
P. corruscans	*P. corruscans*	Paraná, São Francisco	Argentina, Brasil, Paraguay, Uruguay

Figura 5. Distribución de *P. punctifer* en la Amazonía peruana (García-Dávila et al., 2018).

verticales blancas y negras. Posee una aleta caudal con lóbulos redondeados y pequeños puntos oscuros tanto en esta como en la aleta anal. Tiene una cabeza deprimida con ojos en posición superior y la mandíbula superior se proyecta ligeramente sobre la mandíbula inferior. Tiene barbillones maxilares cortos que llegan al origen de las pectorales y boca terminal con dientes pequeños y numerosos disponibles en almohadillas en las mandíbulas y premaxilares. Posee una fontanela corta y poco profunda que no llega a la base de la apófisis occipital. Los barbillones del mentón son más largos que la longitud de la cabeza (García Dávila et al., 2018; Figura 6). Esta especie piscívora se alimenta

principalmente durante la noche de peces más pequeños del orden de los Characiformes e incluso de invertebrados. Está muy extendida en la Amazonía donde habita los principales cauces de ríos y lagunas tanto de aguas blancas (ricas en nutrientes con gran cantidad de sedimentos inorgánicos y arcilla) como negras (pobres en nutrientes con gran cantidad de materia orgánica y apenas minerales). Es una especie migratoria que realiza dos migraciones anuales, la primera en verano para alimentarse y la segunda al inicio del periodo de lluvias para reproducirse (García Dávila et al., 2018).

Figura 6. Espécimen adulto de *P. punctifer* (Foto: IIAP, Iquitos, Perú).

Cultivo de *Pseudoplatystoma punctifer*

En la Amazonía peruana, *P. punctifer* sufre de una elevada presión pesquera y, actualmente, es la especie de bagre grande más desembarcada en los puertos de la región de Loreto (García Dávila et al., 2018). A parte del interés por su carne de calidad, su piel se utiliza para fabricar accesorios de cuero y, además, es apreciado como pez ornamental en su etapa juvenil (Padilla-Pérez et al., 2001). Es por ello que en el Perú el desarrollo de la acuicultura de esta especie ha despertado un gran interés y ha sido objeto de investigaciones a lo largo de las últimas dos décadas con el fin de desarrollar un paquete tecnológico. Sin embargo, el cultivo comercial no ha sido aun implementado en este país debido a las dificultades de su cultivo. A pesar de que la reproducción en cautiverio está controlada desde hace tiempo (Nuñez et al., 2008; Padilla-Pérez et al., 2001), *P. punctifer* sufre de una alta tasa de mortalidad durante las etapas tempranas de vida debido, principalmente, a una elevada incidencia de canibalismo y a la dificultad de adaptación al alimento balanceado (Baras et al., 2011).

A pesar de ello, las especies del género *Pseudoplatystoma* son las especies de pez gato más cultivadas en Sudamérica, siendo Brasil el principal productor (FAO, 2021; Gisbert et al., 2022). Su producción se ha basado principalmente en híbridos interespecíficos, como, por ejemplo, entre *P. corruscans* y *P. reticulatum*, ya que se considera que tienen un mejor crecimiento que el de las especies

parentales (Crepaldi et al., 2006; Oliveira et al., 2014). En la actualidad, los híbridos más producidos son intergenéricos entre *Pseudoplatystoma* spp. y los bagres omnívoros de la familia Pimelodidae *Leiarius marmoratus* o *Phractocephalus hemioliopterus*, que tienen un menor comportamiento caníbal, aceptan fácilmente los alimentos balanceados y presentan hábitos de alimenticios omnívoros en las etapas juvenil y adulta (Gisbert et al., 2022; Hashimoto et al., 2012). Sin embargo, la producción de híbridos conlleva riesgos para el medio ambiente y para la industria acuícola. Con frecuencia se han detectado híbridos en ambientes naturales y, en el caso de los híbridos interespecíficos, están contaminando las poblaciones naturales debido a su fertilidad (Hashimoto et al., 2013). Además, algunos estudios genéticos han revelado que la producción, el comercio y el manejo de estos híbridos actualmente no están controlados en Brasil, ya que los planteles de reproductores a menudo se componen por error de híbridos interespecíficos e incluso de híbridos post-F1, lo que puede llevar a pérdidas económicas (Hashimoto et al., 2015). En este contexto, el desarrollo de programas de mejora genética y de técnicas de cultivo para especies puras con el objetivo de obtener rendimientos similares al de los híbridos se hace necesario para lograr la sostenibilidad del cultivo de las especies del género *Pseudoplatystoma* (Alves et al., 2014).

Hoy en día, el éxito del cultivo temprano de las especies del género *Pseudoplatystoma* o de sus híbridos interespecíficos depende en gran medida de la pericia del productor para controlar el canibalismo y adaptar a los juveniles al consumo de alimento balanceado. En Brasil, las larvas de *Pseudoplatystoma* spp. suelen ser cultivadas en tanques cilíndricos conectados a un sistema de recirculación y son alimentadas con nauplios de *Artemia* desde los 2-3 días post eclosión (dpe) por 7 a 10 días (Inoue et al., 2009; Silva et al., 2013). A continuación, son cultivadas en tanques conectados a un sistema de recirculación o en estanques fertilizados al aire libre durante 30 a 40 días hasta que alcanzan una longitud total (LT) de 4 a 5 cm. Durante esta fase, los peces son alimentados con zooplancton (cladóceros y copépodos), a los que algunos productores agregan carne o pescado picados o larvas de peces de las especies *Prochilodus* sp., *Leporinus* sp. o *Piaractus* sp. (Campos, 2013). La supervivencia durante este período es muy variable y depende de la abundancia de zooplancton, las condiciones climáticas o la depredación de insectos. Además, requiere clasificar regularmente los peces por tamaño para reducir el canibalismo (Campos, 2013). La adaptación al alimento balanceado se realiza en la siguiente fase de cultivo (de 4-5 cm a 11-13 cm LT) en la que los juveniles de *Pseudoplatystoma* spp. se cultivan en tanques con renovación constante de agua. El alimento vivo se elimina gradualmente y los alevines se adaptan progresivamente, durante un período de 4 a 6 semanas, a una dieta formulada que incluye ingredientes como sardinas, corazón y pulmón de res, plancton congelado o gónadas de pescado picadas (Campos, 2013; Inoue et al., 2009). Durante este período, los juveniles se siguen clasificando periódicamente por tamaño para reducir el canibalismo.

Con el fin de mejorar el cultivo temprano de *P. punctifer*, las investigaciones realizadas en la Amazonía peruana han buscado mejorar aspectos zootécnicos centrándose en los efectos maternos y paternos en la tasa de eclosión y las variaciones del crecimiento larvario (Núñez et al., 2011), el efecto

del número de raciones diarias de alimento sobre la incidencia del canibalismo (Baras et al., 2011), la tasa de ingestión y evacuación de *Artemia* (Baras et al., 2012) o el efecto de un alimento seco o húmedo sobre el crecimiento y la supervivencia (Fernández-Méndez et al., 2015). Sin embargo, la incidencia del canibalismo en esta fase temprana del desarrollo ha seguido siendo elevada.

Canibalismo en peces

El canibalismo es una estrategia de alimentación depredadora que implica matar y comer individuos de la misma especie, fenómeno que puede ocurrir tanto entre padres e hijos, entre hermanos en un grupo de edad determinado o entre individuos de poblaciones separadas (Naumowicz et al., 2017). El canibalismo es considerado un mecanismo ecológico con valor adaptativo, ya que conduce a una mejor aptitud individual, parece estabilizar o mejorar las poblaciones en algunas circunstancias y puede actuar como un mecanismo de supervivencia cuando los recursos alimenticios alternativos escasean (Baras y Jobling, 2002). En condiciones de cultivo, el canibalismo intracohorte representa un problema mayor en el cultivo de numerosas especies de peces, especialmente en aquellas con alta fecundidad, desarrollo altricial y hábitos depredadores, y puede causar grandes mortalidades en las etapas larvaria y juvenil temprana (Baras y Jobling, 2002). Existen dos tipos de canibalismo intracohorte, el tipo I, en el que la víctima no es ingerida o consumida por completo y que ocurre en una fase más temprana del desarrollo y es independiente de la variabilidad de tamaños de los peces, y el tipo II, en el que la víctima es consumida entera y que ocurre en una fase posterior y está asociada a un crecimiento heterogéneo (Naumowicz et al., 2017; Figura 7).

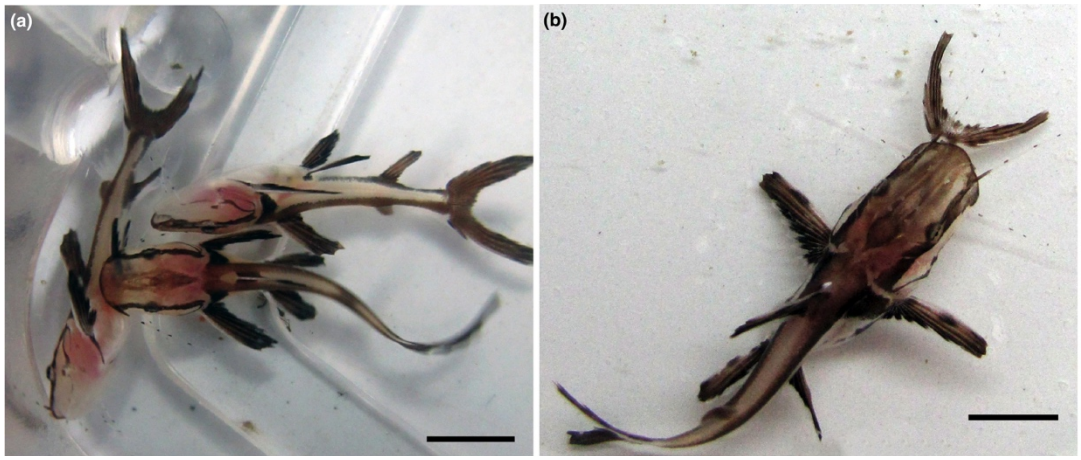

Figura 7. Canibalismo intracohorte en *P. punctifer* (a) tipo I, (b) tipo II (Darias et al., 2015).

En acuicultura, la intensidad del canibalismo está directamente influenciada por factores poblacionales y abióticos. Los factores poblacionales incluyen, entre otros, la densidad de cultivo, el tamaño de las larvas o el sexo, especialmente en las especies con dimorfismo sexual del crecimiento durante la ontogenia temprana (Naumowicz et al., 2017). Además, algunos estudios han demostrado que hay una cierta determinación genética en el canibalismo en peces, si bien aún no está claro si existe un ecofenotipo caníbal con una mayor propensión o capacidad para ejercer el canibalismo o si, por el

contrario, los caníbales son solamente el resultado de una intensa competencia inicial (Baras y Jobling, 2002). Entre los factores abióticos que influyen en el canibalismo se encuentran el protocolo de alimentación, la temperatura y claridad del agua, la intensidad de la luz, el fotoperiodo y las características de los tanques de cultivo (Naumowicz et al., 2017).

En este sentido, para reducir la incidencia del canibalismo intracohorte en cultivo, se hace necesario determinar las condiciones óptimas de estos factores para cada especie. Así, por ejemplo, en algunas especies como *Esox lucius*, *Perca fluviatilis* o *Clarias gariepinus* se ha observado un aumento proporcional del canibalismo como resultado del aumento de la densidad de cultivo, lo que puede estar relacionado con una mayor probabilidad de interacción caníbal-presa. Sin embargo, en otras especies como *Gadus morhua*, *Heterobranchus longifilis* o *Squalius cephalus*, el uso de densidades de cultivo altas resultan en una reducción del canibalismo, especialmente cuando está asociado con el comportamiento territorial, ya que se sugiere que el espacio limitado resulta en un menor número de individuos agresivos protegiendo el territorio, además de que, a una cierta densidad de cultivo, los peces comienzan a formar cardúmenes y los caníbales se confunden perceptivamente y no pueden seleccionar una presa (Baras y Jobling, 2002; Naumowicz et al., 2017). De igual manera, un periodo de iluminación más largo reduce el comportamiento agresivo de algunas especies, especialmente de aquellas que dependen principalmente de la vista para la búsqueda de alimentos, como *Esox lucius*, *Perca fluviatilis* o *Sander lucioperca*. Al contrario, en especies como *O. niloticus*, *Lates calcarifer* o *C. gariepinus* una intensidad de luz excesiva puede ser una fuente de estrés e intensificar el comportamiento agresivo (Naumowicz et al., 2017). Un protocolo de alimentación que satisfaga los requisitos nutricionales de la especie, favorezca un crecimiento homogéneo de las larvas y elimine la competencia por el alimento constituye también un elemento importante en las estrategias de reducción del canibalismo en peces. Para ello, es necesario determinar para cada especie tanto el tipo de alimento adecuado (composición nutricional, digestibilidad, textura, tamaño, forma, etc.), como la frecuencia de alimentación, el tamaño de la ración o el momento adecuado de transición de la presa viva al alimento balanceado (Naumowicz et al., 2017).

En *P. punctifer*, las investigaciones relacionadas con la incidencia de canibalismo han mostrado que, por ejemplo, cultivar las larvas en completa oscuridad aumenta la supervivencia (Nuñez et al., 2008). El uso de una frecuencia de alimentación de 6 raciones al día repartidas en 24 h dio también mejores resultados a nivel de supervivencia que cuando éstas fueron repartidas en 12 h (Nuñez et al., 2008). Al contrario, no se encontró ningún efecto paternal o maternal, ni de densidad de cultivo (10, 30 o 100 larvas L^{-1}) en la supervivencia (Núñez et al., 2011; Nuñez et al., 2008). Sin embargo, en estos estudios no se analizó qué porcentaje de dicha mortalidad fue debida al canibalismo. En Baras et al. (2011), los autores mostraron que es necesario una frecuencia de alimentación mínima de 6 raciones al día para mantener el canibalismo a niveles bajos, pero que éste aumenta casi exponencialmente por cada ración que falte. En otro estudio, Baras et al. (2012) sugieren que las larvas de *P. punctifer* sean alimentadas cada 2.5 a 3 h a 28 °C. Sin embargo, hasta la realización de esta tesis, ningún estudio se

había enfocado en estudiar la fisiología digestiva, ni en determinar las necesidades nutricionales de *P. punctifer* durante las primeras etapas de vida con el fin de desarrollar un protocolo de alimentación adaptado a la especie que pueda contribuir a reducir la incidencia de canibalismo.

La nutrición de peces durante las etapas tempranas de vida

En acuicultura, una dieta equilibrada en términos de macro- y micronutrientes es esencial para asegurar la supervivencia, así como un desarrollo, salud y crecimiento óptimos de los peces, especialmente durante las primeras etapas de la vida, cuando los órganos y sistemas están en desarrollo. Los requisitos nutricionales son específicos para cada especie, varían a lo largo del desarrollo y dependen de las condiciones de cultivo. Por tanto, este conocimiento es clave para formular dietas adaptadas para cada especie. El costo de las dietas formuladas suele representar el gasto operativo más alto en el cultivo de peces, siendo las proteínas el componente principal y más caro de los alimentos. Con el fin de ahorrar el uso de proteínas en el metabolismo energético y, por tanto, reducir los costos de producción, los esfuerzos de investigación se han centrado principalmente en reemplazar parte del contenido de proteínas con fuentes de energía no proteicas. Sin embargo, es necesaria una proporción equilibrada de energía:proteína (E:P) para regular la ingesta de alimentos y el metabolismo de los nutrientes, incluidos los carbohidratos y los lípidos (Alam et al., 2019; El-Sayed y Teshima, 1992; Winfree y Stickney, 1981). Cuando la energía no proteica es inadecuada y/o no se suministra en cantidades suficientes, las proteínas pueden catabolizarse para satisfacer las necesidades energéticas en detrimento del crecimiento (Boonanuntanasarn et al., 2018). Las dietas más eficientes contienen tanto lípidos como carbohidratos en una composición y cantidad adecuadas, lo que permite ahorrar proteínas para satisfacer las necesidades energéticas globales y proporcionar proteínas para el crecimiento somático del organismo. La composición y el contenido de estas fuentes de energía tienen un efecto directo sobre el crecimiento, la eficiencia de conversión alimenticia, la retención de nutrientes y la composición corporal (Castro et al., 2016; El-Sayed y Teshima, 1992; Enes et al., 2006; Erfanullah y Jafri, 1998; Francis et al., 2007; Gao et al., 2010; Hemre et al., 1995; Li et al., 2019; Taj et al., 2020; Torstensen et al., 2000; Winfree y Stickney, 1981).

Los lípidos son la fuente de energía preferida para muchas especies de peces, especialmente las carnívoras. Sin embargo, un contenido excesivo de lípidos puede provocar un crecimiento reducido y un aumento de la deposición de lípidos corporales (Erfanullah y Jafri, 1998). En general, los carbohidratos no son la principal fuente de energía o de carbono de los peces y son poco utilizados por la mayoría de los peces carnívoros (Halver y Hardy, 2003; Hemre et al., 1995), aunque pueden mejorar la utilización de proteínas en algunas especies (Enes et al., 2006; García-Meilán et al., 2014). Dado que los carbohidratos son menos costosos que los lípidos, se busca un aumento en el nivel de inclusión en las dietas, especialmente en especies herbívoras y omnívoras que utilizan el almidón de manera más eficiente que los peces carnívoros (Halver y Hardy, 2003; Wilson, 1994). Al igual que con los lípidos, un contenido excesivo de carbohidratos en la dieta puede alterar el metabolismo energético, lo que lleva

a la deposición de grasa corporal y, en última instancia, afecta al crecimiento (Brauge et al., 1994; Wang et al., 2005). Por lo tanto, las proporciones óptimas entre proteínas y energía, así como entre carbohidratos y lípidos en los alimentos para peces deben definirse cuidadosamente para promover un desarrollo saludable y un crecimiento y supervivencia óptimos.

Durante las primeras etapas de la vida de los peces, una composición óptima de ácidos grasos en la dieta, especialmente ácidos grasos poliinsaturados (PUFA), es esencial para promover un desarrollo y crecimiento adecuados (Lund et al., 2012; Mourente, 2003; Watanabe, 1993). La ausencia de PUFA en la dieta conduce a síntomas de deficiencia, que incluyen un crecimiento reducido y un aumento de la mortalidad (Glencross, 2009; Tocher, 2010). Los peces de agua dulce pueden mantener un cierto grado de actividades D6 y D5 desaturasas y elongasas para sintetizar ácido docosahexaenoico (DHA, 22:6n-3), ácido eicosapentaenoico (EPA, 20:5n-3) y ácido araquidónico (ARA, 20:4n-6) de sus precursores, los ácidos linoleico (LA, 18:2n-6) y linolénico (ALA, 18:3n-3), presentes en la dieta. Así, LA y ALA son considerados los ácidos grasos esenciales (AGE) en especies de agua dulce (Izquierdo et al., 2008; Satoh et al., 1989; Watanabe, 1982; Figura 8), las cuales poseen menores requisitos de ácidos grasos altamente insaturados (HUFA) n-3 que las larvas de peces marinos (Verreth et al., 1994). Sin embargo, la composición de ácidos grasos de la *Artemia*, que naturalmente carece de HUFA n-3

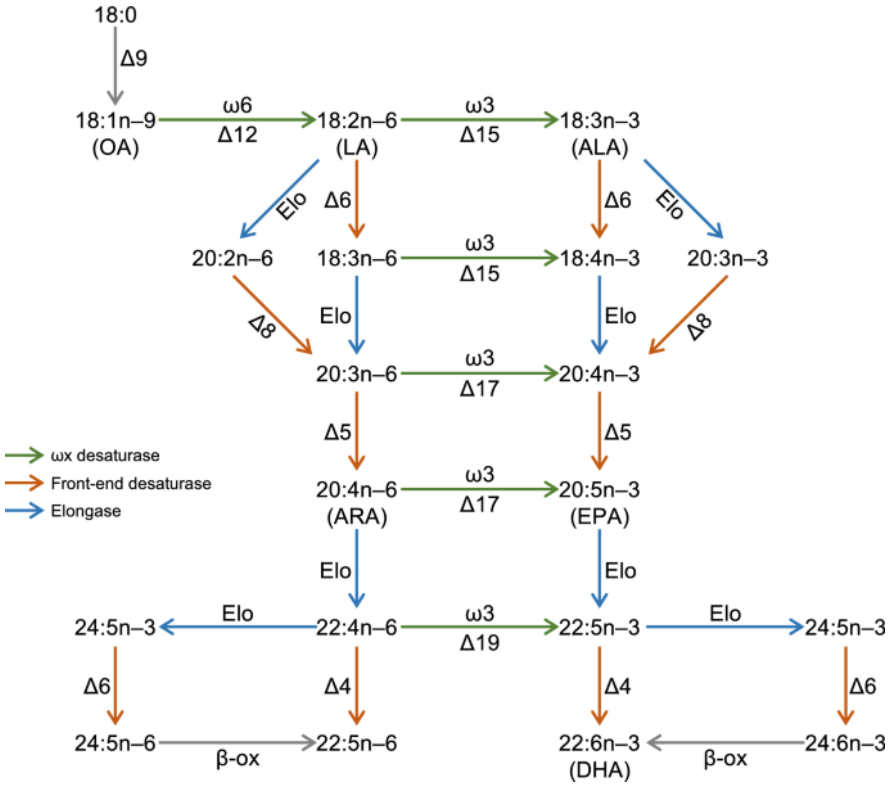

Figura 8. Vías biosintéticas de ácidos grasos poliinsaturados en organismos acuáticos. Las reacciones de desaturación se indican como "ω" de "Δ" para referirse, respectivamente, a la posición del carbono en la que se ubica la insaturación dentro de los extremos metilo (ω; flechas verdes) y "front-end" (Δ; flechas naranjas) de las cadenas de acilos grasos. Las reacciones de elongación "Elo" se indican con flechas azules. β-ox, β-oxidación; OA, ácido oleico; LA, ácido linoleico; ALA, ácido α-linolénico; ARA, ácido araquidónico; EPA, ácido eicosapentaenoico; DHA, ácido docosahexaenoico (Monroig y Kabeya, 2018).

(Sargent et al., 1999), también puede afectar al crecimiento de las especies de agua dulce (Bengtson et al., 1991). El DHA es particularmente importante durante el desarrollo larvario debido a su papel estructural en las membranas celulares, especialmente en los tejidos neuronales, como la retina y el cerebro (Bell et al., 1996; Mourente, 2003; Wassall y Stillwell, 2008). Además, es conocido que una deficiencia de DHA en la dieta promueve el estrés (Lund et al., 2012), el cual, a su vez, puede inducir cambios en el comportamiento (Lund et al., 2014). Proporcionar cantidades suficientes de HUFA en la dieta es clave para asegurar una supervivencia, crecimiento y metamorfosis exitosos en larvas de peces (Izquierdo et al., 2000; Sargent et al., 1999).

Las etapas tempranas de vida de los peces constituyen una fase muy sensible durante la cual la morfogénesis ocurre en un período de tiempo muy corto y una alimentación y nutrición óptimas al momento de la apertura de la boca son claves para la supervivencia y el crecimiento (Yúfera y Darias, 2007). Sin embargo, poco se sabe sobre las necesidades nutricionales de las larvas de peces (Holt, 2011), ya que el estudio de sus dietas en el medio natural puede resultar muy difícil. Si bien las larvas de peces pueden ser morfológicamente capaces de capturar diferentes alimentos (p.e., organismos zooplanctónicos y microdietas), su sistema digestivo sufre una serie de cambios en el desarrollo antes de ser completamente funcional poco después de la eclosión (Gisbert et al., 2022; Rønnestad et al., 2013). En condiciones de cultivo, se han utilizado ampliamente métodos indirectos, como la caracterización de la ontogenia del sistema digestivo, para comprender mejor sus capacidades digestivas en desarrollo y los requerimientos y condiciones nutricionales (Gisbert et al., 2008; Lazo et al., 2011; Rønnestad et al., 2013; Zambonino Infante et al., 2008; Zambonino Infante y Cahu, 2001). Aunque los mecanismos básicos del desarrollo de órganos y sistemas son similares entre los teleósteos, existen diferencias interespecíficas considerables con respecto al tiempo relativo de diferenciación, desarrollo y funcionalidad durante la ontogenia (Treviño et al., 2011). El desarrollo del sistema digestivo es específico para cada especie y es un proceso genéticamente programado que se ve afectado por la historia de vida general y la estrategia reproductiva de cada especie, y por una variedad de factores abióticos y bióticos, como la temperatura del agua y la disponibilidad y composición de los alimentos (Lazo et al., 2011; Rønnestad et al., 2013; Zambonino Infante et al., 2008). En el contexto de la acuicultura, el conocimiento de las diferencias interespecíficas a nivel de la diferenciación, el desarrollo y la funcionalidad del tracto digestivo y las glándulas accesorias durante las primeras etapas de la vida es esencial para desarrollar protocolos de alimentación adaptados al desarrollo fisiológico específico de cada especie. Para ello, el enfoque más común es el estudio de la morfología del sistema digestivo y de la actividad de las enzimas digestivas a lo largo del desarrollo a mediante análisis histológicos, histoquímicos y bioquímicos. Los hitos generalmente utilizados para evaluar el desempeño digestivo de las larvas incluyen la aparición de gránulos de zimógeno en el páncreas exocrino y de la actividad de las enzimas pancreáticas antes del inicio de la alimentación exógena, la maduración enzimática del borde en cepillo de los enterocitos y el desarrollo del estómago, el cual está determinado generalmente por la aparición de las glándulas gástricas y de la actividad de la pepsina en los peces gástricos, que marcan la

transición del modo de digestión larvario al juvenil (Lazo et al., 2011; Rønnestad et al., 2013; Yúfera et al., 2018; Zambonino Infante et al., 2008). Sin embargo, el inicio de la digestión ácida puede estar sincronizado o no con el desarrollo morfológico de las glándulas gástricas. Así, por ejemplo, está sincronizada en *Rhamdia quelen* (Silveira et al., 2013) o en *C. gariepinus* (Nattabi, 2018), pero ocurre después de 67 grados día (gd) en *Melanogrammus aeglefinus* (Perez-Casanova et al., 2006), 147 gd en *Paralichthys dentatus* (Huang et al., 1998), 154 gd en *Hippoglossus hippoglossus* (Murray et al., 2006), 195 gd en *Pagrus pagrus* (Darias et al., 2005) o 233 gd en *Gadus morhua* (Perez-Casanova et al., 2006). El inicio de la digestión ácida también se considera generalmente un punto óptimo para la transición de la presa viva al alimento formulado (destete), momento a partir del cual las proteínas complejas de la dieta se digieren con más facilidad (Lazo et al., 2011; Rønnestad et al., 2013; Zambonino Infante et al., 2008). La morfología y la funcionalidad del sistema digestivo se pueden utilizar como indicador no sólo del desarrollo y la maduración intestinal, sino también del estado nutricional de peces al evaluar distintos dietas o protocolos alimentarios, lo que eventualmente conduce a mejorar las tasas de crecimiento, la supervivencia y la calidad de los peces (Gisbert et al., 2008; Hamre et al., 2013; Rønnestad et al., 2013; Zambonino Infante et al., 2008).

Entre las diferentes enzimas involucradas en la digestión, la α-amilasa (EC 3.2.1.1) se sintetiza en el páncreas exocrino y es clave para la digestión de carbohidratos complejos en los peces (Cahu y Zambonino Infante, 1994; Darias et al., 2006; Ma et al., 2005; Moyano et al., 1996). Las fosfolipasas A2 (PLA2, EC 3.1.1.4) son enzimas lipolíticas esenciales que hidrolizan los fosfolípidos para generar ácidos grasos libres y lisofosfolípidos (Dennis, 1994). La fosfolipasa pancreática A2-IB (PLA2-IB) es uno de los diferentes tipos de PLA2 secretora (sPLA2) y se considera la enzima digestiva más importante en los peces marinos (Cahu et al., 2003; Rønnestad et al., 2013). La enzima pancreática tripsina (EC 3.4.21.4) se considera la enzima proteolítica alcalina más importante en las primeras etapas de la vida de los peces y también desempeña un papel clave en la activación de otras enzimas pancreáticas en la luz intestinal como la quimiotripsina (Rønnestad et al., 2013). La quimotripsina (EC 3.4.21.1) es otra enzima proteolítica pancreática importante, cuya actividad es complementaria a la de la tripsina. Durante la ontogenia del sistema digestivo de los peces gástricos, la tripsina y la quimotripsina son responsables de la digestión de proteínas en el ambiente alcalino del intestino hasta que se forma el estómago. En ese momento, aparece una tercera enzima proteolítica, la pepsina. Entre las dos clases principales identificadas, la pepsina A y C, la primera es la forma predominante y existen varias isoformas en los peces gástricos (Kapoor et al., 1976). La pepsina es responsable de la hidrólisis inicial y parcial de las proteínas en el estómago en presencia de un ambiente ácido. Su precursor, el pepsinógeno, es producido y secretado por las glándulas gástricas del estómago, donde es activado por el ácido clorhídrico (Darias et al., 2005, 2007a; Douglas et al., 1999; Gawlicka et al., 2001). La lipasa activada por sales biliares (BAL; E.C. 3.1.1) es considerada una de las lipasas más importantes en peces (Murray et al., 2003; Patton et al., 1977), ya que actúa sobre una amplia gama de sustratos de ésteres de cera y triacilgliceroles ricos en PUFAs. Estos sustratos son más resistentes a la hidrólisis por otras lipasas pancreáticas (Chen

et al., 1990). Para la hidrólisis de lípidos, la BAL se secreta a la luz intestinal y, como su propio nombre indica, se activa mediante sales biliares. Posteriormente, el intestino puede absorber las sustancias resultantes (Lazo et al., 2011). La lipoproteína lipasa (EC 3.1.1.34) es un regulador clave del metabolismo de los lípidos que hidroliza las lipoproteínas ricas en triglicéridos transportadas en el torrente sanguíneo como quilomicrones y lipoproteínas de muy baja densidad, y los ácidos grasos liberados son absorbidos por los tejidos para su oxidación o almacenamiento (Mead et al., 2002). Además de la actividad enzimática pancreática se suele medir la actividad de enzimas citosólicas (p.ej., leucina-alanina peptidasa–LAP) y del borde en cepillo (p. ej., fosfatasa alcalina–AP, maltasa–MAL, aminopeptidasa N–AN) de los enterocitos para complementar en análisis del desarrollo y maduración del tracto digestivo (Cahu y Zambonino Infante, 2001).

Contrariamente a la actividad de las principales enzimas digestivas, el perfil de expresión ontogenética de los genes que codifican estas enzimas se ha estudiado en relativamente pocas especies de peces, aunque los patrones de expresión temporal y espacial de los genes implicados en el desarrollo y la funcionalidad del tracto digestivo, así como los conocimientos sobre los mecanismos moleculares subyacentes a la función y modulación de la hidrólisis enzimática de los distintos macronutrientes dietéticos son necesarios para comprender mejor el proceso de digestión en los peces, especialmente durante la ontogenia (Lazo et al., 2011; Yúfera et al., 2018).

OBJETIVOS

OBJETIVO GENERAL

El objetivo general de esta tesis es generar conocimientos sobre la fisiología digestiva de *Pseudoplatystoma punctifer* (Castelnau, 1855) a través del estudio de la ontogenia funcional del sistema digestivo, así como de sus necesidades nutricionales, con el fin de contribuir a desarrollar protocolos de alimentación adaptados a las etapas tempranas de vida de esta especie amazónica.

OBJETIVOS ESPECÍFICOS

1. Estudiar la ontogenia funcional del aparato digestivo en *P. punctifer*.
2. Estudiar las necesidades nutricionales de *P. punctifer* durante las primeras etapas de vida con el fin de mejorar las técnicas de cultivo.

Con el fin de alcanzar los objetivos específicos arriba mencionados, el presente trabajo de investigación está organizado en dos capítulos:

Capítulo 1: Estudio de la ontogenia del aparato digestivo de *Pseudoplatystoma punctifer*

1.1. Aislamiento, identificación y análisis de la expresión de los principales precursores enzimáticos digestivos de *P. punctifer*.

1.2. Análisis de la actividad enzimática intestinal, pancreática y estomacal de *P. punctifer*.

Capítulo 2: Estudio de las necesidades nutricionales de *Pseudoplatystoma punctifer* durante las primeras etapas de vida

2.1. Influencia de diferentes proporciones de proteínas y lípidos (y sus ratios) en la dieta en la función digestiva, el crecimiento y la supervivencia.

2.2. Influencia del enriquecimiento con DHA de la *Artemia* y del alimento balanceado en la incidencia de canibalismo, la función digestiva y el crecimiento.

INFORME DEL FACTOR DE IMPACTO

La Dra. María J. Darias y el Dr. Enric Gisbert Casas, respectivamente como directora y co-director de la tesis titulada "Fisiología digestiva y nutrición durante las primeras etapas de vida del pez gato amazónico *Pseudoplatystoma punctifer* (Castelnau, 1855) en cultivo" realizada por Diana Castro Ruiz, manifiestan la veracidad del factor de impacto y la implicación de la doctoranda en cada artículo científico realizado en coautoría que se presenta en esta Tesis, y que ninguno de los artículos ha sido utilizado para la elaboración de alguna otra tesis doctoral, como a continuación se expone:

1.1. Isolation, identification, and gene expression analysis of the main digestive enzymes during ontogeny of the Neotropical catfish *Pseudoplatystoma punctifer* (Castelnau, 1855)

AUTORES/AS (p.o. de firma): D. Castro-Ruiz, K. B. Andree, E. Blondeau-Bidet, C. Fernández-Méndez, C. García-Dávila, E. Gisbert, M. J. Darias

REF. REVISTA/LIBRO: *Aquaculture (2021), 543, 737031.*

IF: 4.242 (Q1 – FISHERIES)

Diana Castro Ruiz participó en la preparación del diseño experimental, realizó el cultivo de *Pseudoplatystoma punctifer*, producción de la presa viva (*Artemia*), muestreos, procesado y análisis de las muestras biológicas (expresión génica), interpretación de los datos y redacción final del artículo científico.

1.2. Ontogeny of the digestive enzyme activity of the Amazonian pimelodid catfish *Pseudoplatystoma punctifer* (Castelnau, 1855)

AUTORES/AS (p.o. de firma): D. Castro-Ruiz, M. T. Mozanzadeh, C. Fernández-Méndez, K. B. Andree, C. García-Dávila, C. Cahu, E. Gisbert, M. J. Darias

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FI: 3.225 (Q1 – FISHERIES)

Diana Castro Ruiz participó en la preparación del diseño experimental, realizó el cultivo de *Pseudoplatystoma punctifer*, producción de la presa viva (*Artemia*), muestreos y participó en el procesado y análisis de las muestras biológicas (actividad enzimática), interpretación de los datos y redacción final del artículo científico.

2.1. The digestive function of *Pseudoplatystoma punctifer* early juveniles is differentially modulated by dietary protein, lipid and carbohydrate content and their ratios

AUTORES/AS (p.o. de firma): D. Castro-Ruiz, K. B. Andree, M. M. Solovyev, C. Fernández-Méndez, C. García-Dávila, C. Cahu, E. Gisbert, M. J. Darias

REF. REVISTA/LIBRO: *Animals (2021), 11(2), 369.*

IF: 2.752 (Q1 – AGRICULTURE, DAIRY & ANIMAL SCIENCE)

Diana Castro Ruiz participó en la preparación del diseño experimental, realizó el cultivo de *Pseudoplatystoma punctifer*, producción de la presa viva (*Artemia*), muestreos y participó en el procesado y análisis de las muestras biológicas (expresión génica, actividad enzimática, histología, bioquímica), interpretación de los datos y redacción final del artículo científico.

2.2. DHA-enrichment of live and compound feeds influences the incidence of cannibalism, digestive function, and growth in the Neotropical catfish *Pseudoplatystoma punctifer* (Castelnau, 1855) during early life stages

AUTORES/AS (p.o. de firma): D. Castro-Ruiz, K. B. Andree, J. Magris, C. Fernandez-Méndez, C. García-Dávila, E. Gisbert, M. J. Darias

REF. REVISTA/LIBRO: *Aquaculture (2021), en revisión*

IF: 4.242 (Q1 – FISHERIES)

Diana Castro Ruiz participó en la preparación del diseño experimental, cultivo de *Pseudoplatystoma punctifer*, producción y enriquecimiento de la presa viva (*Artemia*), muestreos, procesado y análisis de las muestras biológicas (expresión génica, bioquímica, histología, crecimiento, canibalismo, supervivencia), interpretación de los datos y redacción final del artículo científico.

María J. Darias

Enric Gisbert

CAPÍTULO 1

Estudio de la ontogenia del aparato digestivo de *Pseudoplatystoma punctifer*

1.1

Isolation, identification, and gene expression analysis of the main digestive enzymes during ontogeny of the Neotropical catfish *Pseudoplatystoma punctifer* (Castelnau, 1855)

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Resumen

La ontogenia de las capacidades digestivas de los peces es específica a cada especie y su conocimiento es esencial para comprender los requisitos nutricionales de las larvas de peces. Si bien la cuenca del Amazonas alberga la mayor biodiversidad de peces del mundo, las bases moleculares de la fisiología digestiva temprana de ninguna especie amazónica han sido aún reportadas. Con el fin de aumentar los conocimientos básicos sobre la ontogenia molecular del sistema digestivo de una especie de pez amazónico de importancia comercial, se aislaron e identificaron seis genes de enzimas digestivas (α-amilasa, fosfolipasa A2, lipoproteína lipasa, tripsina, quimotripsina y pepsina) y se analizó su expresión de 3 a 24 días post-fertilización (dpf) en *Pseudoplatystoma punctifer*, una especie de bagre con alto potencial para la diversificación acuícola. Los presentes resultados, junto con los obtenidos previamente sobre el desarrollo de la actividad enzimática digestiva, mostraron que la expresión génica y las actividades enzimáticas están sincronizadas y que la maquinaria enzimática de *P. punctifer* estaba completamente preparada para el inicio de la alimentación exógena (4 dpf, 6 mm de longitud total, LT) y alcanzó su madurez entre 10 y 13 dpf (11-14 mm LT). Esto indicó que la transición del modo de digestión larval al juvenil se había completado y que los especímenes eran aptos para el destete en condiciones de cultivo. Además, los análisis de expresión génica sugieren que esta especie muestra un comportamiento omnívoro con preferencia carnívora durante el desarrollo temprano. El presente estudio proporciona el primer análisis ontogenético integral de la función digestiva desde un punto de vista molecular de una especie del género *Pseudoplatystoma* y contribuye al desarrollo de estrategias de alimentación en el contexto de la diversificación acuícola sudamericana.

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Isolation, identification, and gene expression analysis of the main digestive enzymes during ontogeny of the Neotropical catfish *Pseudoplatystoma punctifer* (Castelnau, 1855)

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ABSTRACT

The ontogeny of the digestive capacities in fish is species-specific and its knowledge is essential for understanding the nutritional requirements of fish larvae. While the Amazon basin contains the world's highest fish biodiversity, the molecular basis of the early digestive physiology has not yet been reported in any Amazonian fish species. In order to increase basic knowledge on the molecular ontogeny of the digestive system of a commercially important Amazonian fish species, six digestive enzyme genes (*α-amylase*, *phospholipase A2*, *lipoprotein lipase*, *trypsin*, *chymotrypsin,* and *pepsin*) were isolated and identified, and their expression analyzed from 3 to 24 days post fertilization (dpf) in *Pseudoplatystoma punctifer*, a catfish species with high potential for aquaculture diversification. The present results, together with those previously obtained on the development of the digestive enzyme activity, showed that gene expression and enzymatic activities are synchronized and that the enzymatic machinery of *P. punctifer* was completely prepared for the onset of exogenous feeding (4 dpf, 6 mm total length, TL) and had reached its maturity between 10 and 13 dpf (11–14 mm TL). This indicated that the transition from the larval to the juvenile mode of digestion had been completed and they were suitable for weaning under culture conditions. Furthermore, the gene expression analyses suggest that this species displays an omnivorous behavior with a preference towards carnivory during early development. The present study provides the first comprehensive ontogenetic analysis of the digestive function from a molecular point of view of a species of the genus *Pseudoplatystoma*, and contributes to the development of feeding strategies in the context of South American aquaculture diversification.

1. Introduction

The early life stages of fish constitute a very sensitive phase during which morphogenesis occurs in a very short time period and optimal feeding and nutrition at mouth opening are key for survival and growth (Yúfera and Darias, 2007); however, very little is known about the nutritional requirements of fish larvae (Holt, 2011). Studying the natural diets of developing larvae can be difficult, and indirect approaches under culture conditions, such as the characterization of the ontogeny of the digestive system, have been widely used to better understand their developing digestive capacities, and nutritional requirements and conditions (Gisbert et al., 2008; Lazo et al., 2011; Rønnestad et al., 2013; Zambonino Infante et al., 2008; Zambonino Infante and Cahu, 2001). The development of the digestive system is species-specific and is a genetically programmed process that is affected by the general life history and reproductive strategy of each species, and by a variety of abiotic and biotic factors, such as water temperature and food availability and composition (Lazo et al., 2011; Rønnestad et al., 2013;

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Zambonino Infante et al., 2008). In the context of aquaculture, knowledge of the interspecific differences in the relative timing of the differentiation, development, and functionality of the digestive tract and accessory glands during early life stages is essential to develop feeding protocols adapted to the physiological stages of development of each species. The most common approach to estimate the digestive capacities of fish has been the determination of the activity of digestive enzymes by biochemical analysis. Studies evaluating larval digestive performance are generally focused on the appearance of the activity of pancreatic enzymes before the onset of exogenous feeding, the enzymatic maturation of the brush border of enterocytes, and the appearance of pepsin activity in gastric fish, which mark the transition from the larval to the juvenile mode of digestion (Lazo et al., 2011; Rønnestad et al., 2013; Yúfera et al., 2018; Zambonino Infante et al., 2008). Among the different enzymes involved in digestion, α-amylase (EC 3.2.1.1) is synthesized in the exocrine pancreas and is key for the digestion of complex carbohydrates in fish (Cahu and Zambonino Infante, 1994; Darias et al., 2006; Ma et al., 2005; Moyano et al., 1996). The phospholipases A2 (PLA2, EC 3.1.1.4) are essential lipolytic enzymes that hydrolyze phospholipids to generate free fatty acids and lysophospholipids (Dennis, 1994). The pancreatic phospholipase A2-IB (PLA2-IB) is one of the different types of secretory PLA2 (sPLA2) and it is considered the most important digestive enzyme in marine fish (Cahu et al., 2003; Rønnestad et al., 2013). The pancreatic enzyme trypsin (EC 3.4.21.4) is considered the most important alkaline proteolytic enzyme in early life stages of fish and it also plays a key role in activating other pancreatic enzymes in the gut lumen (Rønnestad et al., 2013). Chymotrypsin (EC 3.4.21.1) is another important pancreatic proteolytic enzyme, whose activity is complementary to that of trypsin. During the digestive system ontogeny of gastric fish, trypsin, and chymotrypsin are responsible of protein digestion in the alkaline environment of the intestine until the stomach is formed. At that time, a third proteolytic enzyme, pepsin, appears. Among the two main classes identified, pepsin A and C, the first one is the predominant form, and several isoforms exist in gastric fish (Kapoor et al., 1976). They are responsible for the initial and partial hydrolysis of proteins in the stomach in the presence of an acidic environment. Its precursor, *pepsinogen*, is produced and secreted by the gastric glands of the stomach, where it is activated by hydrochloric acid (Darias et al., 2005; Darias et al., 2007a; Douglas et al., 1999; Gawlicka et al., 2001). Lipoprotein lipase (LPL, EC 3.1.1.34) is a key regulator of lipid metabolism that hydrolyzes triglyceride-rich lipoproteins transported in the bloodstream as chylomicrons and very-low-density lipoproteins, and the released fatty acids are taken up by the tissues for oxidation or storage (Mead et al., 2002). Contrary to the activity of the main digestive enzymes, the ontogenetic expression pattern of the genes encoding for these enzymes has been studied in relatively few fish species, even though basic knowledge on the molecular mechanisms underlying the function and modulation of the enzymatic hydrolysis of the various dietary macronutrients is necessary to better understand the process of digestion in fish (Yúfera et al., 2018).

Covering more than 6,000,000 km^2, the Amazon basin is home to the richest fish fauna in the world with 2406 valid species, 1402 of which are endemic (Jézéquel et al., 2020); however, to our knowledge, no data has been reported on the molecular basis of the early digestive physiology of any Amazonian fish species. Fish is the main source of proteins, essential fatty acids, and micronutrients for the local population, especially for low-income families, and per capita fish consumption is one of the highest in the world (Isaac and de Almeida, 2011). Fish populations are increasingly faced with numerous threats such as pollution, deforestation, hydropower dams, invasive species, and overfishing (Carolsfeld et al., 2003; Winemiller et al., 2016). To counter-balance these effects, aquaculture has been developing steadily for the last decades to contribute to the food needs of a fast growing population (FAO, 2020). Among the cultured species, the highly prized species of the genus *Pseudoplatystoma* Bleeker, 1862 (maximum total lengths of up to 140 cm (Buitrago-Suárez and Burr, 2007)) are the most produced catfish species in South America, and Brazil is the largest producer (IBGE, 2020; Valladão et al., 2018). Production mostly relies on interspecific hybrids (e.g., *P. reticulatum* x *P. corruscans*) for their better growth performance, and more recently, on intergeneric hybrids between *Pseudoplatystoma* spp. and omnivorous catfish species such as *Leiarius marmoratus* or *Phractocephalus hemioliopterus*, since they are less cannibalistic during early life stages, readily accept compound diets, and exhibit faster growth rates than the *Pseudoplatystoma* spp. parent species (Hashimoto et al., 2012). However, the production of hybrids entails risks for the environment and the aquaculture industry. Hybrids have been frequently detected in natural environments and, in the case of the interspecific hybrids, are contaminating natural stocks due to their fertility (Hashimoto et al., 2013). Additionally, some genetic monitoring studies have revealed that the production, trade, and management of these hybrids are currently uncontrolled in Brazil, as broodstocks are often mistakenly composed of interspecific hybrids and even post-F1 hybrids, causing economic losses (Hashimoto et al., 2015). In this context, in order to achieve sustainability in *Pseudoplatystoma* spp. aquaculture, genetic improvement programs and culture techniques should be developed for pure species seeking to obtain similar performances as those of hybrids (Alves et al., 2014).

In order to increase basic knowledge on the molecular basis of the ontogeny of the digestive system of commercially important Amazonian fish species, the aim of this study was to understand the molecular phylogeny of the main digestive enzyme precursors and to analyze their ontogenetic expression pattern in *Pseudoplatystoma punctifer* (Castelnau, 1855). This is a carnivorous migratory catfish species widely distributed in the Amazon basin in Bolivia, Brazil, Colombia, Ecuador, Peru, and Venezuela (Buitrago-Suárez and Burr, 2007) with high potential for aquaculture diversification in the region. We previously analyzed the histological development of the digestive system (Gisbert et al., 2014) and the ontogeny of the digestive enzyme activity (Castro-Ruiz et al., 2019) of this species and in this study we focused on the molecular ontogeny. For that purpose, the digestive enzyme precursors of *α-amylase* (*amy*), *phospholipase A2* (*sPLA2-IB*), *lipoprotein lipase* (*lpl*), *trypsinogen* (*try*), *chymotrypsinogen* (*ctr*), and *pepsinogen* (*pga*) of this species were isolated, partially sequenced, and identified, with gene expression patterns characterized from 3 to 24 days post-fertilization (dpf).

2. Materials and methods

2.1. Fish rearing and feeding protocol

Pseudoplatystoma punctifer larvae were obtained by hormonal-induced spawning of a sexually mature couple of genitors (♀: 4.73 kg; ♂: 1.15 kg) maintained in captivity at the Instituto de Investigaciones de la Amazonia Peruana (IIAP, Iquitos, Peru). The female and male were injected intramuscularly with the synthetic hormone Conceptal® (Intervet, Huixquilucan, México) at 2.6 ml kg^{-1} and 1 ml kg^{-1} BW, respectively. Hormone injections were administered in two doses: a first one at 10% and 50% of the total dose, and a second one 12 h later at 90% and 50% of the total dose for female and male, respectively. After ovulation, the female was stripped and the obtained eggs (400 g, ca. 800,000 eggs) were distributed in batches of 100 g and gently mixed with 5 ml of physiological serum and 300 μl of previously collected sperm for 1 min. Then, 30 ml of distilled water was added with constant gentle stirring for another 1 min. Fertilized eggs were rinsed three times with 100 ml of water from the incubators and transferred thereafter to 60 l cylindroconical incubators connected to a recirculation water system at 28 °C. The larvae hatched 18 ± 2 h later (hatching rate = 84%) and were transferred at 3 dpf to three 30 l fiberglass tanks connected to a water recirculation system provided with mechanical and biological filters. The rearing conditions were as follows: temperature, 28.3 ± 0.4 °C; pH, 6.9 ± 0.2; dissolved oxygen, 8.2 ± 0.5 mg l^{-1}; NO_2^-, 0.04 ± 0.02 mg l^{-1}, NH_4^+, 0.14 ± 0.05 mg l^{-1}, and water flow rate of 0.2 l

min^{-1}. The larvae were reared in triplicate (initial density 90 larvae l^{-1}) under a photoperiod of 0 L:24 D and fed six times a day from 4 to 17 dpf with non-enriched *Artemia* spp. nauplii (37% proteins, 14% lipids, and 11% carbohydrates) in slight excess (0.4 to 17 nauplii ml^{-1}) considering the larval density, the weight increase of the larvae, and the daily food ration and weaned onto a commercial inert diet (BioMar®, Nersac, France; proximate composition: 58% proteins, 15% lipids, 20% carbohydrates, 11% ash; particle size: 0.5 mm) within 4 days. Once weaned, individuals were fed five times a day at 5% of the larval wet weight until the end of the experiment at 24 dpf.

In the absence of an ad hoc ethical committee at the IIAP where this trial was conducted, the animal experimental procedures were conducted in compliance with the Guidelines of the European Union Council (2010/63/EU) on the protection of animals used for scientific purposes.

2.2. Sampling and growth measurements

Whole larvae and early juveniles were collected at 3, 10, 13, 18, and 24 dpf and euthanized with an overdose of Eugenol (0.05 µl ml^{-1}; Moyco®, Moyco, Lima, Peru). The sampling criterion was based both on the developmental stage and the feeding protocol used (Fig. 1). Sampling was done in the morning before the first feeding of the day. For total length (TL) measurements, 15–30 individuals, depending on the size, were placed in a Petri dish, photographed using a scale bar and TL was measured on the pictures using ImageJ software (Schneider et al., 2012). For evaluating the expression of the digestive enzyme genes throughout development, 100 mg of pooled individuals (125 to 6 individuals, depending on size) at each sampling point were preserved in RNA*later* (1:10 dilution) at −20 °C until further analyses. Survival was evaluated by counting the individuals surviving at the end of each feeding period and considering the number of sampled individuals.

2.3. Partial mRNA isolation, identification and phylogenetic analysis

Total RNA was extracted using TRIzol™ (Invitrogen, San Diego, CA, USA) according to manufacturer's protocol. RNA concentration and quality were determined by spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain) measuring the absorbance at 260 and 280 nm and a denaturing electrophoresis in TAE agarose gel (1.5%). For the preparation of cDNA, total RNA was treated with DNAse I Amplification Grade (Invitrogen) according to manufacturer's protocol to remove genomic DNA traces. Total RNA was then reverse transcribed in 10 µl reaction volume containing 3 µg total RNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) with oligo (dT) $_{(12–18)}$ (0.5 µg/ul) and random hexamers primers (50 ng µl^{-1}), 10× RT buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCL) 25 mM $MgCl_2$, 0.1 M DTT, 10 mM dNTP mix, SuperScript™ II RT (50 U µl^{-1}), RNaseOUT™ (40 U µl^{-1}) followed by RNase H (2 U µl^{-1}) (Invitrogen) treatment. Reverse transcription reactions were carried out in a thermocycler (Mastercycle R nexus GSX1, Eppendorf AG, Hamburg, Germany) and run according to manufacturer's protocol. Samples were diluted 1:20 in molecular biology grade water and stored at −20 °C until further analyses. To obtain the specific sequences of *try*, *ctr*, *amy*, *lpl*, *sPLA2-IB*, *pga*, and Gliceraldehide-3-fosphate deshydrogenase (*gadph*) genes, alignments of teleost homologs for these genes' sequences obtained from GenBank were made using BioEdit Sequence Alignment Editor ver. 7.0.5.2 (Hall, 1999). Consensus primers designed from conserved regions identified in these alignments were used for amplification of *P. punctifer* specific gene sequences. The fragments amplified were separated in 2% agarose gel electrophoresis and resulting bands of the expected size were excised, isolated, purified (QIAQuick PCR purification kit, Qiagen, Hilden, Germany) and sequenced. The identity of each sequence was verified using the NCBI Blast analysis tool (www.ncbi.nlm.nih.gov/BLAST) and sequences were deposited in Genbank (Table 1).

For the phylogenetic analyses, protein sequences from different species coding for each gene were obtained at NCBI (Tables A1–6). Multiple protein alignments were performed with MAFFT (Katoh et al., 2002) and ambiguous regions were removed with Gblocks V0.91b (Talavera and Castresana, 2007). The phylogenetic trees were inferred using the maximum likelihood (ML) method implemented in the PhyML

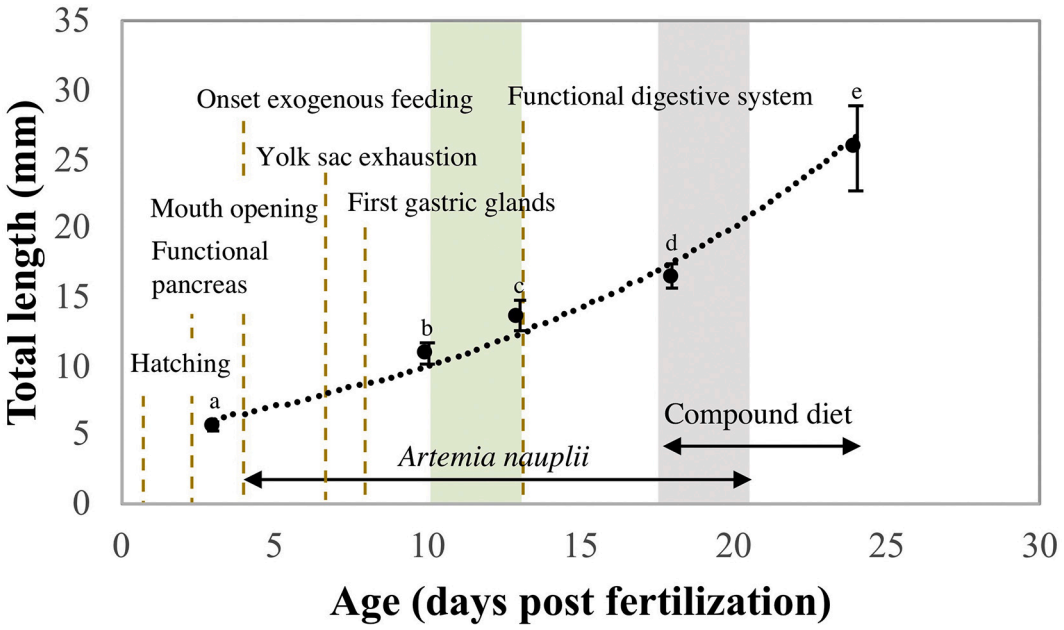

Fig. 1. Larval and early juvenile growth in total length (mm) of *P. punctifer* from 0 to 24 days post fertilization. Data are represented as means ± S.D. ($n = 45$). Values with a different letter denote significant differences during development (one-way ANOVA, $P < 0.05$).
The ochre dashed lines indicate some key events in the ontogeny of the digestive system. The green area indicates the transition period from the larval to the juvenile mode of digestion, and the gray area indicates the weaning period. The feeding protocol is indicated by horizontal arrows below the growth curve. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Accession numbers and oligonucleotide primers used for PCR and relative quantification of gene expression of six digestive enzymes during development of *P. punctifer*. *Gadph* was used as reference gene. The amplification efficiency of each gene was close to 100%.

Gene name	Genbank accession number	Primer	Nucleotide sequence (5′ – 3′)	Tm (°C)	Product size (bp)
Amy	MT006358	PpuAmy F	CAACAACGYTGGGGTCAACATC	56.7	300
		PpuAmy R	GTCRATCAGCTTGTTCADGAAG	54.8	
		qpAMYPf F	CATGTGGCCTGGAGATTTACAGGC	60.3	113
		qpAMYPf R	CCACCCAGATCAATAACCTCCTGG	58.9	
Try	MT006359	Tryp1 F2	TGTGTCTGCTGCTCACTGC	57.9	384
		Tryp1 R2	GTCACCCTGGCAAGAGTCC	57.2	
		qpTRYPf F	TATGACTCCTGGACCATTGACAATG	57.0	190
		qpTRYPf R	CAGACACTGCAGCTTGTTGCCATC	61.4	
Ctr	MT006344	LgChym2F	GCGGTGCCTCACTCCTGGCCC	64.1	250
		LgChym2R	AGSRSGATGTCGTTGTTGATGGTG	59.1	
		qpCHTPf F	TGCCCACTGCAATGTCAACACTTTC	61.0	100
		qpCHTPf R	ACCTTGGCAATCCTCATGACCTGG	61.2	
sPlA2-IB	MT006345	LgPhLipF	RRATGATCCTGTGYGTGATGCC	58.6	230
		LgPhLipR	CTCRCAGATGAACATCTCRCATTC	57.4	
		qpPLPPf F	ATGCAACATGATGCATGCTGGCC	61.6	100
		qpPLPPf R	GTTGTTGCTCTTGCAGGTGATTGTG	59.9	
Lpl	MT006346	LgLip-qF	AAGCTGGTGTCTGCCCTCTACG	61.0	250
		LgLip-qR	AGCCACATGTGCTCCCAGACTG	61.5	
		qpLLPPf F	TGCCAACGTCATAGTGGTGGACTG	59.1	130
		qpLLPPf R	GTAATCGAGTTCCATCATAAGCCAG	56.0	
Pga	MT006343	PEP 2F	GATGCTGACCTGTCCTACTA	52.8	600
		PEP 2R	TTGATGGTAACACTGTCCAT	51.3	
		qpPEPPf F	TGTCTACCTAAGCAGCAACTCTC	56.4	160
		qpPEPPf R	ATGACAGAGGGATCCAGACCAGAG	59.4	
Gadph	MT006341	GAPD2HF	TATCAATGGATTCGGCCGCA	56.9	500
		GAPD2HR	TGGCAGTGATGGCATGAACT	57.0	
		qpGAPDHPf F	GGTCTTGAGGGCCTGCCTGCAG	64.3	160
		qpGADPHPf R	CGATGAGCTTGCCATCCTCGTG	60.6	

program (v3.1/3.0 aLRT) (Guindon and Gascuel, 2003). Best model of evolution was selected using Modelgenerator V.85 (Keane et al., 2006) following the corrected Akaike Information Criterion (with four discrete gamma categories) and used to construct a phylogenetic tree. Bayesian posterior probabilities were computed with MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003). Two different runs with four incrementally heated simultaneous Monte Carlo Markov chains were conducted over one million generations. Trees were sampled every 100 generations to produce 10,000 trees. In order to estimate posterior probabilities, 25% of the trees were discarded as a burn-in stage, observing when average standard deviation of split frequency (ASDSF) values dropped below 0.01. Trees were generated using MEGA 10.1.7 software and robustness of the phylogeny assumption was evaluated by the bootstrapping procedure from 1000 data-set replicates and with posterior probabilities (PP). The nodes supported at or above the 50% level in the bootstrap analysis were emphasized.

2.4. Gene expression analyses of digestive enzymes during development

The transcript sequences were used as templates to design specific primers with the primer 3 software (Table 1). Quantitative PCR analyses for each gene were performed in triplicate in a 7300 Real-Time PCR System (Applied Biosystems, Roche, Barcelona, Spain). The amplification mix contained 1 μl cDNA, 0.5 μl primers (20 μM) and 10 μl SYBR Green Supermix (Life Technologies, Carlsbad, CA, USA) in a total volume of 20 μl. A negative control was included for each set of reactions on each 96-well plate. The amplification conditions were: 10 min at 95 °C, 40 cycles of 20 s at 95 °C and 1 min at 65 °C, followed by 15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C, and finally 15 s at 60 °C. A standard curve was obtained by amplification of a dilution series of cDNA to calculate the amplification efficiency (E) for each set of primers. Real-time PCR efficiencies were determined for each gene from the slopes obtained with Applied Biosystems software, applying the eq. $E = 10[-1/\text{slope}]$, where E is PCR efficiency. The relative gene expression ratio (R) for each gene was calculated according to Pfaffl's (2001) formula: $R = (E_{\text{target gene}})^{\Delta Cq \text{ target gene (mean sample}-\text{mean reference sample)}} / (E_{\text{reference gene}})^{\Delta Cq \text{ reference gene (mean sample} - \text{mean reference sample)}}$, where ΔCq is the deviation of the target sample minus the reference sample. The initial time point (3 dpf) was chosen as the reference sample and the relative gene expression was normalized using *gadph* as the reference gene since it did not exhibit any significant variation in expression between the samples.

2.5. Statistics

Results of gene expression were expressed as mean ± SD ($n = 9$). All data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett's test). One-Way ANOVA was performed to analyze differences in gene expression during development. All Pairwise Multiple Comparisons were performed using the Holm-Sidak method when significant differences were found at $P < 0.05$. Statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, VA, USA).

3. Results

3.1. Growth and survival

Growth during the ontogeny of *P. punctifer* followed an exponential curve TL (mm) = $4.181\ e^{0.23\times T}$ ($r^2 = 0.97$, $P < 0.05$) (Fig. 1). Survival rate was 95% and 49% at the end of the *Artemia* feeding period (17 dpf) and at the end of the experiment (24 dpf), respectively.

3.2. Sequences and phylogenetic analyses

The size of the partial nucleotide sequences isolated for *P. punctifer* is indicated in Table 1. Since the aim of this work was to analyze the expression patterns of these genes during ontogeny, obtaining the full-length cDNAs was not pursued.

3.2.1. Alpha amylase

Phylogenetic analysis of 14 teleost α-amylase protein sequences revealed a broad congruence among the results of the Maximum

Likelihood (ML) and Bayesian analyses (Fig. 2). Seven nodes received strong support in each (ML bootstrap supports between 67 and 100%, Bayesian posterior probabilities of 1.0), whereas 6 nodes received no support (ML bootstrap between 28 and 41%, Bayesian posterior probabilities between 0.6 and 0.8). The phylogenetic analysis revealed two main clades. The first one was composed of two clusters, one containing 4 species from the order Siluriformes (*Tachysurus fulvidraco*, *Bagarius yarelli*, *P. punctifer*, *Pangasianodon hypophthalmus*) and the other including two species from the order Cypriniformes (*Ctenopharyngodon idella*, *Labeo rohita*). The second clade was also divided in two other clusters. The first one was represented by Salmoniformes (*Salmo salar*) and the second one included different orders of Eupercaria represented by Tetraodontiformes (*Tetraodon nigroviridis*), Caragiformes (*Seriola lalandi dorsalis*), Perciformes (*Epinephelus coioides*, *Siniperca chuatsi*), Spariformes (*Sparus aurata*, *Pagrus pagrus*), and Pleuronectiformes (*Pseudopleuronectes americanus*). *Pseudoplatystoma punctifer* was grouped within the catfish (Siluriformes) clade (Bayesian = 1), where the analyzed sequences displayed the highest percentage of identity with *P. punctifer* that ranged between 85 and 87% (Table A1). The following groups with higher identity percentage with *P. punctifer* were species from the order Spariformes, Perciformes, Carangiformes and Pleuronectiformes (78–77%), and the lowest percentage of identity corresponded to species from the orders Cypriniformes, Perciformes and Tetraodontiformes (76%).

3.2.2. Phospholipase A2

Phylogenetic analysis of 18 teleost phospholipase A2 protein sequences revealed a broad consistency among the results of the ML bootstrap and Bayesian posterior analyses (Fig. 3). Four nodes received strong support in each (ML bootstrap supports between 85 and 96%, Bayesian posterior probabilities of 0.9–1.0), 4 nodes received medium support for ML bootstrap (50–70%) but high support by Bayesian posterior probabilities (0.92–0.99) and 6 nodes received no support (ML bootstrap support between 28 and 55%, Bayesian posterior probabilities between 0.6 and 0.9). The phylogenetic analysis revealed two main clades.

The first one was represented by *Oncorhynchus mykiss* (Salmoniformes) and the second one was composed of two clusters. One of them contained species from the orders Gobiiformes (*Parambassis ranga*), Cichliformes (*Oreochromis niloticus*, *Neolamprologus brichardi*), and Carangiformes (*Seriola dumerili*), and the other one was divided into two more clusters. The first cluster was represented by a species from the order Osteoglossiformes (*Scleropages formosus*) and the second included two clades: one was composed of species from the orders Siluriformes (*Ictalurus punctatus*, *P. hypophthalmus*, *P. punctifer*, *T. fulvidraco*), Gymnotiformes (*Electrophorus electricus*), and Characiformes (*Astyanax mexicanus*, *Colossoma macropomum*); and the other was composed of species belonging to the orders Cyprinodontoformes (*Kryptolebias marmoratus*, *Xiphophorus couchianus*), Beloniformes (*Oryzias melastigma*, *O. latipes*), and Esociformes (*Esox lucius*). The node of the clade Siluriformes was supported by the Bayesian posterior analysis but not by the ML bootstrap, and the only supported classification within the clade was that grouping *P. punctifer* and *T. fulvidraco* (Fig. 3). The phospholipase A2 of *P. punctifer* presented the highest percentage of identity with the sequences of the Siluriformes species (97%, Table A2). The following groups with higher identity percentage with *P. punctifer* corresponded to species from the order Characiformes and Gymnotiformes (89–92%), and the lowest percentage of identity corresponded to the species from the orders Cyprinodontiformes, Beloniformes, and Osteoglossiformes (66–69%, Table A2).

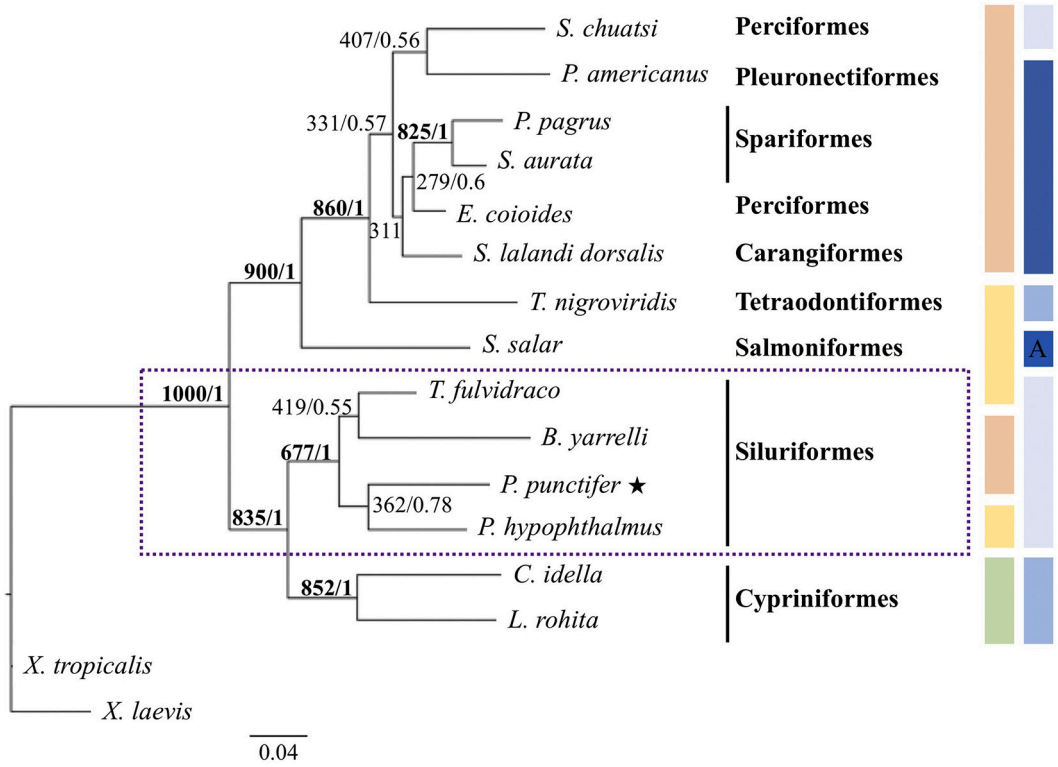

Fig. 2. Phylogenetic tree for α-amylase protein sequences from several teleosts, inferred from the Maximum Likelihood method (ML) and rooted on Anura species as outgroups. Numbers close to nodes display branch support values for ML (based on 1000 bootstrapping) followed by Bayesian posterior analysis. Full name of the species, GenBank accession numbers and identity percentage of the α-amylase sequences with respect to that of *P. punctifer* are shown in Table A1. The *amy* obtained in this study from *P. punctifer* is marked with a black star. The clade of the order Siluriformes is demarcated by a violet dotted rectangle. The two-colored columns on the right correspond to feeding habits and water environments of the analyzed species. Code of colors is as follows: salmon pink, carnivorous; yellow, omnivorous; green, herbivorous; dark blue, marine water; medium blue, freshwater/brackish; light blue, freshwater. A, anadromous. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

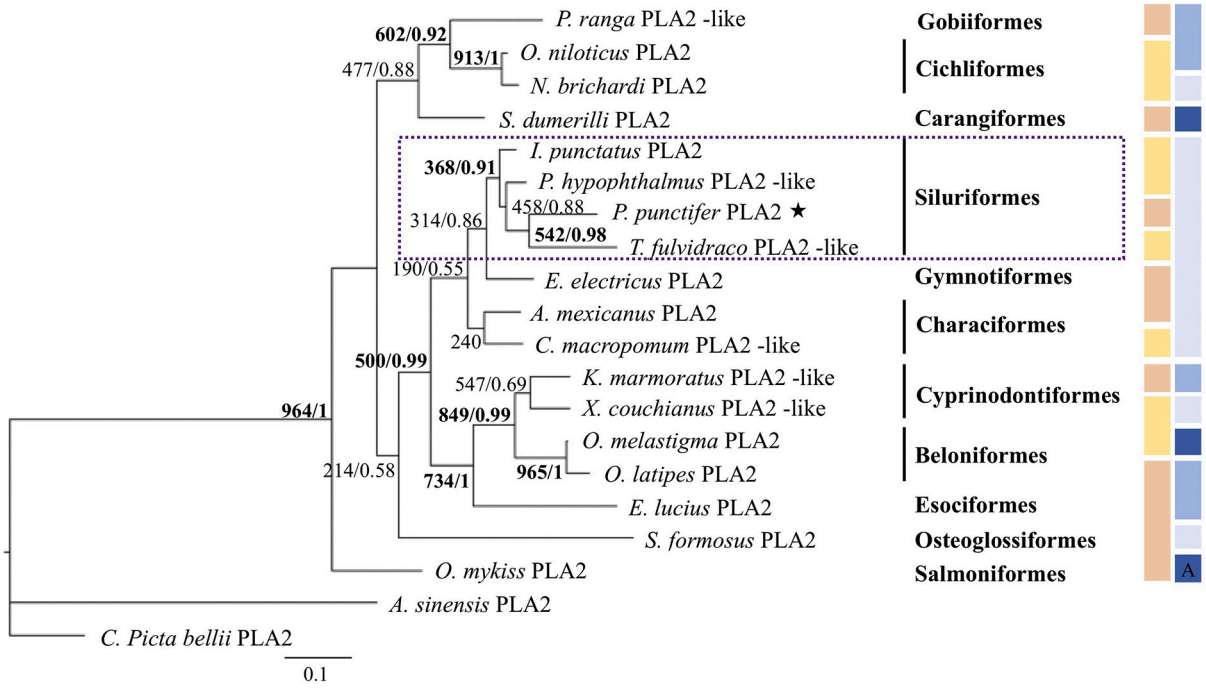

Fig. 3. Phylogenetic tree for phospholipase A2 (PLA2) protein sequences from several teleosts, inferred from the Maximum Likelihood method (ML) and rooted on Testudines and Crocodilia species as outgroups. Numbers close to nodes display branch support values for ML (based on 1000 bootstrapping) followed by Bayesian posterior analysis. Full name of the species, GenBank accession numbers and identity percentage of the PLA2 sequences with respect to that of *P. punctifer* are shown in Table A2. The *plA2* obtained in this study from *P. punctifer* (*sPLA2-IB)* is marked with a black star. The clade of the order Siluriformes is demarcated by a violet dotted rectangle. The two-colored columns on the right correspond to feeding habits and water environments of the analyzed species. Code of colors is as follows: salmon pink, carnivorous; yellow, omnivorous; dark blue, marine water; medium blue, freshwater/brackish; light blue, freshwater. A, anadromous. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2.3. Lipoprotein lipase

Phylogenetic analysis of 19 teleost lipoprotein lipase protein sequences revealed a very broad congruence among the results of the ML bootstrap and Bayesian posterior analyses (Fig. 4). Fourteen nodes received strong support in each (ML bootstrap supports between 77 and 100%, Bayesian posterior probabilities of 0.93–1.0), one node received medium support for ML bootstrap (59%) but good support by Bayesian posterior probabilities (0.93) and two nodes received support by bootstrap (60 and 64%) but not by Bayesian analysis (0.6 and 0.8). The phylogenetic analysis revealed two main clades. The first clade was composed of two clusters. The first one included species from the order Salmoniformes (*Oncorhynchus clarkia*, *O. mykiss*) and second one was represented by species from the orders Moroniformes (*Dicentrarchus labrax*), Pleuronectiformes (*Scophthalmus maximus*, *Paralichthys olivaceus*), Perciformes (*S. chuatsi*), and Spariformes (*Pagrus major*, *S. aurata*). The second clade was also divided into two clusters. One grouped species from the orders Characiformes (*A. mexicanus*), Gymnotiformes (*E. electricus*), and Siluriformes (*I. punctatus*, *P. hypophthalmus*, *P. punctifer*, *T. fulvidraco*), and the other was represented by several species of the order Cypriniformes (*C. idella*, *Danio rerio*, *Carassius auratus*, *Cyprinus carpio*). The node of the clade Siluriformes was supported by both the ML and the Bayesian posterior analyses (Fig. 4). The lipoprotein lipase sequence of *P. punctifer* showed the highest percentage of identity with the sequences of omnivorous species of the order Cypriniformes (91–89%), followed by species from the orders Salmoniformes, Characiformes, Siluriformes, and Cypriniformes (88%). The lowest percentage of identity corresponded to species from the orders Pleuronectiformes, Moroniformes, and Spariformes (82–79%, Table A3).

3.2.4. Trypsin

Phylogenetic analysis of 16 teleost trypsin protein sequences revealed three main clusters that were fully supported by the ML (100%) and Bayesian posterior analyses (1.0) (Fig. 5). A first cluster was composed of species represented by the orders Pleuronectiformes (*P. americanus*), Tetraodontiformes (*Takifugu flavidus*), Perciformes (*Gymnodraco acuticeps*), Salmoniformes (*Oncorhynchus nerka*, *Salmo trutta*), Esociformes (*E. lucius*), Gobiiformes (*P. ranga*), and Spariformes (*S. aurata*, *P. pagrus*). The only supported node of this clade was that grouping the Salmoniformes species (ML 65%, Bayesian 0.97) and the Spariformes species (ML 79%, Bayesian 0.99) together. A second cluster was represented by the orders Cypriniformes (*C. carpio*) and Clupeiformes (*Denticeps clupeoides*), and a third cluster grouped species from the order Siluriformes (*Clarias magur*, *I. punctatus*, *P. hypophthalmus, P. punctifer*, and *T. fulvidraco*). Regarding the latter, two clades were observed, one represented by *T. fulvidraco,* and the other composed by *C. magur* and another group of species including *I. punctatus*, *P. hypophthalmus*, and *P. punctifer*. The only supported nodes were those that grouped the last three species together (ML 59%, Bayesian 0.94; ML 33%, Bayesian 0.92). The sequence of *P. punctifer* showed the highest percentage of identity with that of the species of the order Siluriformes (86–82%) and also with *D. clupeoides* (84%). The lowest percentage of identity corresponded to species from the orders Pleuronectiformes and Spariformes (73%, Table A4).

3.2.5. Chymotrypsin

The phylogenetic tree of 14 teleost chymotrypsin protein sequences is shown in Fig. 6. The ML and Bayesian posterior analyses revealed 7 nodes that received strong support in each (ML 69–99%, Bayesian 1.0), one node only supported by the Bayesian posterior probabilities (0.97) and 5 nodes that were not supported (28–48%, 0.5–0.8). The phylogenetic analysis revealed two first clades that were fully supported. One of them was represented by the order Clupeiformes (*D. clupeoides*), whose sequences corresponded to the isoform B, and the other one grouped the isoform A of the sequences and was divided in two clusters. The first one

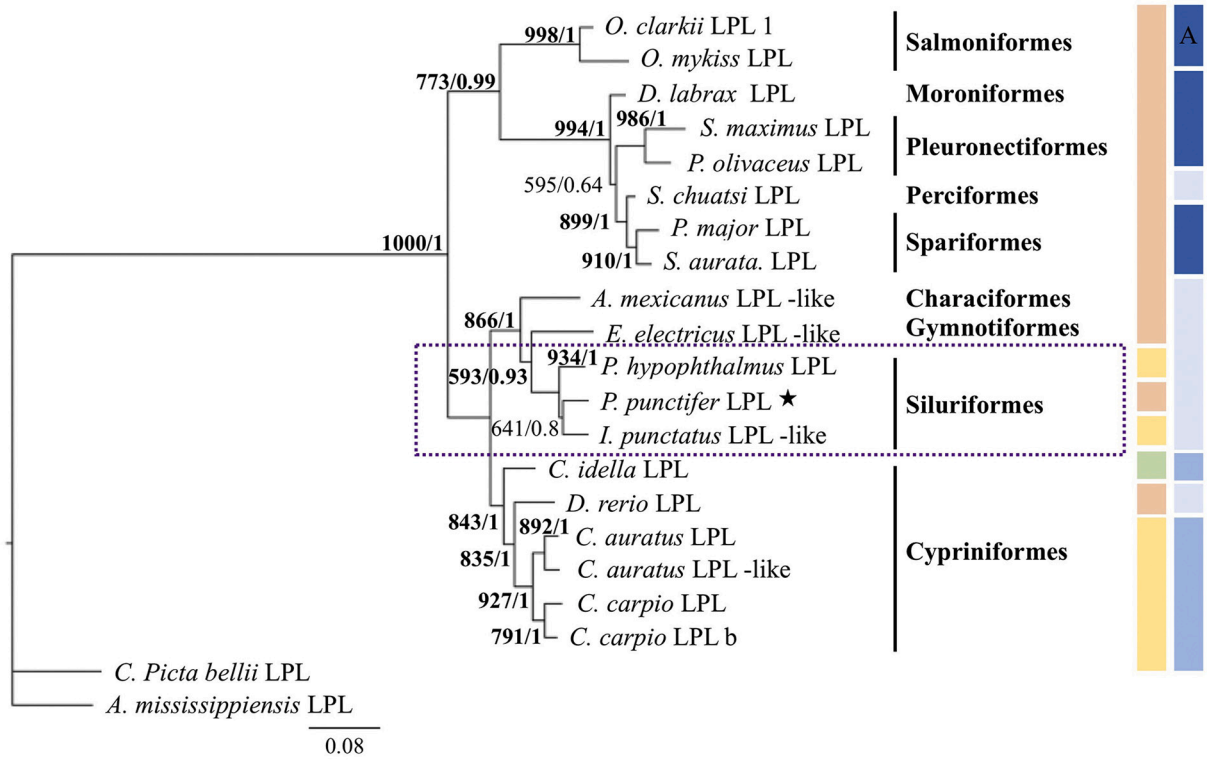

Fig. 4. Phylogenetic tree for lipoprotein lipase (LPL) protein sequences from several teleosts, inferred from the Maximum Likelihood method (ML) and rooted on Testudines and Crocodilia species as outgroups. Numbers close to nodes display branch support values for ML (based on 1000 bootstrapping) followed by Bayesian posterior analysis. Full name of the species, GenBank accession numbers and identity percentage of the LPL sequences with respect to that of *P. punctifer* are shown in Table A3. The *lpl* obtained in this study from *P. punctifer* is marked with a black star. The clade of the order Siluriformes is demarcated by a violet dotted rectangle. The two-colored columns on the right correspond to feeding habits and water environments of the analyzed species. Code of colors is as follows: salmon pink, carnivorous; yellow, omnivorous; dark blue, marine water; medium blue, freshwater/brackish; light blue, freshwater. A, anadromous. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

included species from the order of Siluriformes (*P. hypophthalmus*, *P. punctifer*) and the second one contained two more clusters: one included species from the order Cypriniformes (*D. rerio*, *C. auratus*, *L. rohita*) and the other was represented by species from the order Carangiformes (*S. lalandi dorsalis*), Perciformes (*Perca flavescens*, *Lutjanus guttatus*), Scombriformes (*Thunus orientalis*), and Clupeiformes (*Clupea harengus*). The node of the clade Siluriformes that grouped together *P. punctifer* and *P. hypophthalmus* was not supported (ML 47%, B 0.6) and their sequences displayed an 81% identity (Table A5). Similar percentage of identity as with the Siluriformes species was observed for species from the order Cypriniformes, Perciformes and Clupleiformes (81%), and the lowest percentage of identity corresponded to species from the order Cypriniformes and Carangiformes (74–76%, Table A5).

3.2.6. Pepsin

Phylogenetic analysis of 22 teleost pepsinogen protein sequences revealed a broad congruence among the results of the ML bootstrap and Bayesian posterior analyses (Fig. 7). Twelve nodes received strong support by both analyses (ML 73–100%, Bayesian 0.94–1.0), 3 nodes received medium or low support by ML (39–64%) but high support by Bayesian posterior analysis (0.94–0.99), and 6 nodes were not supported (ML 31–40%, Bayesian 0.58–0.86). The phylogenetic tree of pepsinogen revealed two first clusters. The first one was represented by one species from the order Osteoglossiformes (*S. formosus*) and the second was divided in two main clades. The first clade grouped together species from the order Spariformes (*P. pagrus*, *S. aurata*), Cichliformes (*Cichlasoma trimaculatum*, *O. niloticus*), Pleuronectiformes (*P. olivaceus*, *P. americanus*, *Solea senegalensis*), and Scombriformes (*T. orientalis*). The second clade was divided into two clusters, one of them was represented by species from the orders Siluriformes (*P. hypophthalmus*, *T. fulvidraco*, *Bagarius yarrelli*, *P. punctifer*) and Gymnotiformes (*E. electricus*), and the other one grouped together species from the orders Perciformes (*S. chuatsi*, *Siniperca scherzeri*, *Micropterus salmoides*), Pleuronectiformes (*P. americanus*), Scombriformes (*T. orientalis*), Gadiformes (*Gadus macrocephalus*), Esociformes (*E. lucius*), and Clupeiformes (*D. clupeoides*). According to the phylogenetic tree, the sequences of the species from the order of Siluriformes, including *P. punctifer*, corresponded to pepsinogen A1. The node of the clade Siluriformes was highly supported and grouped *P. punctifer* and *B. yarrelli* together (ML 89%, Bayesian 0.98). They displayed the highest identity percentage (100%) of all sequences analyzed, followed by *T. fulvidraco* and *P. hypophthalmus* (93%), and then by *E. electricus,* in accordance with the tree classification. The following groups with higher identity percentage with *P. punctifer* were the species from the order Clupeiformes and Perciformes (75%) and the lowest percentage of identity corresponded to the species from the order Osteoglossiformes (65%) and Chicliformes (64%) (Table A6).

3.3. Gene expression analyses of digestive enzymes during early stages of development

The expression profile of the analyzed digestive genes during the early development of *P. punctifer* is shown in Fig. 8. The expression of all genes was detected from 3 dpf (6 mm TL at 28 °C). The level of *amy* expression increased 8-fold from 3 dpf (0.17 ± 0.004 relative expression units, reu) to 10 dpf (11 mm TL at 28 °C) (1.4 ± 0.39 reu, $P < 0.05$), then decreased at 13 dpf (14 mm TL at 28 °C) (1.07 ± 0.133 reu, $P < 0.05$) and remained constant until 18 dpf (18 mm TL at 28 °C) (1.10 ± 0.003 reu, $P > 0.05$) to increase again 3-fold at 24 dpf (26 mm TL at 28 °C) (2.76 ± 0.05 reu, $P < 0.05$). *Try* expression increased from 3 dpf (3.15 ± 0.22 reu) to 10 dpf (5.12 ± 0.43 reu, $P < 0.05$), then decreased 2.5-fold

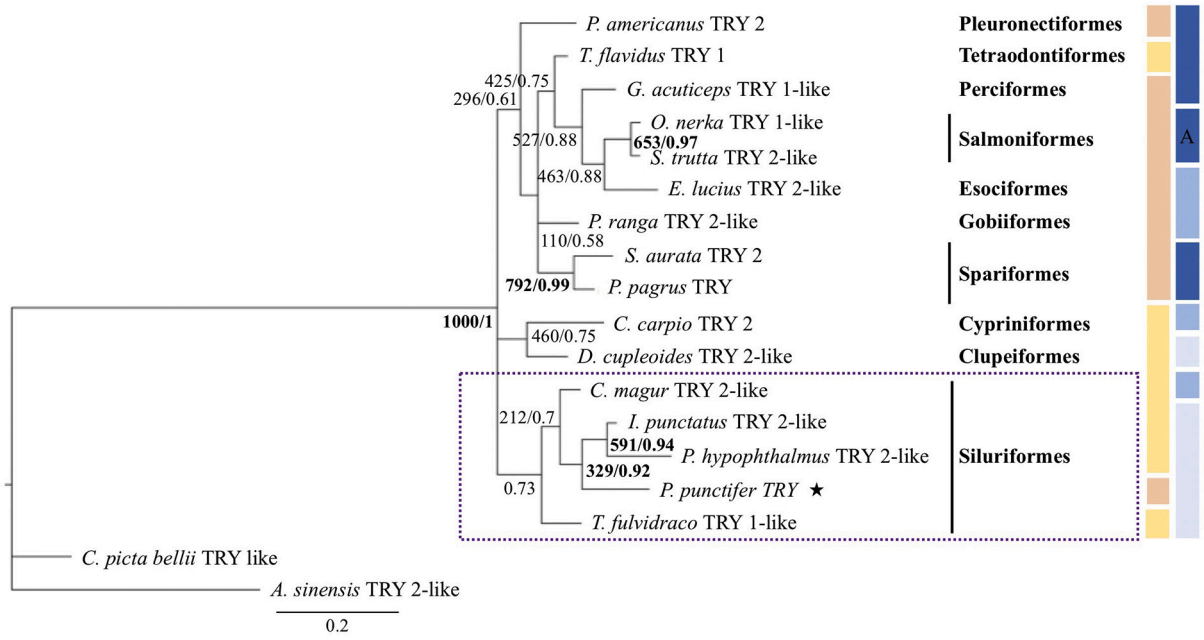

Fig. 5. Phylogenetic tree for trypsin (TRY) protein sequences from several teleosts, inferred from the Maximum Likelihood method (ML) and rooted on Testudines and Crocodilia species as outgroups. Numbers close to nodes display branch support values for ML (based on 1000 bootstrapping) followed by Bayesian posterior analysis. Full name of the species, GenBank accession numbers and identity percentage of the TRY sequences with respect to that of *P. punctifer* are shown in Table A4. The *try* obtained in this study from *P. punctifer* is marked with a black star. The clade of the order Siluriformes is demarcated by a violet dotted rectangle. The two-colored columns on the right correspond to feeding habits and water environments of the analyzed species. Code of colors is as follows: salmon pink, carnivorous; yellow, omnivorous; dark blue, marine water; medium blue, freshwater/brackish; light blue, freshwater. A, anadromous. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

at 13 dpf (2.05 ± 0.07 reu, $P < 0.05$) and progressively increased until the end of the trial (6.65 ± 0.53 reu; $P < 0.05$). *Ctr* expression increased 6-fold from 3 dpf (0.14 ± 0.02 reu) to 10 dpf (0.88 ± 0.14 reu, $P < 0.05$) to abruptly decrease at 13 dpf (0.24 ± 0.04 reu, $P < 0.05$). *Ctr* expression continued to decrease until 18 dpf (0.04 ± 0.0003 reu, $P < 0.05$) to remain constant thereafter (0.02 ± 0.005 reu, $P > 0.05$). The level of *pga* expression significantly increased from 10 dpf (0.71 ± 0.11 reu) to 13 dpf (237.23 ± 59.06 reu, $P < 0.05$) and continued to increase in an exponential manner until the end the trial (1496.7 ± 619.9 reu, $P < 0.05$). The *sPLA2-IB* expression significantly increased from 3 dpf (0.23 ± 0.02 reu) to 10 dpf (0.94 ± 0.15 reu, $P < 0.05$), and progressively decreased until 18 dpf (0.55 ± 0.24 reu, $P < 0.05$) to remain invariable until the end of the study (0.84 ± 0.08 reu, $P > 0.05$). The level of *lpl* expression significantly increased from 3 dpf (0.03 ± 0.01 reu) to 10 dpf (0.15 ± 0.004 reu, $P < 0.05$), remained constant until 13 dpf (0.17 ± 0.05 reu, $P > 0.05$), and increased again from 13 to 18 dpf (0.33 ± 0.12 reu, $P < 0.05$) to remain constant until the end of the study (0.37 ± 0.23 reu, $P > 0.05$).

4. Discussion

This study provides the first comprehensive analysis of the transcriptional ontogeny of some of the most important digestive enzymes of an Amazonian fish species of the genus *Pseudoplatystoma* and gives insights into the molecular phylogeny of the digestive enzymes and the development of the digestive capacities and feeding preferences during the early life stage of *P. punctifer*.

Overall, the phylogenetic relationships of the protein sequences of the studied digestive genes of *P. punctifer* corresponded to the phylogenetic classification of bony fishes (Betancur-R et al., 2017). As expected, the protein sequences of *P. punctifer* were clustered together with those of other catfish species in all the phylogenetic trees, and were mostly grouped with the protein sequences of species belonging to the primarily freshwater clade of the Otophysi (Siluriformes, Cypriniformes, Characiformes, and Gymnotiformes) (Betancur-R et al., 2017; Nelson et al., 2016), except in those trees with a higher number of less supported nodes (*try*, *ctr*). Similarly, the protein sequences of the species contained in the Superorder Acanthopterygii (Beloniformes, Carangiformes, Cichliformes, Cyprinodontiformes, Gobiiformes, Moroniformes, Perciformes, Pleuronectiformes, Scombriformes, Spariformes, Tetraodontiformes (Betancur-R et al., 2017; Nelson et al., 2016)) were grouped together in most trees. In addition, most of the studied protein sequences of *P. punctifer* (*amy*, *plA2*, *try*, and *pga*) also showed maximum homology with those of other catfish species, with the exception of *lpl* and *ctr*, probably due to the use of partial sequences and/or problems in the annotation of the isoforms. Nevertheless, the phylogenetic relationships allowed identifying some isoforms of the protein sequences. The *ctr* and *pga* sequences isolated from *P. punctifer* were located in clades with sequences of the isoforms *ctr*A and *pga*A1, respectively. In contrast, it was difficult to identify the isoform of *try* as the sequences of the other catfish species were all predicted coding sequences.

The expression of the different genes analyzed in *P. punctifer* was detected before the onset of exogenous feeding, denoting that it is a genetically programmed process. Similar results have been found in other reared fish species (Cahu et al., 2004; Darias et al., 2006; Darias et al., 2007a, 2007b; Galaviz et al., 2015; Mata-Sotres et al., 2016; Péres et al., 1998; Zambonino Infante et al., 2008).

In fish, the stomach is one of the last digestive organs to develop during ontogeny. The onset of the acidic digestion shows the switch from the larval to the juvenile mode of digestion, characterized by a notable improvement of the digestion of complex proteins. This moment is usually considered the transition from the larval to the juvenile stage from a digestive physiology perspective and a suitable moment for weaning onto compound feeds under culture conditions (Lazo et al., 2011; Rønnestad et al., 2013; Zambonino Infante et al., 2008). The development of the stomach is generally determined by the appearance of gastric glands, while stomach functionality is characterized by the expression of pepsinogen and proton pump (H^+/K^+-ATPase) genes in

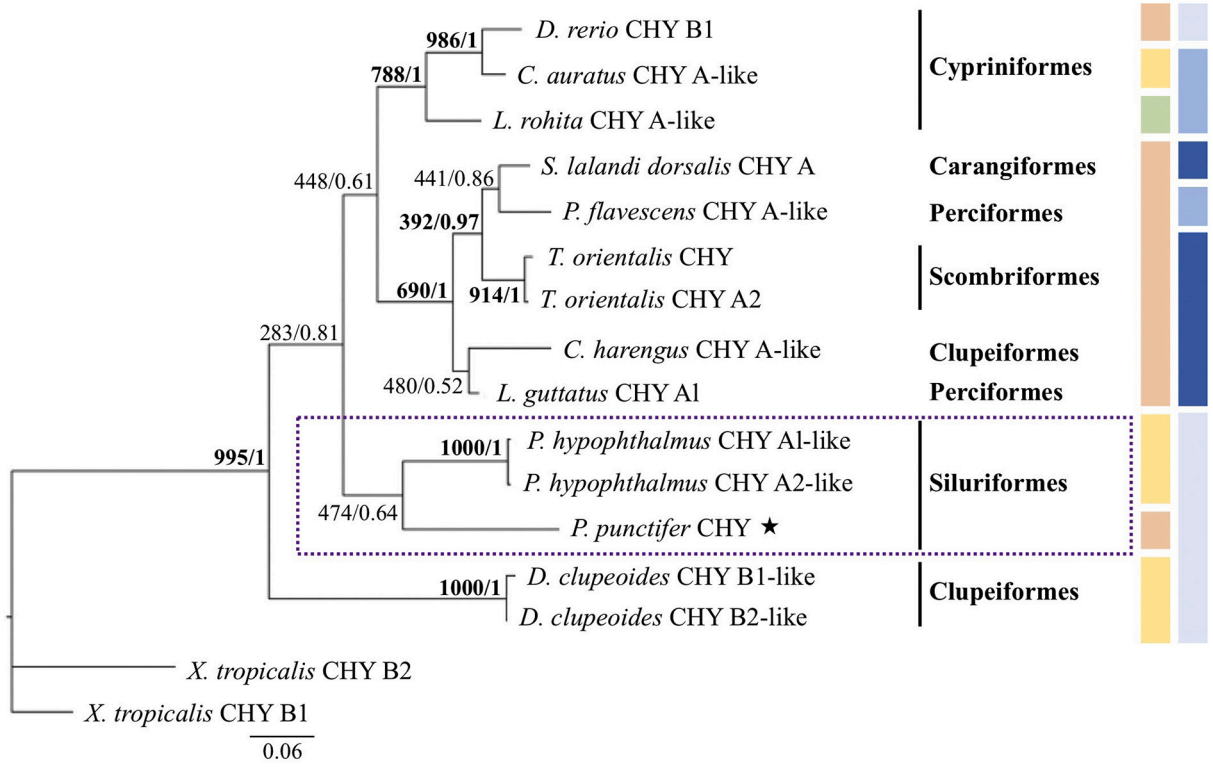

Fig. 6. Phylogenetic tree for chymotrypsin (CTR) protein sequences from several teleosts, inferred from the Maximum Likelihood method (ML) and rooted on Anura species as outgroups. Numbers close to nodes display branch support values for ML (based on 1000 bootstrapping) followed by Bayesian posterior analysis. Full name of the species, GenBank accession numbers and identity percentage of the CTR sequences with respect to that of *P. punctifer* are shown in Table A5. The *ctr* obtained in this study from *P. punctifer* is marked with a black star. The clade of the order Siluriformes is demarcated by a violet dotted rectangle. The two-colored columns on the right correspond to feeding habits and water environments of the analyzed species. Code of colors is as follows: salmon pink, carnivorous; yellow, omnivorous; green, herbivorous; dark blue, marine water; medium blue, freshwater/brackish; light blue, freshwater. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the gastric glands, which are responsible for the production of pepsin and hydrochloric acid, respectively (Darias et al., 2005; Darias et al., 2007a, 2007c; Douglas et al., 1999; Gawlicka et al., 2001). However, the onset of acidic digestion may or may not be synchronized with the morphological development of the gastric glands. For instance, stomach functionality has shown to be delayed by 67 degree days (DD) in haddock, *Melanogrammus aeglefinus* (Perez-Casanova et al., 2006), 147 DD in summer flounder, *Paralichthys dentatus* (Huang et al., 1998), 154 DD in Atlantic halibut, *Hippoglossus hippoglossus* (Murray et al., 2006), 195 DD in red porgy, *Pagrus pagrus* (Darias et al., 2005), or 233 DD in Atlantic cod, *Gadus morhua* (Perez-Casanova et al., 2006). In *P. punctifer*, the increase of *pga* expression observed in *P. punctifer* between 10 and 13 dpf was synchronized with the appearance of the gastric glands (Gisbert et al., 2014)—as has also been observed in other fish species, such as in winter flounder, *Pleuronectes americanus* (Douglas et al., 1999), orange-spotted grouper, *Epinephelus coioides* (Feng et al., 2008), spotted rose snapper, *Lutjanus guttatus* (Galaviz et al., 2012), or Sobaity sea bream, *Sparidentex hasta* (Nazemroaya et al., 2020)—and indicated the achievement of the functional maturation of the gastric glands, and hence marked the transition from the larval to the juvenile stage. These results coincide with those we observed at enzymatic activity levels in this species (Castro-Ruiz et al., 2019). The level of *pga* expression generally increases with development and larval growth, which is associated with the increasing number of gastric glands in the stomach (Darias et al., 2005; Darias et al., 2007c; Galaviz et al., 2012; Gao et al., 2013; Mir et al., 2018; Moguel-Hernández et al., 2016; Murray et al., 2006), leading to full acidification capacity and hence a more efficient protein digestion that can be attained several weeks after the onset of acidic digestion (Darias et al., 2005; Hoehne-Reitan et al., 2001; Yúfera et al., 2004). In *P. punctifer*, the exponential increase of *pga* expression after 13 dpf (14 mm TL) is in accordance with the greater development both in number and size of the gastric glands of the stomach observed histologically (Gisbert et al., 2014).

Although the expression profile of *pga* observed in *P. punctifer* is common to carnivorous fish, *amy* expression did not follow the usual ontogenetic pattern observed in carnivorous species, characterized by an initial high level of expression that gradually decreases until the end of the larval stage (Cahu et al., 2004; Darias et al., 2006; Galaviz et al., 2015; Moguel-Hernández et al., 2016; Péres et al., 1998; Srichanun et al., 2013; Zambonino Infante and Cahu, 1994). Instead, *amy* expression in *P. punctifer* gradually increased until the end of the larval development (10 dpf, 11 mm TL) and remained relatively high afterwards. The capacity to synthesize α-amylase at larval stages is not only considered an indicator of the maturation of the exocrine pancreas (Cahu et al., 2004; Cahu and Zambonino Infante, 1994), but is also related to feeding habits (Kuz'mina, 1996). In particular, it has been reported that α-amylase activity is higher in omnivorous than in carnivorous fish (Fernández et al., 2001; Hidalgo et al., 1999; Kim et al., 2014). An increasing pattern of *amy* expression during development has also been observed in the omnivorous thick lipped gray mullet, *Chelon labrosus* (Zouiten et al., 2008), and it has been suggested that constitutive expression of *amy* may represent a true dietary specialization for herbivory and omnivory in prickleback fishes (Kim et al., 2014). The feeding habits of *P. punctifer* larvae and early juveniles in the wild are unknown; however, considering its *amy* expression profile, carbohydrates are probably important in their larval diet. During this developmental period, it seems plausible that this species displays an omnivorous feeding behavior with preference to carnivory and that

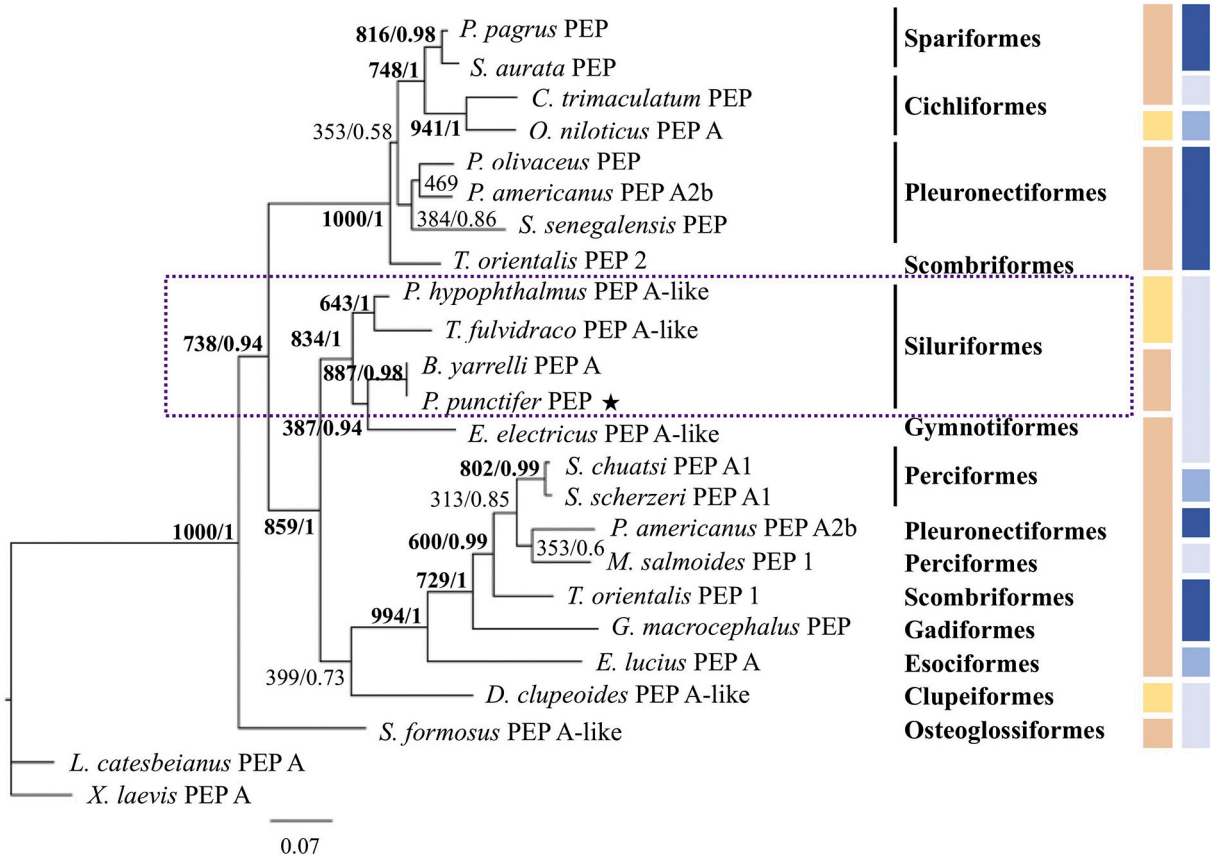

Fig. 7. Phylogenetic tree for pepsin (PGA) protein sequences from several teleosts, inferred from the Maximum Likelihood method (ML) and rooted on two Anura species as outgroups. Numbers close to nodes display branch support values for ML (based on 1000 bootstrapping) followed by Bayesian posterior analysis. Full name of the species, GenBank accession numbers and identity percentage of the PGA sequences with respect to that of *P. punctifer* are shown in Table A6. The *pga* obtained in this study from *P. punctifer* is marked with a black star. The clade of the order Siluriformes is demarcated by a violet dotted rectangle. The two-colored columns on the right correspond to feeding habits and water environments of the analyzed species. Code of colors is as follows: salmon pink, carnivorous; yellow, omnivorous; dark blue, marine water; medium blue, freshwater/brackish; light blue, freshwater. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phytoplankton and invertebrates may be a significant component of the diet. Although *P. punctifer* also presents histological and biochemical digestive characteristics of a carnivorous species (Castro-Ruiz et al., 2019; Gisbert et al., 2014), this species has the ability to produce high levels of amylase throughout the onset of acidic digestion, as has been observed later on during the early juvenile stage (Castro-Ruiz et al., 2021). Similar findings were reported in *Odax pullus*, an herbivorous fish that consumes starch-rich red algae as a juvenile, but not much starch as an adult, and yet presents an elevated amylase activity in adulthood (Skea et al., 2005). Between 18 and 24 dpf (18 and 26 mm TL, respectively), *P. punctifer* showed a drastic increase in *amy* expression coinciding with the weaning period, in order to adapt the enzymatic activity to the carbohydrate content (11% in *Artemia* vs. 20% in compound diet) of the new diet supplied. This increase in *amy* expression resulted in an increase of the activity of its enzyme (Castro-Ruiz et al., 2019), showing that the dietary modulation of the α-amylase activity occurs at a transcriptional level during the juvenile stage in this species, as has been observed in other fish species such as European sea bass, *Dicentrarchus labrax* (Péres et al., 1998). The ability of *P. punctifer* to digest carbohydrates during the larval stage could represent an advantage from an industrial perspective since this would permit the formulation of larval feeds with lower protein content if the total replacement of live prey by compound diets is achieved for this species (Darias et al., 2015). However, an increase in gene expression does not necessarily mean that the fish is adequately using this source of energy. Indeed, a recent study performed on the nutritional needs of *P. punctifer* during the early juvenile stage (from 13 to 26 dpf) showed that a dietary carbohydrate content higher than 25% induced the production of α-amylase at higher levels than diets with lower carbohydrate content, but reduced growth performance and induced fatty livers (Castro-Ruiz et al., 2021). Therefore, results of digestive enzymes gene expression and activity should be considered along with additional physiological responses, including nutrient interaction.

The importance of phospholipids in fish larval nutrition is widely recognized (reviewed in Cahu et al., 2009). The secretory sPLA2-IB does not discriminate fatty acid species and needs bile acid for full enzymatic activity in the intestinal lumen (Murakami et al., 2015). As with α-amylase, an increase in *sPLA2-IB* gene expression during larval development is an indicator of the maturation of the exocrine pancreas (Cahu et al., 2003). Similarly to our results, an increase in sPLA2 gene expression and/or activity during development has also been observed in other fish species such as in turbot, *Scophthalmus maximus* (Hoehne-Reitan et al., 2003), Atlantic cod, *Gadus morhua* (Sæle et al., 2011), spotted rose snapper (Moguel-Hernández et al., 2016), gilthead seabream, *Sparus aurata* (Mata-Sotres et al., 2016), large yellow croaker, *Larimichthys crocea* (Cai et al., 2017), and California halibut, *Paralichthys californicus* (Fuentes-Quesada and Lazo, 2018). Moreover, phospholipid content can also affect the maturation of both the pancreas and the intestine, and the regulation of PLA2 activity mainly occurs at the transcriptional level (Cahu et al., 2003). For instance, the regulation of *sPLA2-IB* expression by the exogenous diet has been observed in large yellow croaker (Cai et al., 2017). The fact that the *PLA2-IB* gene

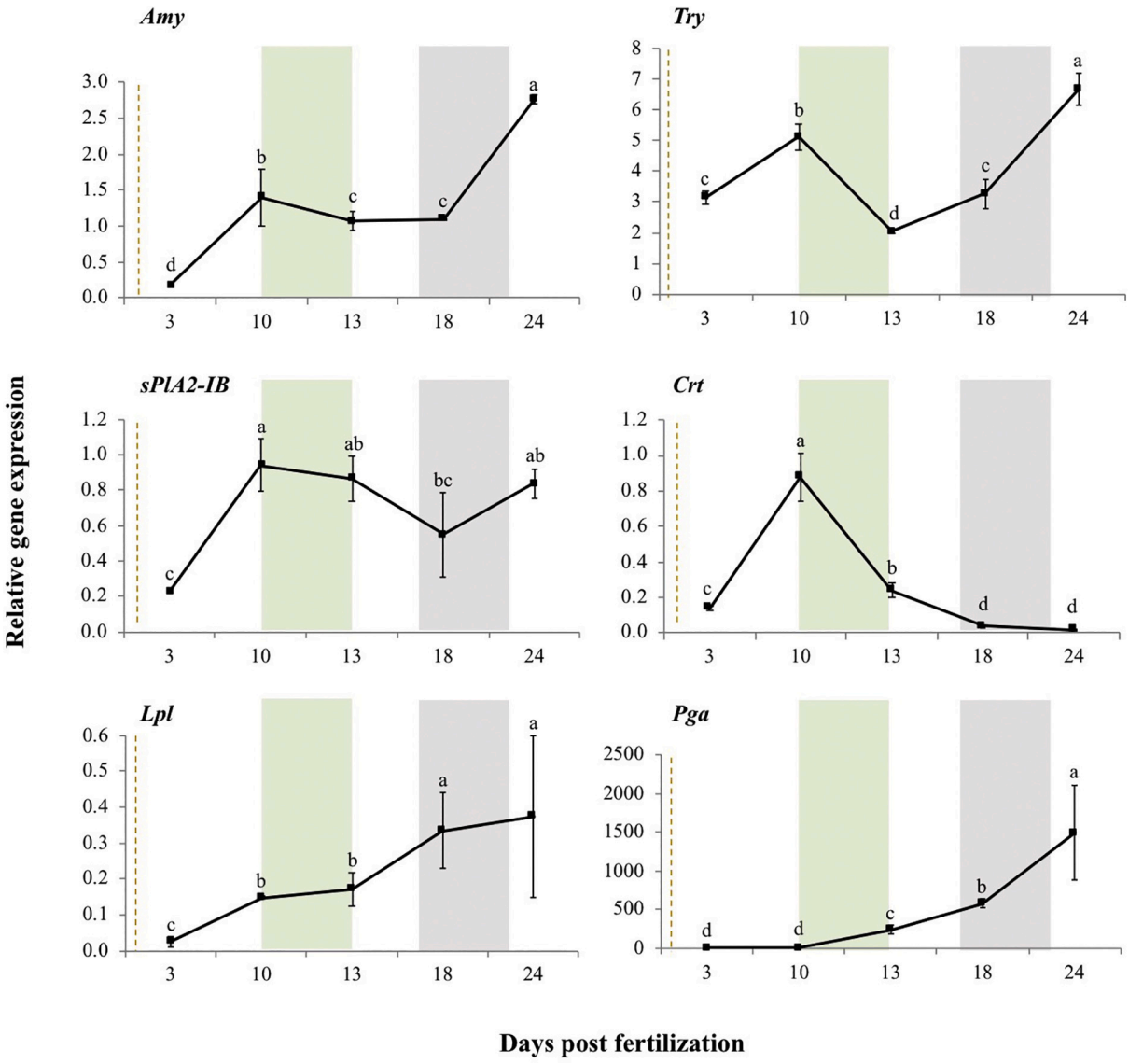

Fig. 8. Relative expression of α-amylase (*amy*), phospholipase A2 (*sPlA2-IB*), lipoprotein lipase (*lpl*), trypsinogen (*try*), chymotrypsin (*ctr*), and pepsinogen (*pga*) genes during the development of *P. punctifer* reared at 28 °C. The ochre dashed line indicates hatching (*ca.* 18 h post fertilization). The green area indicates the transition period from the larval to the juvenile mode of digestion, and the gray area indicates the weaning period. Data are represented as means ± S.D. ($n = 9$). Values with a different letter denote significant differences during development (one-way ANOVA, $P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expression in *P. punctifer* remained high and constant after the onset of acidic digestion indicates that the expression levels were adequate to digest the phospholipids present in the compound diet supplied at weaning. The level of *sPLA2-IB* expression could be also considered an indicator of the nutritional condition of fish, as a down-regulation of the expression of *sPLA2* has been observed in fasted specimens (Benedito-Palos et al., 2014).

In the present study, *lpl* gene expression increased between 3 and 10 dpf (6 and 11 mm TL, respectively), which coincides with the complete maturation of the intestine (Castro-Ruiz et al., 2019; Gisbert et al., 2014), and continued to increase thereafter in response to the switch of diet at weaning. A transcriptional regulation of this gene by the diet has also been found in other fish species such as orange-spotted grouper (Li et al., 2016), spotted rose snapper (Moguel-Hernández et al., 2016), and large yellow croaker (Cai et al., 2017).

Try expression in *P. punctifer* followed an expression profile that has been observed in several fish species, which is characterized by an increase of its expression until the complete formation and functionality of the stomach (Darias et al., 2007b; García-Gasca et al., 2006; Kortner et al., 2011; Kurokawa et al., 2002; Mir et al., 2018; Murray et al., 2004; Parma et al., 2013; Péres et al., 1998; Srichanun et al., 2013; Srivastava et al., 2002). Similar to what has been reported for several fish species (Darias et al., 2005; Darias et al., 2007b; Galaviz et al., 2011; Mir et al., 2018; Suzer et al., 2006), *try* expression in *P. punctifer* decreased from 10 dpf (11 mm TL) concomitant with the increase of *pga* expression, indicating the change from the basic and less efficient digestion of proteins in the anterior intestine to the acidic digestion in the stomach that allows a more efficient digestion of complex proteins from this point onward. However, this pattern can be modulated by the nutritional composition of the diet, as the present study showed at weaning, where both *try* and *pga* expressions increased. This is not surprising considering that both enzymes work together: pepsin makes a first hydrolysis of protein molecules by selective cleavage at Phe, Tyr, and other aromatic amino acids, and trypsin completes the process acting on Lys and Arg residues. As occurred with *amy*, the expression profile of *try* coincided with that of the trypsin activity (Castro-Ruiz et al., 2019), showing that the switch

from *Artemia* to the compound diet induced a modulation of the activity of this enzyme at the transcriptional level, as has also been observed in other species (Péres et al., 1998).

Ctr followed a similar expression profile to *try* during the larval stage of *P. punctifer*, denoting the complementary action of their corresponding enzymes trypsin and chymotrypsin until the onset of acidic digestion (Rønnestad et al., 2013). However, the opposite pattern observed after 13 dpf (decrease in *ctr* expression) did not coincide with the increase in the activity of chymotrypsin (Castro-Ruiz et al., 2019), which would indicate that the activity of this enzyme was regulated at a post-transcriptional level during the juvenile stage in response to the dietary change. Alternatively, this difference in the expression profile between *try* and *ctr* could be related to the role of trypsin in the activation of chymotrypsin. Indeed, knowledge about the chymotrypsin dynamics and the importance of this enzyme in the digestive processes during the early life stages of fish is still limited (Rønnestad et al., 2013).

5. Conclusions

The gene expression of the digestive enzymes analyzed during the development of *P. punctifer* followed the typical profile of a carnivorous species with the exception of *amy*, which increased during development. Based on this, it is suggested that *P. punctifer* displays an omnivorous feeding behavior with a preference towards carnivory during the early life stage. The gene expression results, together with those previously obtained at the protein activity level (Castro-Ruiz et al., 2019), showed that the enzymatic machinery of *P. punctifer* is completely prepared before the onset of exogenous feeding at 4 dpf (6 mm TL) and reaches its maturity between 10 and 13 dpf (11–14 mm TL), when individuals present a digestive system with the adult mode of digestion. This indicates, from a digestive physiology perspective, the transition from the larval to the juvenile stage, and the suitable moment for weaning under culture conditions. Overall, these results contribute to the understanding of the molecular basis of the ontogeny of the digestive system of a commercially important Amazonian fish species and to the development of feeding strategies for fish species of interest for aquaculture diversification in the region.

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Declaration of Competing Interest

None.

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Appendix A. Appendix

Table A1
Sequence ID of the teleost species used for the phylogenetic analysis of α-amylase. Information on the feeding habits of the species as well as on the percentage of identity at amino acid level among *amy* sequences of *P. punctifer* and different species is also provided. Species from the order Anura were used as outgroups.

Sequences ID	Species	Family	Order	Feeding habit	Identity (%)
AAX37668.1	*Tachysurus fulvidraco*	Bagridae	Siluriformes	Omnivorous	86.82
XP_026767155.1	*Pangasianodon hypophthalmus*	Pangasiidae	Siluriformes	Omnivorous	86.13
TST47702.1	*Bagarius yarrellii*	Sisoridae	Siluriformes	Carnivorous	84.54
ALB35087.1	*Sparus aurata*	Sparidae	Spariformes	Carnivorous	78.32
ACJ26844.1	*Epinephelus coioides*	Serranidae	Perciformes	Carnivorous	77.73
AAU93830.1	*Pagrus pagrus*	Sparidae	Spariformes	Carnivorous	77.53
XP_023263181.1	*Seriola lalandi dorsalis*	Carangidae	Carangiformes	Carnivorous	77.34
AAF65827.1	*Pseudopleuronectes americanus*	Pleuronectidae	Pleuronectiformes	Carnivorous	76.95
ACX35465.1	*Ctenopharyngodon idella*	Cyprinidae	Cypriniformes	Herbivorous	76.56
ACJ06746.1	*Siniperca chuatsi*	Sinipercidae	Perciformes	Carnivorous	76.37
AHY00275.1	*Labeo rohita*	Cyprinidae	Cypriniformes	Herbivorous	76.03
CAD20312.1	*Tetraodon nigroviridis*	Tetraodontidae	Tetraodontiformes	Omnivorous	75.83
XP_002938902.1	*Xenopus tropicalis*	Pipidae	Anura	Omnivorous	75.59
AAL87102.1	*Xenopus laevis*	Pipidae	Anura	Omnivorous	74.46
ABD13895.1	*Salmo salar*	Salmonidae	Salmoniformes	Carnivorous	72.07

Table A2
Sequence ID of the teleost species used for the phylogenetic analysis of phospholipase A2. Information on the feeding habits of the species as well as on the percentage of identity at amino acid level among *sPLA2-IB* sequences of *P. punctifer* and different species is also provided. Species from the orders Testudines and Crocodilia were used as outgroups.

Sequences ID	Species	Family	Order	Feeding habit	Identity (%)
XP_017345520	*Ictalurus punctatus*	Ictaluridae	Siluriformes	Omnivorous	97.22
XP_026765864	*Pangasianodon hypophthalmus*	Pangasiidae	Siluriformes	Omnivorous	97.22
XP_036451599	*Colossoma macropomum*	Serrasalmidae	Characiformes	Omnivorous	91.67
XP_026852122	*Electrophorus electricus*	Gymnotidae	Gymnotiformes	Carnivorous	91.43
XP_007241794	*Astyanax mexicanus*	Characidae	Characiformes	Carnivorous	88.89
XP_026988397.1	*Tachysurus fulvidraco*	Bagridae	Siluriformes	Omnivorous	86.11
XP_022609172.1	*Seriola dumerili*	Carangidae	Carangiformes	Carnivorous	80.56
XP_028270170.1	*Parambassis ranga*	Ambassidae	Gobiiformes	Carnivorous	75.00

(*continued on next page*)

Table A2 (*continued*)

Sequences ID	Species	Family	Order	Feeding habit	Identity (%)
XP_003445720.1	*Oreochromis niloticus*	Cichlidae	Cichliformes	Omnivorous	72.22
XP_006805482.1	*Neolamprologus brichardi*	Cichlidae	Cichliformes	Omnivorous	72.22
XP_010875989.1	*Esox lucius*	Esocidae	Esociformes	Carnivorous	72.22
XP_021461418.1	*Oncorhynchus mykiss*	Salmonidae	Salmoniformes	Carnivorous	72.22
XP_017280986.1	*Kryptolebias marmoratus*	Rivulidae	Cyprinodontiformes	Carnivorous	69.44
XP_024155431.1	*Oryzias melastigma*	Adrianichthyidae	Beloniformes	Omnivorous	69.44
XP_023816872.1	*Oryzias latipes*	Adrianichthyidae	Beloniformes	Carnivorous	69.44
XP_027881920.1	*Xiphophorus couchianus*	Poeciliidae	Cyprinodontiformes	Omnivorous	66.67
XP_018587154.2	*Scleropages formosus*	Osteoglossidae	Osteoglossiformes	Carnivorous	65.71
XP_005288773.1	*Chrysemys picta bellii*	Emydidae	Testudines		56.25
XP_014373187.1	*Alligator sinensis*	Alligatoridae	Crocodilia		45.16

Table A3
Sequence ID of the teleost species used for the phylogenetic analysis of lipoprotein lipase. Information on the feeding habits of the species as well as on the percentage of identity at amino acid level among *lpl* sequences of *P. punctifer* and different species is also provided. Species from the orders Testudines and Crocodilia were used as outgroups.

Sequences ID	Species	Family	Order	Feeding habit	Identity (%)
XP_026091567.1	*Carassius auratus*	Cyprinidae	Cypriniformes	Omnivorous	91.07
ACN66300.1	*Ctenopharyngodon idella*	Cyprinidae	Cypriniformes	Herbivorous	89.29
ACN37860.1	*Carassius auratus*	Cyprinidae	Cypriniformes	Omnivorous	89.29
AFL69952.1	*Oncorhynchus clarkii*	Salmonidae	Salmoniformes	Carnivorous	87.50
XP_007240188.2	*Astyanax mexicanus*	Characidae	Characiformes	Carnivorous	87.50
XP_026786482.1	*Pangasianodon hypophthalmus*	Pangasiidae	Siluriformes	Omnivorous	87.50
NP_571202.1	*Danio rerio*	Cyprinidae	Cypriniformes	Carnivorous	87.50
AIU47021.1	*Cyprinus carpio*	Cyprinidae	Cypriniformes	Omnivorous	87.50
CAB40545.1	*Oncorhynchus mykiss*	Salmonidae	Salmoniformes	Carnivorous	85.71
XP_026861273.1	*Electrophorus electricus*	Gymnotidae	Gymnotiformes	Carnivorous	85.71
NP_001316205.1	*Ictalurus punctatus*	Ictaluridae	Siluriformes	Omnivorous	85.71
ACN66301.1	*Cyprinus carpio*	Cyprinidae	Cypriniformes	Omnivorous	85.71
AFH75405.1	*Scophthalmus maximus*	Scophthalmidae	Pleuronectiformes	Carnivorous	82.14
ADY05335.1	*Paralichthys olivaceus*	Paralichthyidae	Pleuronectiformes	Carnivorous	82.14
CAL69901.1	*Dicentrarchus labrax*	Moronidae	Moroniformes	Carnivorous	80.36
ACI32420.1	*Siniperca chuatsi*	Sinipercidae	Perciformes	Carnivorous	80.36
BAE95413.1	*Pagrus major*	Sparidae	Spariformes	Carnivorous	78.57
AAS75120.1	*Sparus aurata*	Sparidae	Spariformes	Carnivorous	78.57
KYO35095.1	*Alligator mississippiensis*	Alligatoridae	Crocodilia		67.86
XP_005278929.1	*Chrysemys picta bellii*	Emydidae	Testudines		66.07

Table A4
Sequence ID of the teleost species used for the phylogenetic analysis of trypsin. Information on the feeding habits of the species as well as on the percentage of identity at amino acid level among *try* sequences of *P. punctifer* and different species is also provided. Species from the orders Testudines and Crocodilia were used as outgroups.

Sequences ID	Species	Family	Order	Feeding habit	Identity (%)
XP_017311586.1	*Ictalurus punctatus*	Ictaluridae	Siluriformes	Omnivorous	86.18
KAF5908450.1	*Clarias magur*	Clariidae	Siluriformes	Omnivorous	85.37
XP_028837466.1	*Denticeps clupeoides*	Denticipitidae	Clupeiformes	Omnivorous	83.74
XP_026779498.1	*Pangasianodon hypophthalmus*	Pangasiidae	Siluriformes	Omnivorous	82.93
XP_027022007.1	*Tachysurus fulvidraco*	Bagridae	Siluriformes	Carnivorous	82.11
TWW54618.1	*Takifugu flavidus*	Tetraodontidae	Tetraodontiformes	Omnivorous	79.67
XP_034077433.1	*Gymnodraco acuticeps*	Bathydraconidae	Perciformes	Carnivorous	79.67
XP_028282177	*Parambassis ranga*	Ambassidae	Gobiiformes	Carnivorous	79.67
XP_029535066.1	*Oncorhynchus nerka*	Salmoninae	Salmoniformes	Carnivorous	78.86
ABE68639.1	*Sparus aurata*	Sparidae	Spariformes	Carnivorous	78.05
XP_029593925.1	*Salmo trutta*	Salmonidae	Salmoniformes	Carnivorous	78.05
XP_034144643	*Esox lucius*	Esocidae	Esociformes	Carnivorous	78.05
BAL04386.1	*Cyprinus carpio*	Cyprinidae	Cypriniformes	Omnivorous	76.42
AAC32752.1	*Pseudopleuronectes americanus*	Pleuronectidae	Pleuronectiformes	Carnivorous	73.17
AAX39390.1	*Pagrus pagrus*	Sparidae	Spariformes	Carnivorous	72.88
XP_023969437	*Chrysemys picta bellii*	Emydidae	Testudines		44.96
XP_025055986	*Alligator sinensis*	Alligatoridae	Crocodilia		41.54

Table A5
Sequence ID of the teleost species used for the phylogenetic analysis of chymotrypsin. Information on the feeding habits of the species as well as on the percentage of identity at amino acid level among *ctr* sequences of *P. punctifer* and different species is also provided. Species from the order Anura were used as outgroups.

Sequences ID	Species	Family	Order	Feeding habit	Identity (%)
BAL72705.1	*Thunnus orientalis*	Scombridae	Scombriformes	Carnivorous	82.09
BAL14137.1	*Thunnus orientalis*	Scombridae	Scombriformes	Carnivorous	82.09
XP_026793160.1	*Pangasianodon hypophthalmus*	Pangasiidae	Siluriformes	Omnivorous	80.88
XP_026793159.2	*Pangasianodon hypophthalmus*	Pangasiidae	Siluriformes	Omnivorous	80.88
RXN21076.1	*Labeo rohita*	Cyprinidae	Cypriniformes	Herbivorous	80.60
XP_028434889.1	*Perca flavescens*	Percidae	Perciformes	Carnivorous	80.60
XP_031424552.1	*Clupea harengus*	Clupeidae	Clupeiformes	Carnivorous	80.60
AAH55574.1	*Danio rerio*	Cyprinidae	Cypriniformes	Carnivorous	76.12
XP_026071043.1	*Carassius auratus*	Cyprinidae	Cypriniformes	Omnivorous	76.12
XP_023261815.1	*Seriola lalandi dorsalis*	Carangidae	Carangiformes	Carnivorous	74.60
AAH89075.1	*Xenopus tropicalis*	Pipidae	Anura		73.13
NP_001011477.1	*Xenopus tropicalis*	Pipidae	Anura		73.12
XP_028812135.1	*Denticeps clupeoides*	Denticipitidae	Clupeiformes	Omnivorous	69.12
AIS23637.1	*Lutjanus guttatus*	Lutjanidae	Perciformes	Carnivorous	64.71
XP_028812134.1	*Denticeps clupeoides*	Denticipitidae	Clupeiformes	Omnivorous	63.01

Table A6
Sequence ID of the teleost species used for the phylogenetic analysis of pepsin. Information on the feeding habits of the species as well as on the percentage of identity at amino acid level among *pga* sequences of *P. punctifer* and different species is also provided. Species from the order Anura were used as outgroups.

Sequences ID	Species	Family	Order	Feeding habit	Identity (%)
TUC86171.1	*Bagarius yarrelli*	Sisoridae	Siluriformes	Carnivorous	100.00
XP_027011481.1	*Tachysurus fulvidraco*	Bagridae	Siluriformes	Omnivorous	93.18
XP_026771141.1	*Pangasianodon hypophthalmus*	Pangasiidae	Siluriformes	Omnivorous	93.18
XP_026864186.1	*Electrophorus electricus*	Gymnotidae	Gymnotiformes	Carnivorous	88.64
XP_028822570.1	*Denticeps clupeoides*	Denticipitidae	Clupeiformes	Omnivorous	75.00
ACF18588.1	*Siniperca scherzeri*	Siniperciidae	Perciformes	Carnivorous	75.00
ACF18587.1	*Siniperca chuatsi*	Siniperciidae	Perciformes	Carnivorous	75.00
XP_010890229.2	*Esox lucius*	Esocidae	Esociformes	Carnivorous	72.50
AAD56287.1	*Pseudopleuronectes americanus*	Pleuronectidae	Pleuronectiformes	Carnivorous	71.05
BAM76489.1	*Gadus macrocephalus*	Gadidae	Gadiformes	Carnivorous	70.45
BAG48263.1	*Thunnus orientalis*	Scombridae	Scombriformes	Carnivorous	70.45
AAD56288.1	*Pseudopleuronectes americanus*	Pleuronectidae	Pleuronectiformes	Carnivorous	70.27
ABX89618.1	*Sparus aurata*	Sparidae	Spariformes	Carnivorous	68.89
AAZ29603.1	*Pagrus pagrus*	Sparidae	Spariformes	Carnivorous	68.89
BAC87742.1	*Paralichthys olivaceus*	Paralichthyidae	Pleuronectiformes	Carnivorous	68.29
ASW27226.1	*Solea senegalensis*	Soleidae	Pleuronectiformes	Carnivorous	68.29
BAU37037.1	*Micropterus salmoides*	Centrarchidae	Perciformes	Carnivorous	68.18
BAG48264.1	*Thunnus orientalis*	Scombridae	Scombriformes	Carnivorous	66.67
KPP65830.1	*Scleropages formosus*	Osteoglossidae	Osteoglossiformes	Carnivorous	65.12
XP_003444873.1	*Oreochromis niloticus*	Cichlidae	Cichliformes	Omnivorous	64.44
AXB22642.1	*Cichlasoma trimaculatum*	Cichlidae	Cichliformes	Carnivorous	64.44
BAB20798.1	*Xenopus laevis*	Pipidae	Anura		60.00
BAB20092.1	*Lithobates catesbeianus*	Ranidae	Anura		60.00

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1.2

Ontogeny of the digestive enzyme activity of the Amazonian pimelodid catfish *Pseudoplatystoma punctifer* (Castelnau, 1855)

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Resumen

El objetivo del estudio fue evaluar la ontogenia funcional del sistema digestivo de *Pseudoplatystoma punctifer* mediante el análisis de la actividad de las principales enzimas intestinales (fosfatasa alcalina, aminopeptidasa N, maltasa y leucina-alanina peptidasa), pancreáticas (tripsina, quimotripsina, proteasas alcalinas totales, lipasa activada por sales biliares y α-amilasa) y gástricas (pepsina). Las larvas fueron cultivadas por triplicado en un sistema de recirculación de 4 a 27 días post-fertilización (dpf) a una densidad inicial de 90 larvas L^{-1}, 27,8 ± 0,7 °C y un fotoperiodo de 0L:24O. Las larvas fueron alimentadas de 4 a 17 dpf con nauplios de *Artemia* y fueron destetadas con un alimento formulado experimental (contenido de proteína cruda ~45%; contenido de grasa cruda ~10%; carbohidratos crudos ~8%) en 3 días, y luego siguieron siendo alimentadas con la misma dieta hasta el final del experimento. *P. punctifer* mostró un patrón de crecimiento exponencial con dos tasas de crecimiento diferentes: una más lenta desde la eclosión hasta 12 dpf seguida de una más rápida de 12 a 27 dpf. Las actividades específicas y totales de las enzimas pancreáticas e intestinales se detectaron a partir de la eclosión. El sistema digestivo fue funcional a los 12 dpf, lo que indica la transición de la etapa larvaria a la juvenil (digestión alcalina a ácida). Por lo tanto, los individuos podrían ser destetados a partir de ese momento. Las variaciones observadas en la actividad enzimática a partir de los 17 dpf reflejó la adaptación de la maquinaria enzimática a la nueva dieta suministrada. Las larvas de *P. punctifer* mostraron un rápido desarrollo del sistema digestivo con un perfil enzimático típico de una especie tropical y carnívora.

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Ontogeny of the digestive enzyme activity of the Amazonian pimelodid catfish *Pseudoplatystoma punctifer* (Castelnau, 1855)

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ABSTRACT

The aim of the study was to evaluate the functional ontogeny of the digestive system of *Pseudoplatystoma punctifer* through the analysis of the activity of the main intestinal (alkaline phosphatase, aminopeptidase N, maltase and leucine-alanine peptidase), pancreatic (trypsin, chymotrypsin, total alkaline proteases, bile-salt activated lipase and amylase) and gastric (pepsin) enzymes. Larvae were raised in triplicate in a recirculation system from 4 to 27 days post fertilization (dpf) at an initial density of 90 larvae L^{-1}, 27.8 ± 0.7 °C and 0 L: 24D photoperiod. Larvae were fed from 4 to 17 dpf with *Artemia* nauplii and weaned onto an experimentally formulated feed (crude protein content ~ 45%; crude fat content ~ 10%; crude carbohydrate ~ 8%) within 3 days, then continued with the same diet until the end of the trial. *P. punctifer* showed an exponential growth pattern with two different growth rates: a slower one from hatching to 12 dpf followed by a faster one from 12 to 27 dpf. The specific and total activities of the pancreatic and intestinal enzymes were detected from hatching. The digestive system was functional at 12 dpf, indicating the transition from the larval to the juvenile stage (alkaline to acid digestion). Therefore individuals could be weaned from that day onwards. The variations observed in the enzymatic activity from 17 dpf reflected the adaptation of the enzymatic machinery to the new diet supplied. *P. punctifer* larvae showed a fast digestive system development with an enzymatic profile typical of a tropical and carnivorous species.

1. Introduction

Over the last two decades, an increasing number of studies have been focused on the ontogeny of digestive enzyme activities in both marine and freshwater fishes considered as candidates for aquaculture. In fact, the knowledge of the digestive enzyme profiles during early life stages and their adaptation to the diet in a given species is essential for understanding its nutritional physiology. This is particularly important during ontogeny as the constantly changing digestive capacities determine the types of nutrients that can be digested and absorbed throughout its development (Gisbert et al., 2013; Hamre et al., 2013; Rønnestad et al., 2013). Moreover, considering the high cost of *Artemia* cysts and the associated costs of nauplii production, the replacement of live food by formulated feeds has become one of the main objectives in fish larviculture hence, identifying the type of digestive enzymes and the variation of their specific activities during ontogeny can be used as an indicator not only of larval development and gut maturation but also of survival and performance (Zambonino-Infante et al., 2008) when testing different feeding regimes. Notably, the transitions from endogenous to exogenous feeding as well as from the larval to the juvenile stage are considered key events during which feeding protocols should be adapted to the digestive capacities of a developing organism. Therefore, understanding of the ontogeny of digestive enzymes is critical for determining the most appropriate time for weaning and also for optimizing compound diets as well as feeding protocols, eventually leading to improve growth rates, survival and larval quality (Zambonino-Infante et al., 2008; Hamre et al., 2013; Rønnestad et al., 2013).

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Among South American catfish species (Siluridae), the species of the pimelodid genus *Pseudoplatystoma* (Bleeker, 1862) are highly prized for human consumption due to their excellent flesh quality and lack of intra-muscular spines. In Brazil, the country producing most of *Pseudoplatystoma* reared fish, commercial aquaculture is mostly centered on producing hybrids of *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum* (Valladão et al., 2018), which accept formulated feeds during early life stages more easily than pure species and hence present less cannibalism and a better growth performance (Porto-Foresti et al., 2013). Taking into consideration the risk of genetic variability loss through genetic introgression caused by the escape of interspecific hybrids to the natural environment (Vaini et al., 2014; Lima et al., 2018), research efforts have been made in Amazonia to develop the culture of *Pseudoplatystoma punctifer* (Castro-Ruiz, et al., 2009, 2016; Núñez et al., 2011; Baras et al., 2011, 2012; Gilles et al., 2014; Gisbert et al., 2014; Darias et al., 2015a; Fernández-Méndez et al., 2015; among others). This carnivorous migratory tropical catfish species, native to the Amazon basin, can attain 140 cm of total length (Buitrago-Suárez and Burr, 2007). It is worth noting that the last taxonomic revision of the genus *Pseudoplatystoma* (Buitrago-Suárez and Burr, 2007) recognized eight species instead of the previous three species and redefined their geographic distribution, so attention should be paid when comparing studies done before and after this revision. However, inconsistencies between the taxonomy of the genus proposed by Buitrago-Suárez and Burr (2007) and subsequent molecular and morphological studies (e.g. García-Dávila et al., 2013; Estivals et al., 2015) highlight the need to reexamine the classification of the *Pseudoplatystoma* species.

Although captive-breeding methodology is well established under controlled conditions (Padilla et al., 2001; Núñez et al., 2008), large-scale aquaculture of *P. punctifer* has not yet been implemented due to high rates of cannibalism resulting in increased mortality during early life stages. Although a genetic component cannot be neglected, several studies have found a clear correlation between the feeding protocol and the incidence of cannibalism in *P. punctifer* (Gisbert et al., 2014; Darias et al., 2015a; Fernández-Méndez et al., 2015) and that it could be reduced using feeds having an adequate texture and meeting the specific nutritional requirements of larvae and early juveniles (Darias et al., 2015a; Fernández-Méndez et al., 2015). Further studies on the digestive physiology and nutrition in this species are still needed for improving current rearing strategies and removing bottlenecks associated to mass culture of fingerlings of *P. punctifer*. According to Gisbert et al. (2014), the histological development of the digestive system of *P. punctifer* followed the same general pattern reported in most studied silurid species, such as African catfish (*Clarias garipinus*; Verreth et al., 1992), silver catfish (*Rhamdia quelen*; De Amorim et al., 2009), slender walking catfish (*Clarias nieuhohofii*; Saelee et al., 2011) or yellow catfish (*Pelteobagrus fulvidraco*; Yang et al., 2010). However, different reproductive guilds, egg and larval size or even different larval rearing practices might lead to some species-specific differences in the timing of differentiation and functionality of various digestive structures among these species (Gisbert et al., 2014). In addition, attention should be paid when extracting conclusions about the functionality of the digestive system (i.e. beginning of acid digestion and the onset of weaning of larvae) using only histological data, since morphology does not always match functionality (Darias et al., 2005; Solovyev et al., 2016). Thus, in order to complement the information about the histological development of the digestive system of *P. punctifer* (Gisbert et al., 2014), the present study aimed to evaluate its functional ontogeny through evaluating the activity of the main intestinal (alkaline phosphatase, aminopeptidase N, maltase and leucine-alanine peptidase), pancreatic (trypsin, chymotrypsin, total alkaline proteases, bile-salt activated lipase and amylase) and gastric (pepsin) enzymes.

2. Material and methods

2.1. Larval and early juvenile rearing and sampling

Larvae were obtained by hormonally-induced spawning of a sexually mature pond-reared pair of *P. punctifer* (♀: 3.60 kg; ♂: 1.85 kg) at the Instituto de Investigaciones de la Amazonía Peruana (IIAP, Iquitos, Peru). Female and male were transferred to 500-L tanks and injected intramuscularly with carp pituitary extract (Argent Chemical Laboratories, Inc., Redmond, WA) at 5 mg kg^{-1} and 1 mg kg^{-1} of body weight, respectively, according to Darias et al. (2015a); then, eggs were fertilized according to Núñez et al. (2008). Fertilized eggs (fertilization rate ~99.9%) were incubated at 27.7 ± 0.6 °C in 40-L cylindroconical tanks connected to a freshwater recirculating system; hatching took place after 18.0 ± 2.0 h (hatching rate ~96%). Mouth opening occurred at day 1 post fertilization (dpf) and exogenous feeding started at 4 dpf. At 4 dpf (6.05 ± 0.28 mm total length-TL, $n = 45$; mean ± standard deviation) larvae were transferred into 40-L square tanks made of fiberglass (functional volume = 30 L), which were connected to a water recirculating system with mechanical and biological filters. Water quality parameters throughout the experimental period were as follows: temperature, 27.8 ± 0.7 °C; pH, 7.0 ± 0.5; dissolved oxygen, 7.4 ± 0.2 mg L^{-1}; NO_2-, 0.38 ± 0.27 mg L^{-1}, NH_4+, 0.26 ± 0.13 mg L^{-1} and water flow rate of 0.2 L min^{-1}. Larvae were reared in triplicate (initial density = 90 larvae L^{-1}) under 0 L:24D photoperiod (< 0.001 Lx at the water surface) and fed in slight excess with newly-hatched *Artemia* sp. nauplii (crude protein content ~37%; crude fat content ~15%; crude carbohydrate content ~10%; 0.6–12.2 nauplii mL^{-1}; five times a day from 4 to 17 dpf). At 17 dpf, larvae were weaned onto an experimentally formulated feed (crude protein content ~45%; crude fat content ~10%; crude carbohydrate content ~8%) within 3 days according to Darias et al. (2015a), whereas larvae were solely fed with the same formulated feed from 20 dpf to the end of the experiment at 27 dpf. In order to monitor larval growth (total length, TL and wet body weight, WW), triplicate groups of larvae ($n = 15$) were sampled from each rearing tank at 4, 8, 12, 20 and 27 dpf and euthanized with an overdose of Eugenol (Moyco®, Moyco, Lima, Peru). Then, larvae were placed in a Petri dish, photographed using a scale bar and TL was measured on the digital images (300 dpi) using ImageJ software (Rasband, 1997–2012). Individual WW was determined using an analytic microbalance (Sartorius BP 211 D, Data Weighing Systems, Inc., Elk Grove, IL, ± 0.01 mg). Specific growth rate (SGR, in % day^{-1}) was calculated as $SGR = (\ln WW_f - \ln WW_i)/(t_f - t_i) \times 100$; where WW_f, WWi, t_f and t_i represented final and initial WW and time of the experiment, respectively. Survival was calculated at 17 dpf (onset of weaning) and 27 dpf (end of the trial) by counting the individuals surviving at each sampling point and taking into consideration the number of individuals sampled throughout the experimental period. Larvae ($n = 600$ to 15, depending on their WW) were sampled from each tank ($n = 3$) at hatching and at 4, 12, 17, 20, 25 and 27 dpf in order to evaluate ontogenetic changes in digestive enzyme activity patterns, and their relationship to diet and rearing protocol. Sampled larvae were euthanized as previously described, rinsed in distilled water and frozen at −80 °C until their analysis.

2.2. Enzymatic assays

Individuals younger than 12 dpf were completely homogenized for analytical purposes, since they were too small for being dissected. Older fish were dissected in four parts (head, pancreatic segment, intestinal segment and tail) and enzyme assays were performed in the pancreatic and intestinal segments (Cahu and Zambonino-Infante, 1994).

Samples were homogenized in 30 volumes (*v/w*) of Tris-Mannitol buffer (50 mM Mannitol, 2 mM Tris-HCl; pH 7.5) for 30 s (Ultra-Turrax T25, Germany), then 100 µL of 0.1 M $CaCl_2$ was added to the homogenate and the extract was subjected to sonication (Vibra-cell©, Sonics,

Germany) for 1 min. During the homogenizing process, samples were kept on ice (0–4 °C) for reducing the enzymatic activity. An aliquot of homogenate was stored at −80 °C until their analysis for determining activities of pancreatic (trypsin, chymotrypsin, total alkaline proteases, bile-salt activated lipase and amylase), gastric (pepsin) and intestinal cytosolic (leucine-alanine peptidase) enzymes. Processed samples were analyzed within the first two months after their homogenization in order to prevent a loss of activity of the pancreatic and gastric digestive enzymes (Solovyev and Gisbert, 2016). The remaining homogenate was processed for intestinal brush border purification according to the recommendations of Gisbert et al. (2018) in order to properly determine alkaline phosphatase, maltase and aminopeptdiase N activities. To initiate this process, the homogenate was centrifuged (9000 *g* for 10 min at 4 °C), the precipitate discarded, then the supernatant centrifuged once again (34,000 *g* for 30 min at 4 °C). The pellet, containing the brush border (BB) of enterocytes, was re-suspended in 1 mL of buffer (0.1 M KCl, 5 mM Tris-Hepes, 1 mM DTT; pH 7.5) and stored at −80 °C until analysis (Crane et al., 1979).

The determination of the activity of pancreatic, gastric and intestinal digestive enzymes was conducted using spectrophotometric analyses as described by Gisbert et al. (2009). In brief, alkaline phosphatase (AP, E.C. 3.1.3.1) activity was quantified using PNPP (4-nitrophenyl phosphate) as substrate in 30 mM Na_2CO_3 buffer (pH 9.8). One unit (U) was defined as 1 µg nitrophenol released per min^{-1} mL^{-1} of BB homogenate at 27 °C and measured using 407 nm (Bessey et al., 1946). Aminopeptidase N (AN, E.C.3.4.11.2) activity was determined according to Maroux et al. (1973) using sodium phosphate buffer 80 mM (pH 7.0) and L-leucine p-nitroanilide as substrate (in 0.1 mM DMSO). One unit of enzyme activity (U) was defined as 1 µg nitroanilide released per min^{-1} mL^{-1} of BB homogenate at 27 °C and measured using 410 nm. Maltase (MAL, E.C.3.2.1.20) activity was determined at 27 °C using D(+)-maltose as substrate in 100 mM sodium maleate buffer (pH 6.0) (Dahkqvist, 1970); one unit of maltase (U) was defined as µmol of glucose liberated min^{-1} mL^{-1} of homogenate and measured at 420 nm. Leucine-alanine peptidase (LAP, E.C. 3.4.11) was performed using leucine-alanine as substrate in 50 mM Tris-HCl buffer (pH 8.0); one unit of enzyme activity (U) was defined as 1 nmol of the hydrolyzed substrate per min^{-1} $mL^{-1}$of homogenate at 27 °C and measured at 530 nm (Nicholson and Kim, 1975).

Regarding pancreatic enzymes, trypsin (E.C. 3.4.21.4) activity was assayed at 27 °C and measured using 407 nm using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) as substrate (Holm et al., 1988). One unit of trypsin per mL (U) was defined as 1 µmol BAPNA hydrolyzed per min^{-1} mL^{-1} of enzyme. Chymotrypsin (EC. 3.4.21.1) activity was determined at 27 °C and measured at 256 nm using BTEE (N-benzoyl-L-tyrosine ethyl ester) as substrate in 80 mM Tris-HCl, 100 mM $CaCl_2$ buffer (pH 7.2). Chymotrypsin activity (U) corresponded to the 1 µmol BTEE hydrolyzed per min^{-1} mL^{-1} of homogenate (Worthington, 1991). The total activity of alkaline proteases was determined after 30 min of incubation at 27 °C, using 0.5% (*w/v*) azocasein as substrate in 50 mM Tris–HCl buffer (pH 8.0). One unit of total alkaline proteases per mL (U) was defined as 1 µmol azocasein hydrolyzed per min^{-1} mL^{-1} of homogenate and measured at 366 nm (García-Careño and Haard, 1993). Pepsin assays were performed at 27 °C and 280 nm using hemoglobin as substrate (Worthington, 1991). Pepsin activity (U) was defined as 1 µmol of hemoglobin liberated per min^{-1} mL^{-1} of homogenate after 10 min of incubation. The α-amylase (E.C. 3.2.1.1) activity was measured at 580 nm using soluble starch (0.3%) dissolved in Na_2HPO_4 buffer (pH 7.4) as substrate (Métais and Bieth, 1968); and its activity (U) was defined as the mg of starch hydrolyzed during 30 min per mL^{-1} of homogenate at 27 °C. Bile salt-activated lipase (E.C. 3.1.1) activity was measured using 4 *p*-nitrophenyl myristate as substrate dissolved in 0.25 mM Tris-HCl (pH 9.0), 0.25 mM 2-methoxyethanol and 5 mM sodium cholate buffer. The reaction was stopped with a mixture of acetone:n-heptane (5:2), the extract centrifuged (6080 *g*, 2 min at 4 °C) and the absorbance of the supernatant read at room temperature and measured at 405 nm. Lipase activity (U) was defined as the µmol of substrate hydrolyzed per min^{-1} mL^{-1} of homogenate (Iijima et al., 1998). All enzymatic activities were measured using a microplate scanning spectrophotometer (Synergy HT, Bio-Tech, Germany) and expressed as specific (mU mg^{-1} protein) and total (mU $larva^{-1}$) enzyme activities. Soluble protein in enzyme extracts was quantified using the Bradford technique Bradford (1976) using bovine serum albumin as a standard. All the assays were made in triplicate (methodological replicates).

2.3. Statistical analyses

Activity of digestive enzymes was presented as a mean ± standard deviation of the mean (SD). Statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, USA). Data were checked for normality and heterogeneity of variance, and then analyzed by one-way ANOVA followed by the Holm–Sidak method when significant differences were found. The level of statistical significance was set at $P < .01$ and $P < .05$.

3. Results

3.1. Larval and early juvenile growth and survival

Larval and early juvenile growth in terms of WW (mg) and TL (mm) followed an exponential curve according to the following equations: $WW = 0.1655e^{0.2794T}$ ($r = 0.97$; $n = 45$) and $TL = 4.2914e^{0.0787T}$ ($r = 0.99$; n = 45), where T is the age of larvae in dpf (Fig. 1). Larvae at 4 dpf (WW = 0.22 ± 0.17 mg; TL = 6.05 ± 0.28 mm) grew up to 244.11 ± 4.36 mg and 35.77 ± 1.11 mm in weight and length, respectively, by the end of the study (27 dpf). Two phases of growth in WW were identified during *P. punctifer* development; during the first phase, a gradual growth was observed between 4 (0.22 ± 0.17 mg WW) and 12 dpf (6.56 ± 0.23 mg WW), whereas the second phase started from 12 dpf with a pronounced increase in WW, coinciding with the introduction of the formulated feed at 17 dpf (> 17 mm TL). The

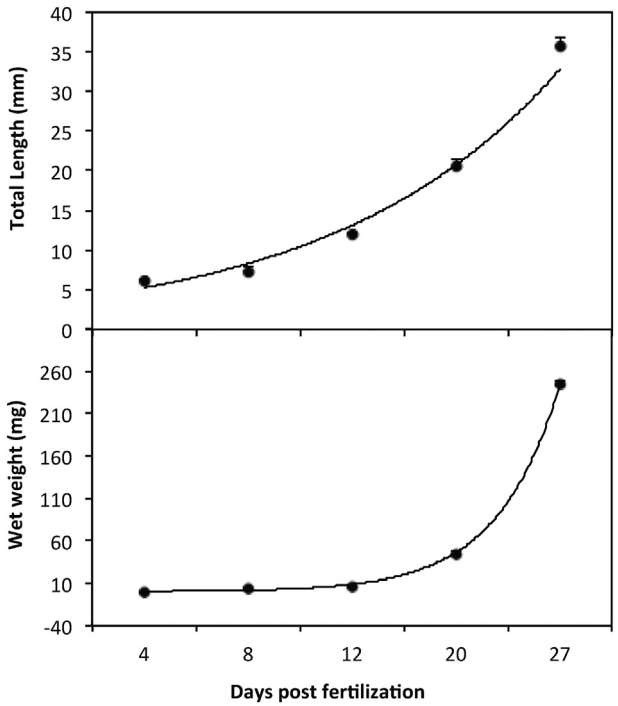

Fig. 1. Larval growth of *P. punctifer* in terms of total length (mm) and wet body weight (mg) from 4 to 27 days post fertilization (dpf). Results are expressed as mean ± SD ($n = 45$).

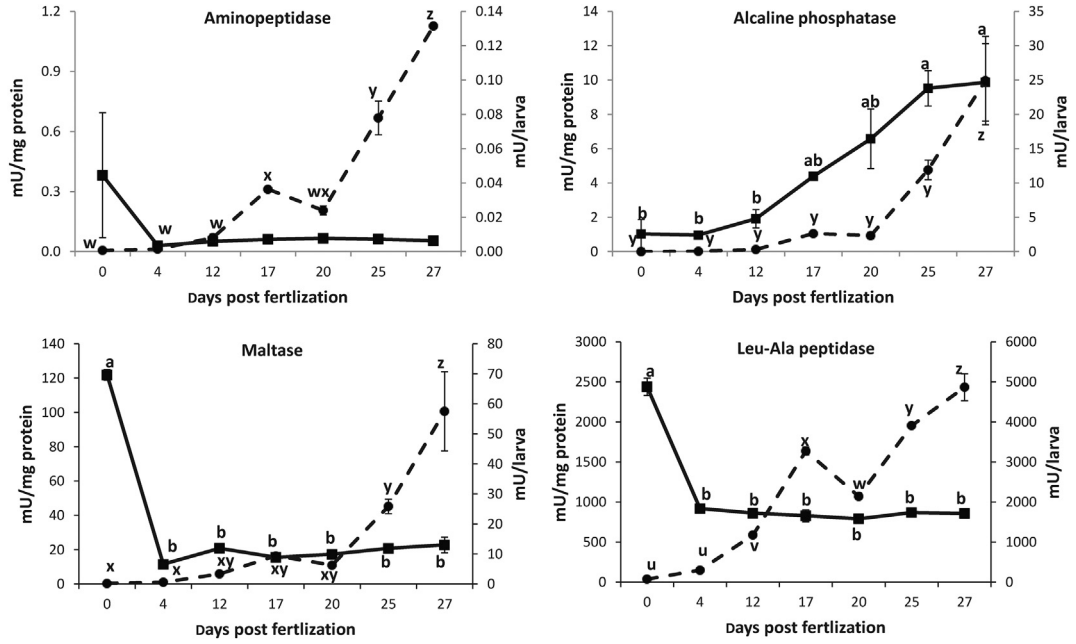

Fig. 2. Specific (squares, mU mg^{-1} protein) and total (circles, mU $larva^{-1}$) activity of brush border (alkaline phosphatase, maltase and aminopeptidase N) and cytosolic (leucine-alanine pepidase) intestinal enzymes in *P. punctifer* from hatching to the juvenile stage (27 dpf). Results are expressed as mean ± SD ($n = 3$). Different letters indicate significant differences (ANOVA, $P < .05$).

SGR of *P. punctifer* was 0.19 ± 0.00 from 4 to 12 dpf and 0.53 ± 0.10 from 12 to 27 dpf. Survival rates at 17 dpf and at the end of the experiment (27 dpf) were 82.7 ± 11.0% and 16.0 ± 0.1%, respectively.

3.2. Activity of BB enzymes

The specific and total activities of the assayed brush border intestinal (AP, AN and MAL) and cytosolic enzymes (LAP) are shown in Fig. 2. The specific activity of AP (Fig. 2) was detected at hatching (1.03 ± 0.83 mU mg $protein^{-1}$) and remained constant until 12 dpf (1.92 ± 0.53 mU mg $protein^{-1}$), then its activity gradually increased until 27 dpf (9.87 ± 2.27 mU mg $protein^{-1}$; $P < .001$). Total activity of AP remained constant from hatching to 25 dpf (0.001 ± 0.001 mU $larva^{-1}$ at 0 dpf and 11.90 ± 1.42 mU $larva^{-1}$ at 25 dpf), which was followed by a significant increase (24.92 mU $larva^{-1}$ ± 6.43) at the end of the experiment ($P < .001$).

The specific activity of AN (Fig. 2) remained constant from hatching (0.38 ± 0.31 mU mg $protein^{-1}$) to the end of the study (0.05 ± 0.004 mU mg $protein^{-1}$; $P > .05$). Moreover, total activity of AN was detected at hatching (0.001 ± 0.001 mU $larva^{-1}$) and remained constant until 12 dpf (0.08 ± 0.002 mU $larva^{-1}$), then increased from 17 dpf (0.04 ± 0.0001 mU $larva^{-1}$) until 27 dpf (0.13 ± 0.0002 mU $larva^{-1}$; $P < .01$).

Maltase specific activity (Fig. 2) was detected at hatching (121.85 ± 3.11 mU mg $protein^{-1}$), decreased abruptly at 4 dpf (11.51 ± 0.41 mU mg $protein^{-1}$) and remained constant until the end of the study (22.81 ± 4.57 mU mg $protein^{-1}$). Total activity of MAL remained statistically constant from hatching (0.19 ± 0.01 mU $larva^{-1}$) until 20 dpf (6.26 ± 0.43 mU $larva^{-1}$) and then gradually increased until 27 dpf (57.51 ± 13.20 mU $larva^{-1}$, $P < .01$).

The specific activity of LAP (Fig. 2) was highest at hatching (2438.96 ± 108.48 mU mg $protein^{-1}$) and it decreased ~3 times at 4 dpf (919.14 ± 27.98 mU mg $protein^{-1}$) and remained constant until 27 dpf (858.2 ± 38.0 mU mg $protein^{-1}$). Regarding total activity values for LAP, the lowest value was found at hatching (75.59 ± 11.0 mU $larva^{-1}$) and its activity gradually increased from 4 dpf to 17 dpf (3275.04 ± 98.62 mU $larva^{-1}$; $P < .01$). A decrease in LAP total activity was detected from 17 to 20 dpf (2142.79 ± 58.12 mU $larva^{-1}$), but it increased again from 20 dpf to the end of the trial (4868.68 ± 336.63 mU $larva^{-1}$; $P < .01$).

The level of intestinal maturation in *P. punctifer* larvae was determined as the ratio between the specific activities of BB enzymes and that of LAP. Differences among these ratios were found depending on the BB enzyme considered (Fig. 3). When considering the ratio MAL/LAP, there was a sharp increase at 4 dpf, whereas the AN/LAP and AP/LAP ratios showed an increase between 12 and 17 dpf ($P < .001$; Fig. 3).

3.3. Activity of pancreatic enzymes

Changes in the specific and total activities of pancreatic (trypsin, chymotrypsin, total alkaline proteases, α-amylase and bile-salt activated lipase) enzymes are shown in Fig. 4. Trypsin specific activity (Fig. 4) was detected from hatching (0.007 ± 0.002 mU mg $protein^{-1}$) and remained constant until the end of the trial (0.012 ± 0.003 mU mg $protein^{-1}$; $P > .05$). Total trypsin activity remained statistically constant from hatching (0.18 ± 0.005 μU $larva^{-1}$) until 20 dpf (0.02 ± 0.001 μU $larva^{-1}$) and sharply increased thereafter until the end of the experiment (0.08 ± 0.02 mU $larva^{-1}$; $P < .001$).

Chymotrypsin specific activity (Fig. 4) remained constant from hatching (0.022 ± 0.04 mU mg $protein^{-1}$) until the end of the experiment (0.18 ± 0.072 mU mg $protein^{-1}$; $P > .05$). Chymotrypsin total activity was statistically constant from hatching (0.006 ± 0.001 mU $larva^{-1}$) to 4 dpf (0.024 ± 0.012 mU $larva^{-1}$), then gradually increased until 25 dpf (0.046 ± 0.024 mU $larva^{-1}$) with an abrupt increase at 27 dpf (1.21 ± 0.544 mU $larva^{-1}$; $P < .05$). Total alkaline proteases (Fig. 4) were detected at hatching and their specific activity increased almost 2-fold from hatching (0.76 ± 0.02 mU mg $protein^{-1}$) to 12 dpf (1.51 ± 0.004 mU mg $protein^{-1}$), but abruptly decreased 6 times at 17 dpf (0.23 ± 0.2 mU mg $protein^{-1}$). However, the activity of total alkaline proteases increased from 20 dpf (0.17 ± 0.02 mU mg $protein^{-1}$) until the end of the trial (0.38 ± 0.01 mU mg $protein^{-1}$; $P < .001$). Total activity of total alkaline proteases increased from hatching (0.02 ± 0.004 μU $larva^{-1}$) to 12 dpf (2.11 ± 0.03 mU $larva^{-1}$) and gradually decreased thereafter until 20 dpf (0.47 ± 0.05 mU $larva^{-1}$). A 4-fold increase of total

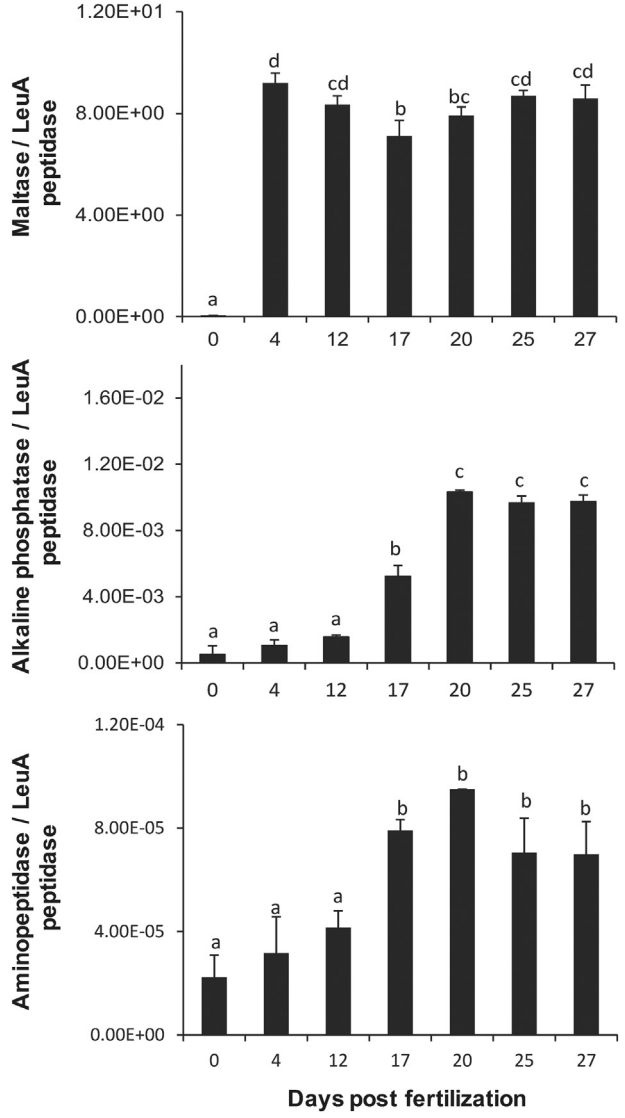

Fig. 3. Intestinal maturation during the larval development of *P. punctifer* measured as the ratio of specific activity of brush border membrane enzymes/cytosolic enzymes: MAL/LAP, AP/LAP and AN/LAP. Results are expressed as mean ± SD (n = 3). Different letters indicate significant differences (ANOVA, $P < .05$).

alkaline proteases activity was observed 5 days later (1.95 ± 0.1 mU larva^{-1}), and continued to increase until the end of the study (2.52 ± 0.1 mU larva^{-1}; $P < .001$).

Alpha-amylase activity (Fig. 4) was detected at hatching (15.4 ± 3.2 mU mg protein^{-1}) and remained constant until the end of the study at 27 dpf (17.78 ± 0.7; $P > .05$). Total α-amylase activity was statistically constant from hatching to 4 dpf (0.39 ± 0.08 mU larva^{-1} and 3.76 ± 1.88 mU larva^{-1}, respectively), then increased from 12 to 17 dpf (11.8 ± 2.3 mU larva^{-1} and 31.23 ± 4.27 mU larva^{-1}), showing a 3-fold increase until the end of the experiment (110.49 ± 2.09 mU larva^{-1}; $P < .001$).

The specific activity of bile-salt activated lipase (Fig. 4) was highest at hatching (7.50 ± 2.0 mU mg protein^{-1}), but its activity abruptly decreased at 4 dpf (1.28 ± 0.11 mU mg protein^{-1}); however, lipase activity gradually increased from 12 dpf (1.81 ± 0.01 mU mg protein^{-1}) until the end of the study (6.50 ± 0.41 mU mg protein^{-1}; $P < .05$). Total activity of lipase remained statistically constant from hatching until 12 dpf (0.37 ± 0.15 mU larva^{-1}) and then sharply increased until 27 dpf (42.12 ± 2.17 mU larva^{-1}; $P < .001$).

3.4. Activity of gastric enzyme

Pepsin specific activity was first detected at 4 dpf (0.12 ± 0.02 μU mg protein^{-1}) and its specific activity remained constant until 12 dpf (0.22 ± 0.08 μU mg protein^{-1}) (Fig. 5). Then, the activity increased abruptly from 12 to 17 dpf (0.7 ± 0.19 μU mg protein^{-1}), whereas it decreased sharply at 20 dpf (0.0003 ± 0.02 μU mg protein^{-1}). Pepsin specific activity increased thereafter until the end of the trial (4.9 ± 0.7 μU mg protein^{-1}; $P < .05$). Total pepsin activity followed a similar pattern to that of the pepsin specific activity ($P < .05$).

4. Discussion

Compared to temperate-water fish, tropical fish have a faster development due to the effect of temperature on metabolic rate (Gillooly et al., 2002). Therefore, comparisons of the present results are made with those obtained for other freshwater and marine tropical fish species, but also for other catfish. Larvae and early juveniles of *P. punctifer* followed a similar previously reported pattern of development and growth (Gisbert et al., 2014, Darias et al., 2015a, Fernández-Méndez et al., 2015). The growth pattern of *P. punctifer* larvae and early juveniles in terms of weight showed an initial slow growth phase (SGR 0.19 ± 0.00) until 12 dpf (12.02 ± 0.18 mm TL), corresponding to the larval stage, followed by a higher exponential growth rate (SGR 0.53 ± 0.10) from 12 dpf onwards, coinciding with the beginning of the juvenile stage. Regardless of their different reproductive guilds, this type of growth pattern has also been reported in both freshwater and marine tropical fish species such as yellow catfish (*Pelteobagrus fulvidraco*; Yang et al., 2010), green catfish (*Mystus nemurus*; Srichanun et al., 2012), common snook (*Centropomus undecimalis*; Jimenez-Martinez et al., 2012), bay snook (*Petenia splendida*; Uscanga-Martínez et al., 2011) and spotted rose snapper (*Lutjanus guttatus*; Moguel-Hernández et al., 2014), among others. The extended low growth rate observed from hatching to 12 dpf (12.02 ± 0.18 mm TL) in *P. punctifer* may be interpreted as an evolutionary strategy for allocating the available energy from yolk-sac reserves and prey items to promote larval physiological changes (i.e. gastrointestinal and other body system development) rather than somatic growth, as it has also been reported in butter catfish (*Ompok bimaculatus;*Pradhan et al., 2013), striped catfish, (*Pangasianodon hypophthalmus;*Rangasin et al., 2012) and tropical gar (*Atractosteus tropicus*; Frías-Quintana et al., 2015). Another reason for the low growth rate of *P. punctifer* could be the fact that *Artemia* nauplii, offered during this developmental period, do not completely satisfy the nutritional needs of larvae or let them fully exploit their growth potential (Gisbert et al. 2014; Darias et al., 2015a). In fact, the nutritional composition of the diet has been shown to modulate the SGR of *P. punctifer* during the early juvenile stage from 12 to 26 dpf (Darias et al., 2015a). Therefore, a higher growth rate during the larval phase could be also expected if a feeding regime fitted to the specific nutritional needs of larvae is offered. Survival rates observed under current experimental conditions were within the range of values reported in other studies for the same species (Núñez et al., 2011; Gisbert et al., 2014; Darias et al., 2015a) and may reflect larval adaptation to the weaning strategy since survival has shown to be affected by the composition (Darias et al., 2015a) and texture (Fernández-Méndez et al., 2015) of compound diets.

The appearance of a functional microvillus membrane in enterocytes constitutes a crucial step during larval development of fish, since this characterizes the maturation of the intestinal mucosa and the acquisition of an adult mode of digestion (Zambonino-Infante et al., 2008; Gisbert et al., 2018). The ratio of BB and cytosolic intestinal enzymes is considered as an indicator of the development of intestinal digestion and describes the change from the larval (intracellular) to the adult (intestinal lumen) mode of digestion. Values of this ratio are considered as important for readiness for introduction of formulated diet into larval rearing protocols (Cahu and Zambonino-Infante, 2001).

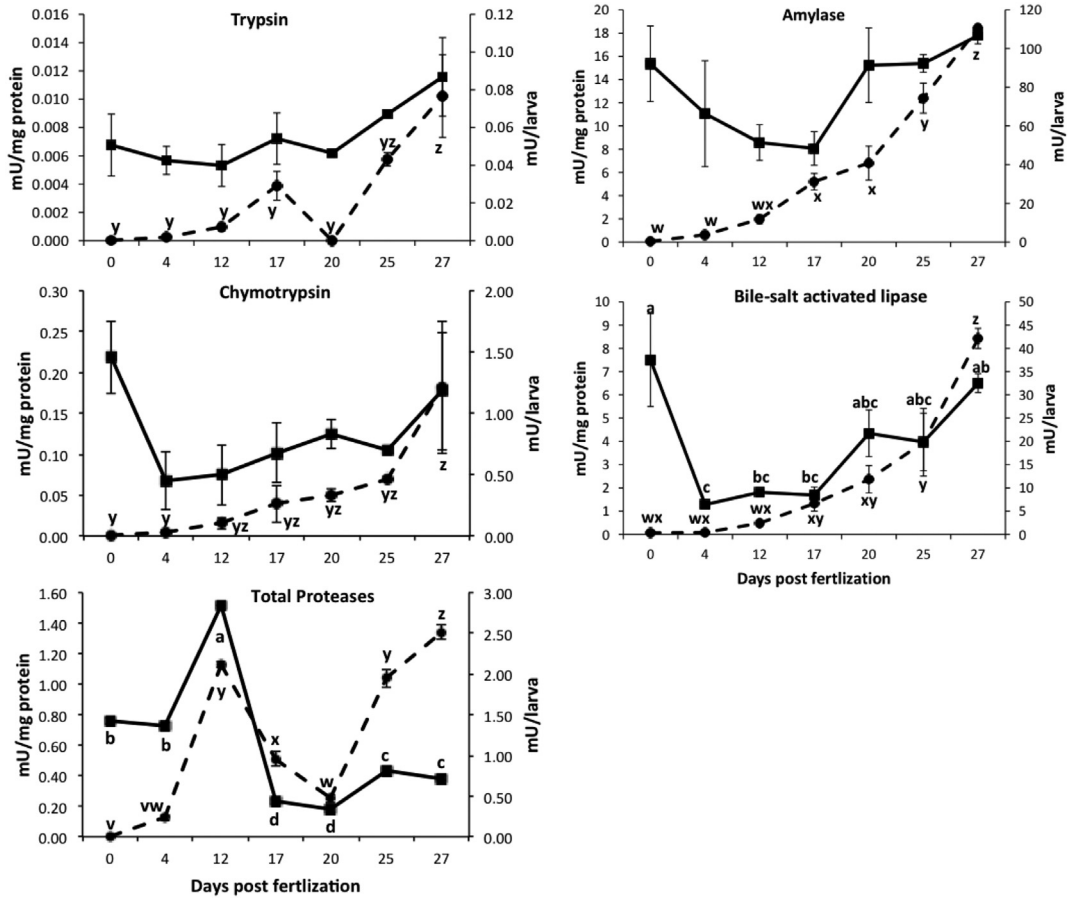

Fig. 4. Specific (squares, mU mg^{-1} protein) and total (circles, mU $larva^{-1}$) activity of pancreatic enzymes during the larval development of *P. punctifer*. Results are expressed as mean ± SD (n = 3). Different letters indicate significant differences (ANOVA, $P < .05$).

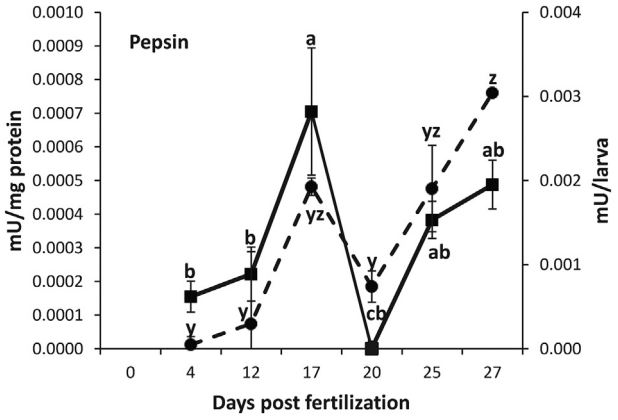

Fig. 5. Specific (squares, mU mg^{-1} protein) and total (circles, mU $larva^{-1}$) activity of pepsin during the larval development of *P. punctifer*. Results are expressed as mean ± SD (n = 3). Different letters indicate significant differences (ANOVA, $P < .05$).

In the present study, different intestinal maturation ratios were low at 4 dpf (6.05 ± 0.28 mm TL), which might be due to the presence of a straight, undifferentiated and unfolded intestine lined by a single layer of columnar epithelial cells (Gisbert et al., 2014). Then, the BB enzymes, and particularly AP and AN activities, increased at 12 dpf (12.02 ± 0.18 mm TL), which indicated the beginning of the enterocytes maturation as well as the increase of their proliferation that resulted in an increase in the intestinal luminal digestion and absorption surface (Gisbert et al., 2014). The increase in activities of BB enzymes up to 20 dpf (20.53 ± 0.98 mm TL) also coincided with the formation of the intestinal loop for accommodating the increasing length of the intestine inside the abdominal cavity as well as the achievement of a morphologically complete digestive system during this stage (Gisbert et al., 2014). Previous histological data (Gisbert et al., 2014) coupled with present results of enzyme activities obtained by spectrophotometric methods indicate that *P. punctifer* larvae could be weaned as early as 12 dpf (12.02 ± 0.18 mm TL). These results explain the successful three-day weaning strategy starting at 12 dpf tested with this species (Darias et al., 2015a). After intestinal maturation, from 12 dpf onwards, BB enzyme activities showed some fluctuations that could be attributed to the switch from live food to the formulated feed. A similar increase and decrease (teeth saw profile) in specific activities of BB and cytosolic enzymes during larval development has also been reported in other fish species such as bay snook (Uscanga-Martínez et al., 2011) and butter catfish (Pradhan et al., 2013), among others.

During the first stages of larval development and before the onset of acidic digestion, the digestion of proteins occurs by the action of alkaline proteases, mainly trypsin and chymotrypsin, in combination with intestinal cytosolic peptidases. In this phase, the larvae have limited capacity of digesting macromolecules that are absorbed by enterocytes (Cahu and Zambonino-Infante, 2001). According to Gisbert et al. (2014), the pancreatic exocrine cells and zymogen granules (precursors of pancreatic digestive enzymes) in *P. punctifer* were detected in the exocrine pancreas before the onset of exogenous feeding. In line with this, the activity of pancreatic enzymes (total alkaline proteases, trypsin, chymotrypsin amylase and lipase) in *P. punctifer* larvae was also detected before the onset of exogenous feeding. The presence of pancreatic enzymes activity before first feeding has also been reported in

many other fish species (Cahu and Zambonino-Infante, 2001; Zambonino-Infante et al., 2008) as it enables fish to be ready to digest exogenous food at mouth opening. The early presence of alkaline proteases in *P. punctifer* larvae coincides with the histological findings regarding the consumption of the yolk sac and the morphogenesis of digestive organs, appearance of the liver and the pancreas, as well as zymogen granules (between hatching and 4 dpf) (Gisbert et al., 2014). These processes have also been noticed in other tropical carnivorous fish species such as butter catfish (Pradhan et al., 2013), common snook (Jiménez-Martínez et al., 2012) and tropical gar (Frías-Quintana et al., 2015). The increment in enzyme activities from 12 dpf (12.02 ± 0.18 mm TL) coincided with the complete morphological formation of the digestive system in *P. punctifer* (Gisbert et al., 2014). In addition, the variations in the level of digestive enzyme activities from 17 to 25 dpf coincided with the transition from live prey to compound diets, revealing a modulation of the activity of pancreatic enzymes (trypsin, chymotrypsin, total alkaline proteases, amylase and lipase) by food composition as has been typically observed for other species (Cahu and Zambonino-Infante, 2001; Uscanga-Martínez et al., 2011). In this study, the activity of trypsin and chymotrypsin was initially detected at hatching. The importance of these enzymes during the first stages of development might be explained by their involvement in the cleavage of proteins contained in the yolk (Zambonino-Infante et al., 2008) and/or digestion and breakage of the egg chorion during the hatching process (Gisbert et al., 2009). Then, the activity of trypsin and chymotrypsin in *P. punctifer* remained constant until the end of the study (27 dpf, 35.77 ± 1.11 mm TL). In a study made with *Pseudoplatystoma fasciatum*, a decrease in pancreatic alkaline protease activities was detected at 10 days after hatching (dah) concomitant with an increase in pepsin-like activity (Dabrowski and Portella, 2006). However, the redescription of the species of the genus *Pseudoplatystoma* (Buitrago-Suárez and Burr, 2007) as well as the contradictions between this taxonomic revision and subsequent genetic and morphological studies (García-Dávila et al., 2013; Estivals et al., 2015) make drawing conclusions difficult at the species level. In any case, the aforementioned differences in digestive enzyme activity could be attributed to differences in the rearing conditions and feeding regimes. Similar profiles of trypsin and chymotrypsin activity to those of *P. punctifer* were observed in tropical gar (Frías-Quintana et al., 2015), both species fed a 45%-protein content diet. In other species, the activities of trypsin and chymotrypsin increased during the larval development and peaked at the transition from the larval to the juvenile stage, such as in the Mayan cichlid (*Cichlasoma urophthalmus*; López-Ramírez et al., 2010) and spotted rose snapper (Moguel-Hernández et al., 2014), which occurred at around 24–25 dah. It seems that a pattern of increasing trypsin and chymotrypsin activity is more easily detectable in species exhibiting a longer larval period and that this increase could be masked by changes in diet composition at weaning in faster-developing species.

In the present study, high values of α-amylase were detected from hatching, which may be due to the presence of glycogen deposits accumulated in the yolk sac (Gisbert and Doroshov, 2006). Moreover, the presence of α-amylase activity before exogenous feeding may be triggered by intrinsic mechanisms rather than by diet stimulation as it has also been reported in other fish species (Lazo et al., 2000; Zambonino-Infante et al., 2008). The α-amylase activity in carnivorous species is typically high at early stages and decreases during development (Cahu and Zambonino-Infante, 2001). Although differences were not statistically significant due to the high individual variability, α-amylase activity in *P. punctifer* tended to decrease during the larval period and to increase in response to changes in the diet composition at weaning. Such response may be related to the type of carbohydrate rather than the content, since the *Artemia* nauplii and the compound diet used in the present study contained similar amounts of carbohydrates (~10% and ~8%, respectively). These results are in contrast with studies made in other tropical fish species like butter catfish (Pradhan et al. 2013) and tropical gar (Frías-Quintana et al., 2015), where α-amylase had low specific activity at hatching and gradually increased after exogenous feeding. Differences in α-amylase activity patterns between species might be linked to differences in their digestive physiology and feeding habits (Solovyev et al., 2014), but also due to variations in rearing protocols among species.

The activity of lipase is modulated by lipid composition (Cahu and Zambonino-Infante, 2001; Morais et al., 2004; Zambonino-Infante et al., 2008) and therefore its analysis is a valuable tool to determine the adequate lipid composition to include in the diet for an optimal utilization (Uscanga-Martínez et al., 2011). The activity of lipase has been shown to be greater in carnivorous than in omnivorous or herbivorous fish species (Chakrabarti et al., 1995; Tengjaroenkul et al., 2000; Solovyev et al., 2014) and generally increases during ontogeny (Zambonino-Infante et al., 2008). In the current study, lipase activity was high at hatching and then decreased at first feeding and increased again from weaning until the end of the study. The early lipase activity observed in *P. punctifer* is in line with the previous histological findings that showed luminal digestion and absorption by the first week of life of larvae, reflecting the functional development of the intestine, and the vacuolization of the hepatocyte cytoplasm and the appearance of lipid droplets in the intermediate regions of the intestine and the liver during the mixed nutritional period (Gisbert et al., 2014). The early activity of lipase has also been reported in tropical gar (Frías-Quintana et al., 2015) and spotted rose snapper (Moguel-Hernández et al., 2014). The lower lipase activity level observed in *P. punctifer* larvae during the *Artemia* feeding period (4–17 dpf) compared to that of the compound diet feeding phase (17–27 dpf) could be related to the different lipid content and/or composition of the *Artemia* (~15% DM) and compound diet (~10% DM) used in this study. The lower lipase activity may indicate an inadequate lipid composition of *Artemia* for this species, since a better growth and survival and a more balanced lipid metabolism of *P. punctifer* larvae and early juveniles were observed when fed enriched *Artemia* and compound diets with an enriching product containing high levels of DHA (Magris et al., 2013; Darias et al., 2015b). This would be in accordance with the previous observation that *Artemia* nauplii do not fully cover the nutritional needs of the larvae (Gisbert et al., 2014; Darias et al., 2015a) and, therefore, this could be also accounting for the lower grow rate found at this developmental stage. Further nutritional studies focused on the larval stage are needed in order to improve growth. The changes in lipase activity after weaning might be indicative of changes in the nutritional requirements, which are reflected in the rate of growth. Moreover, the increase in specific activity of lipase at the end of the experiment (35.77 ± 1.11 mm TL) may be related to the adaption of weaned early juveniles to formulated diet and to the acquisition of the full digestive capacities of this species. The increase in the capacity for digesting lipids during development has been also described for some other catfish species like the sheatfish (*Silurus soldatovi*; Liu et al., 2010), striped catfish (Rangasin et al., 2012) and butter catfish (Pradhan et al., 2013).

Although the appearance of gastric glands was observed at 8 dpf (7.31 ± 0.48 mm TL) in this species (Gisbert et al. 2014), pepsin activity was surprisingly detected at 4 dpf (6.05 ± 0.28 mm TL). However, this activity was not associated to the gastric function, but rather to the presence of other acid proteases such as lysosomal cathepsins, which might be involved in the digestion of yolk proteins (Carnevali et al., 2001). The increase in acid protease activity from 4 dpf (6.05 ± 0.28 mm TL) to 12 dpf (12.02 ± 0.18 mm TL) was related to the appearance of gastric glands as histological data indicated (Gisbert et al., 2014). The onset of acid digestion occurred at the latest at 12 dpf (12.02 ± 0.18 mm TL), after that, the stomach became morphologically complete and the glandular stomach became functional, acquiring thus the adult-like mode of digestion. Therefore, 12 mm TL may be considered as an optimal size for weaning of *P. punctifer* larvae. Fluctuations in pepsin activity after the weaning period could indicate the adaptation of individuals to the feed composition. Similar results were found in other silurid species with several differences in the timing of

organ differentiation and development, such is in butter catfish (Pradhan et al., 2013), *Silurus glanis* (Kozaric et al., 2008) and yellow catfish (Yang et al., 2010).

5. Conclusions

Pseudoplatystoma punctifer showed an exponential growth pattern with two different growth rates: a slower one from hatching to 12 dpf (12.02 ± 0.18 mm TL) followed by a faster one until the end of the study (27 dpf, 35.8 ± 1.11 mm TL). The growth was slow in the initial phase because the energy was used for developing the digestive organs, while after the completion of the digestive system ontogeny the energy was allocated for growth. Also, the use of *Artemia* nauplii during the larval stage could account for this slow growth as this prey has shown to not meet the nutritional requirements for this species. *P. punctifer* larvae showed a fast digestive system development with an enzymatic profile typical of a tropical and carnivorous species. The activity profiles of the intestinal, pancreatic and gastric digestive enzymes reflected the functional changes associated to their ontogeny and to the feeding regime. The digestive system was functional at the latest at 12 dpf when larvae measured 12.02 ± 0.18 mm TL, indicating the transition from the larval to the juvenile stage (alkaline to acid digestion) in this catfish species. Present biochemical data on enzyme activity were in agreement with the previous description of the histological organization of the digestive system. Thus, considering both histological and functional data, it can be concluded that *P. punctifer* larvae can be successfully weaned from 12 dpf onwards. The variations observed in the enzymatic activity from 17 dpf (> 17 mm TL) onwards reflected the adaptation of the enzymatic machinery to the new diet supplied.

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CAPÍTULO 2

Estudio de las necesidades nutricionales de *Pseudoplatystoma punctifer* durante las etapas tempranas de vida

2.1

The digestive function of *Pseudoplatystoma punctifer* early juveniles is differentially modulated by dietary protein, lipid and carbohydrate content and their ratios

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Resumen

Pseudoplatystoma punctifer es un pez gato amazónico muy apreciado por la alta calidad de su carne, su tamaño y valor comercial. Su cultivo se persigue para satisfacer las demandas de una población creciente en la región. Sin embargo, el conocimiento de las necesidades nutricionales durante las primeras etapas de vida es necesario para mejorar el crecimiento y reducir la incidencia del canibalismo, factores que limitan el éxito de su cultivo comercial. Este estudio tuvo como objetivo evaluar el efecto de cuatro dietas con diferentes niveles de proteínas y lípidos (30:15, 30:10, 45:15 o 45:10 en %) en la fisiología digestiva y el rendimiento de los juveniles tempranos. Los resultados mostraron que los niveles y proporciones de proteínas:lípidos y carbohidratos de la dieta influían de manera diferente en la composición proximal de los peces, la fisiología y el desarrollo digestivo y, por lo tanto, el crecimiento y la supervivencia. La dieta 45:15 promovió el mejor crecimiento y supervivencia y el desarrollo más rápido del sistema digestivo, como se muestra a nivel histológico (mayor número de hepatocitos, células caliciformes en el intestino anterior y enterocitos en todas las porciones intestinales, y pliegues más largos en el intestino posterior), molecular (máxima expresión génica de la amilasa, lipoproteína lipasa, fosfolipasa, tripsinógeno y pepsinógeno) y bioquímica (máxima actividad de lipasa y pepsina y mayor proporción de actividad fosfatasa alcalina:leucina alanina peptidasa). Los lípidos se vieron favorecidos sobre los carbohidratos como fuente de energía, y los lípidos promovieron un ahorro de proteínas cuando la proporción de energía:proteína fue la adecuada. Un contenido de carbohidratos superior al 25% fue excesivo para esta especie, lo que provocó un desequilibrio del metabolismo lipídico y la deposición de grasa en el hígado.

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The Digestive Function of *Pseudoplatystoma punctifer* Early Juveniles Is Differentially Modulated by Dietary Protein, Lipid and Carbohydrate Content and Their Ratios

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Simple Summary: The Neotropical catfish *Pseudoplatystoma punctifer* is a promising candidate species for aquaculture diversification in the Amazon basin. However, optimized feeding strategies are needed to improve growth and survival during the early life stages. In order to determine the nutritional needs of early juveniles, the influence of four diets containing different protein and lipid content in their digestive physiology and performance was evaluated. To do so, the gene expression and the activity of the main digestive enzymes, the histology of the digestive organs, and the incorporation of the dietary macronutrients in the body tissue of fish fed the different diets were analyzed. The diet containing the highest protein and lipid content promoted the fastest development of the digestive capacities, resulting in a better growth and survival that increased sixfold and twofold, respectively, in comparison to previous feeding protocols. Lipids were favored over carbohydrates as a source of energy and excessive carbohydrate content led to unbalanced lipid metabolism and fat deposition in the liver. This study provided new knowledge on the molecular and biochemical mechanisms underlying nutrient digestion in this species and on the nutritional needs of early juveniles, which represents a significant contribution towards the establishment of its commercial culture.

Abstract: *Pseudoplatystoma punctifer* is an Amazonian catfish highly appreciated for its high flesh quality, size, and commercial value. Its aquaculture is pursued to satisfy the demands of an increasing population in the region. However, knowledge of the nutritional needs during the early life stages is necessary for improving growth and reducing the incidence of cannibalism, factors that limit the success of its commercial farming. This study aimed at evaluating the influence of four diets containing different protein and lipid levels (30:15, 30:10, 45:15, or 45:10 in %) in the digestive physiology and performance of early juveniles. The results showed that the dietary protein:lipid as well as carbohydrate levels and ratios influenced differently the whole-body proximate composition, the digestive physiology and development, and hence growth and survival. The 45:15 diet promoted the best growth, survival, and the most rapid development of the digestive system, as shown at histological (higher number of hepatocytes, goblet cells in the anterior intestine and enterocytes in all intestinal portions, and longer folds in the posterior intestine), molecular (highest *amylase*, *lipoprotein*

lipase, *phospholipase*, *trypsinogen*, and *pepsinogen* gene expression), and biochemical (highest lipase and pepsin activities and higher alkaline phosphatase:leucine alanine peptidase activity ratio) levels. Lipids were favored over carbohydrates as source of energy, with lipids promoting a protein-sparing effect at adequate energy:protein ratio. Carbohydrate content higher than 25% was excessive for this species, leading to unbalanced lipid metabolism and fat deposition in the liver.

Keywords: neotropical fish; aquaculture; digestive enzymes; gene expression; nutrition; juveniles; histology; development; macronutrients; diet

1. Introduction

In fish farming, a balanced diet in terms of macro- and micronutrients is essential to assure survival, as well as optimal development, health, and growth, especially during the early life stages, when organs and systems are developing. Nutritional requirements are specific for each species, vary throughout development and depend on rearing conditions. Therefore, this knowledge is key to formulate feeds adapted to these specific dietary needs.

Feed costs usually represent the highest operating expense in aquaculture, with dietary proteins being the main and most expensive component of fish feeds. In order to spare proteins from energy metabolism, and hence reduce production costs, research efforts have been largely focused on replacing part of the protein content with nonprotein energy sources. However, a balanced dietary energy:protein (E:P) ratio is necessary for the regulation of food intake and metabolism of nutrients, including carbohydrates and lipids [1–3]. When nonprotein energy is not adequate and/or not supplied in sufficient amounts, proteins can be catabolized to meet the energy requirements in detriment of growth [4]. The most efficient diets contain both lipids and carbohydrates in adequate composition and amount allowing to spare protein to meet the global energy requirements and to provide proteins for somatic growth. The composition and content of these energy sources have a direct effect on growth, feed conversion efficiencies, nutrient retention, and body composition [1,2,5–13]. Lipids are the most preferred energy source for many fish species, especially the carnivorous ones. However, excessive lipid content may lead to reduced growth and increased body lipid deposition [6]. In general, carbohydrates are not the principal source of energy or carbon for fish and are poorly utilized by most carnivorous fish [5,14], although they can improve protein utilization in some species [8,15]. Since carbohydrates are less expensive than lipids, an increase in the level of inclusion in diets is sought, especially in herbivorous and omnivorous species that use starch more efficiently than carnivorous fish [14,16]. As with lipids, an excessive dietary carbohydrate content can alter the energy metabolism, leading to fat body deposition, and ultimately impairing growth [17,18]. Therefore, the optimum dietary ratios between protein and energy as well as between carbohydrate and lipid in fish feeds need to be carefully defined in order to promote a healthy development and an optimal growth and survival.

Pseudoplatystoma punctifer is a piscivorous migratory catfish native to the Amazon basin in Bolivia, Brazil, Colombia, Ecuador, Peru, and Venezuela [19]. Given its high flesh quality, size (up to 1.40 m total length, TL), and commercial value, *P. punctifer* is highly appreciated in the region and suffers from high fishing pressure [20,21], what has led to the development of the aquaculture of this species. However, a high incidence of cannibalism and a low acceptability of compound diets at weaning have been responsible for low survival during early life stages [22,23], hence limiting the success of its commercial farming. Recent studies on the morphological and functional ontogeny of the digestive system of *P. punctifer* [23,24] have shown that the transition from the larval to the juvenile stage from a digestive physiology point of view (transition from alkaline to acid digestion) occurs at around 10–12 days post fertilization (dpf; 12.02 ± 0.18 mm TL) at 28 °C, allowing to successfully wean individuals 1 weak earlier in comparison to previous rearing protocols [25]. In Darias et al. [25], we studied the nutritional needs of *P. punctifer* during the early juvenile

stage (from 13 to 26 dpf) using four compound diets containing different protein:lipid (P:L) levels (30:15, 30:10, 45:15, or 45:10, in %). Low protein diets were formulated to evaluate the potential protein-sparing effect of other energy sources, such as lipids and carbohydrates. The highest growth performance and lowest incidence of cannibalism was observed in individuals fed the 45:15 diet, where the increase in energy from lipids resulted in sparing proteins for growth [25].

With the aim to deepen in the understanding of the nutritional physiology of *P. punctifer* during early life stages, in this paper, we focused on the effect of these diets on the development and function of the digestive system of early juveniles of *P. punctifer* at histological, molecular, and biochemical levels. Regarding the latter, the study focused on the expression of the main digestive genes (trypsinogen (*try*), chymotrypsinogen (*chy*), amylase (*amy*), lipoprotein lipase (*lpl*), phospholipase (*phl*), pepsinogen (*pep*)) and the activity of intestinal (alkaline phosphatase—AP and leucine alanine peptidase—LAP), pancreatic (α-amylase, bile salt-activated lipase, trypsin, and chymotrypsin) and gastric (pepsin) enzymes.

2. Materials and Methods

2.1. Rearing and Feeding Protocol

Larvae were obtained by hormonally induced spawning of a sexually mature couple of *P. punctifer* (♀: 3.6 kg; ♂: 1.85 kg) from a broodstock maintained in captivity at the Instituto de Investigaciones de la Amazonia Peruana (IIAP, Iquitos, Peru). The female and male were injected intramuscularly with carp pituitary extract (Argent Chemical Laboratories, Inc., Redmond, WA, USA) at 5 mg kg^{-1} and 1 mg kg^{-1} of body weight, respectively, according to Darias et al. [25]. Fertilized eggs (fertilization rate ~99.9%) were incubated at 28 °C in 60 L cylindroconical tanks connected to a freshwater recirculating system. Hatching took place 20 ± 2 h later (hatching rate ~96%) and larvae were transferred at 3 dpf into a 30 L tanks connected to a clear water recirculation system with mechanical and biological filters. Water conditions were: temperature: 27.8 ± 0.7 °C, pH: 7.0 ± 0.5, dissolved oxygen: 7.4 ± 0.2 mg L^{-1}, NO_2^-: 0.38 ± 0.27 mg L^{-1}, NH_4^+: 0.26 ± 0.13 mg L^{-1}, and water flow rate: 0.2 L min^{-1}. Larvae and early juveniles were reared in triplicate (initial density 30 larvae L^{-1}) under a photoperiod of 0L:24D and fed 5 times a day with *Artemia* spp. nauplii from 4 to 12 dpf and weaned from 13 dpf within 3 days onto four experimental compound diets containing different P:L levels (30:15, 30:10, 45:15, or 45:10, Table 1). From 16 dpf, early juveniles were fed only these diets until 26 dpf. Once weaned, individuals were fed five times a day at 5% of the fish wet weight.

2.2. Growth and Survival Measurements

The methodology used for growth and survival measurements is described in Darias et al. [25]. In brief, individuals of *P. punctifer* were collected at 12, 20, and 26 dpf and euthanized with an overdose of Eugenol (0.05 µL mL^{-1}; Moyco®, Moyco, Lima, Peru). In order to monitor growth, 15 individuals were placed in a Petri dish, photographed using a scale bar and TL was measured on the digital images (300 dpi) using ImageJ software [26]. Wet weight (WW) was determined using an analytic microbalance (Sartorius BP 211 D, Data Weighing Systems, Inc., Elk Grove, IL, USA, ± 0.01 mg). Survival was calculated at 12 and 26 dpf with respect to the number of individuals at the beginning of each feeding period and calculated considering the number of individuals sampled at each sampling point [25].

2.3. Proximate Composition and Fatty Acid Analyses

Sampled individuals of 26 dpf were washed with distilled water at −80 °C until analysis. Total lipids of the compound diets and *P. punctifer* individuals were extracted in chloroform:methanol (2:1, *v/v*) using the method of Folch et al. [27] and quantified gravimetrically after evaporation of the solvent under a nitrogen flow followed by overnight vacuum desiccation. Total lipids were stored in chloroform:methanol (2:1, 20 mg mL^{-1})

containing 0.01% butylated hydroxytoluene (BHT) at −20 °C until analysis. Acid-catalyzed transmethylation was carried out using the method of Christie [28]. Methyl esters were extracted twice using isohexane:diethyl ether (1:1, *v/v*), purified on thin-layer chromatography plates (Silica gel 60, VWR, Lutterworth, UK), and analyzed by gas–liquid chromatography on a Thermo Electron Trace GC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 × 0.25 mm id; SGE, Milton Keynes, UK), using a two-stage thermal gradient from 50 °C (injection temperature) to 150 °C after ramping at 40 °C min^{-1} and holding at 250 °C after ramping at 2 °C min^{-1}, helium (1.2 mL min^{-1} constant flow rate) as the carrier gas and on-column injection, and flame ionization detection at 250 °C. Peaks of each fatty acid were identified by comparison with known standards (Supelco Inc., Bellefonte, PA, USA) and a well characterized fish oil, and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chrom-Card for Windows (Trace GC, Thermo Finnigan, Milan, Italy). Results of fatty acid content were expressed as a percentage of total fatty acids (TFA). Protein and carbohydrate contents were determined following the Lowry et al. [29] and the DuBois et al. [30] methods, respectively.

Table 1. Composition of the experimental diets. Dietary treatment codes correspond to the protein:lipid level included in tested diets. DM, dry matter.

Ingredients [1] (in % DM)	Dietary Treatments			
	30:15	**30:10**	**45:15**	**45:10**
Fishmeal	36	36	53	53
Hydrolyzed fishmeal (CPSP)	9	9	14	14
Lipids	14	8	12	7
Marine lecithin	3	8	3	7
Soybean lecithin	11	0	9	0
Gelatin	15	15	15	15
Wheat starch	20	26	0	5
Vitamin mix [2] (x4)	2	2	2	2
Mineral mix [3]	3	3	3	3
Betain	1	1	1	1

[1] All dietary ingredients obtained commercially. Fishmeal hydrolysate CPSP 90:10% lipids; soluble fish protein concentrate (Sopropêche, Boulogne sur Mer, France); soy lecithin (Ets Louis François, St Maur des Fosses, France); marine lecithin LC 60 (Phosphotech, St Herblain, France). [2] Composition per kilogram of vitamin mixture: choline chloride 60%, 333 g; vitamin A acetate, (4000 IU g^{-1}) 2 g; vitamin D3 (1920 IU g^{-1}) 0.96 g; vitamin E (40 IU g^{-1}) 20 g; vitamin B3 2 g, vitamin B5 4 g; vitamin B1 200 mg; vitamin B2 80%, 1 g; vitamin B6 600 mg; vitamin B9 80%, 250 mg; vitamin concentrate B12 (10 g kg^{-1}), 0.2 g; biotin, 1.5 g; vitamin K3. 51%, 3.92 g; meso-inositol 60 g; cellulose, 543.3 g. [3] Composition per kilogram of mineral mixture: 90 g KCl, 40 mg KIO_3, 500 g $CaHPO_4$ $2H_2O$, 40 g NaCl, 3 g $CuSO_4$ $5H_2O$, 4 g $ZnSO_4$ $7H_2O$, 20 mg $CoSO_4$ $7H_2O$, 20 g $FeSO_4$ $7H_2O$, 3 g $MnSO_4$ H_2O, 215 g $CaCO_3$, 124 g $MgSO_4$ $7H_2O$, and 1 g NaF.

2.4. Histological Analyses

Individuals of *P. punctifer* (*n* = 10) were sampled at 26 dpf from each tank and fixed in buffered formaldehyde (pH = 7.2) at 4 °C overnight. The day after, individuals were dehydrated with graded series of ethanol and stored in 70% ethanol at 4 °C until further processing. After the dehydration process, individuals were embedded in paraffin with an automatic tissue processor Histolab ZX-60 Myr (Especialidades Médicas MYR SL, Tarragona, Spain). Then, paraffin blocks were prepared in an AP280-2Myr station and cut into serial sagittal sections (3 µm thick) with an automatic microtome Microm HM (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Paraffin cuts were kept at 40 °C overnight. After that, samples were deparaffinized with a graded series of xylene substitute and stained by means of Periodic Acid Schiff (PAS) and Alcian Blue (AB) at pH 2.5 [31]. Histological preparations were observed under a Leica DM2000 LED microscope equipped with a camera Leica MC170 HD (Leica Microsystems Nussloch GmbH, Nussloch, Germany). The number of (i) mucosa folds and goblet cells of the anterior (AI), middle (MI), and posterior (PI) intestine; (ii) lipid vacuoles in the liver and intestine; and (iii) hepatocytes

were counted in 6 randomly chosen fields per specimen (100 μm or 100 μm^2 per field, depending on the case). In addition, the size of (i) mucosa folds and goblet cells of the AI, MI, and PI regions, (ii) epithelium of the intestine, and (iii) lipid droplets in the liver and intestine were measured (in μm or μm^2 depending on the case) in 6 randomly chosen fields per specimen. The surface of lipid droplets was calculated on a total of 40 lipid droplets from five fish per sampling point and tank following the formula $S = \frac{1}{4}\ \pi$ a b; where a and b were the minimum and maximum diameters of the item measured [32]. Measurements on histological slides were performed with the ImageJ software and data expressed as the range comprised between the minimum and maximum values recorded or mean ± S.D.

2.5. RNA Extraction and Gene Expression Analyses

Total RNA from individuals (100 mg) at 12 and 26 dpf was extracted using TRIzol™ (Invitrogen, San Diego, CA, USA) according to manufacturer's protocol. RNA concentration and quality were determined by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Madrid, Spain) measuring the absorbance at λ = 260 and 280 nm and by denaturing electrophoresis in TAE agarose gel (1.5 %), respectively. Total RNA was treated with DNase I Amplification Grade (Invitrogen, San Diego, CA, USA) according to manufacturer's protocol and then reverse transcribed in 10 μL reaction volume containing 3 μg total RNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, San Diego, CA, USA) with oligo (dT) (12-18) (0.5 μg/ul) and random hexamers primers (50 ng μL^{-1}), 10X RT buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCL), 25 mM $MgCl_2$, 0.1 M DTT, 10 mM dNTP mix, SuperScript™ II RT (50 U μL^{-1}), RNaseOUT™ (40 U μL^{-1}), followed by RNase H (2 U μL^{-1}) (Invitrogen, San Diego, CA, USA) treatment. Reverse transcription reactions were carried out in a thermocycler (Mastercycle R nexus GSX1, Eppendorf AG, Hamburg, Germany) and run according to manufacturer's protocol. The samples were diluted 1:20 in molecular biology grade water and stored at −20 °C until further analyses. The expression of *amy* (AC MT006358), *chy* (AC MT006344), *try* (AC MT006359), *lpl* (AC MT006346), *phl* (AC MT006345), and *pep* (AC MT006343) was analyzed in individuals at 12 and 26 dpf fed the four dietary treatments (30:15, 30:10, 45:15, and 45:10). Quantitative PCR analyses were carried out in triplicate in a 7300 Real-Time PCR System (Applied Biosystems, Roche, Barcelona, Spain). The amplification mix contained 1 μL cDNA, 0.5 μL primers (20 μM), and 10 μL SYBR Green Supermix (Life Technologies, Carlsbad, CA, USA) in a total volume of 20 μL. A negative control was included (no template control) for each set of reactions on each 96-well plate. The amplification conditions were as follows: 10 min at 95 °C, 40 cycles of 20 s at 95 °C, and 1 min at 65 °C, followed by 15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C, and finally, 15 s at 60 °C. A standard curve was obtained by amplification of a dilution series of cDNA for calculation of the efficiency (E) for each set of primers. Real-time PCR efficiencies were determined for each gene from the slopes obtained with Applied Biosystems software, applying the equation E = 10[−1/slope], where E is PCR efficiency. The relative gene expression ratio (R) for each gene was calculated according to Pfaffl's [33] formula:

$$R = (E_{\text{target gene}})^{\Delta Cq \text{ target gene (mean sample} - \text{mean reference sample)}} / (E_{\text{reference gene}})^{\Delta Cq \text{ reference gene (mean sample} - \text{mean reference sample)}},$$

where ΔCq is the deviation of the target sample minus the reference sample. The relative expression of the genes was normalized using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, AC MT006341) as the reference gene since it did not exhibit any significant variation in expression between samples.

2.6. Digestive Enzyme Activity Assays

The activity of intestinal (alkaline phosphatase and leucine alanine peptidase), pancreatic (α-amylase, bile salt-activated lipase, trypsin, and chymotrypsin), and gastric (pepsin) enzymes was used to evaluate the impact of the four dietary treatments on the digestive system of *P. punctifer* larvae and early juveniles. Fish pancreatic and intestinal segments

were dissected according to Cahu and Zambonino Infante [34] and homogenized in 30 volumes (*v/w*) of Tris-mannitol buffer (50 mM mannitol, 2 mM Tris-HCl; pH 7.5) for 30 s (Ultra-Turrax T25, Germany). Then, 100 µL of 0.1 M $CaCl_2$ was added to the homogenate and the extract was sonicated (Vibra-cell©, Sonics, Newtown, CT, USA) for 1 min. During the homogenizing process, samples were kept on ice (0–4 °C) to reduce the enzymatic activity. An aliquot of homogenate was stored at −80 °C until analysis. The remaining homogenate was processed for intestinal brush border (BB) purification according to the recommendations of Gisbert et al. [35] in order to properly determine alkaline phosphatase activity. To do so, the homogenate was centrifuged at 9000× *g* and 4 °C for 10 min, the precipitate discarded and the supernatant centrifuged again at 34,000× *g* and 4 °C for 30 min. The pellet containing the BB of enterocytes was resuspended in 1 mL of buffer (0.1 M KCl, 5 mM Tris-HEPES, 1 mM DTT; pH 7.5) and stored at −80 °C until analysis [36].

Enzyme activity was measured at 12, 20, and 26 dpf according to Castro-Ruiz et al. [24]. In brief, alkaline phosphatase (AP, E.C. 3.1.3.1) activity was quantified using PNPP (4-nitrophenyl phosphate) as substrate in 30 mM Na_2CO_3 buffer (pH 9.8); one unit of enzyme activity (U) was defined as 1 µg nitrophenol released per minute and milliliter of BB homogenate at 27 °C and measured at λ = 407 nm [37]. Leucine–alanine peptidase (LAP, E.C. 3.4.11) was performed using leucine–alanine as substrate in 50 mM Tris-HCl buffer (pH 8.0); one unit of enzyme activity (U) was defined as 1 nmol of the hydrolyzed substrate per minute and milliliter of homogenate at 27 °C and measured at λ = 530 nm [38]. Regarding pancreatic enzymes, α-amylase (E.C. 3.2.1.1) activity was measured at λ = 580 nm using soluble starch (0.3%) dissolved in Na_2HPO_4 buffer (pH 7.4) as substrate [39] and its activity was defined as the milligram of starch hydrolyzed during 30 min per milliliter of homogenate at 27 °C. Bile salt-activated lipase (E.C. 3.1.1) activity was measured using 4 p-nitrophenyl myristate as substrate dissolved in 0.25 mM Tris-HCl (pH 9.0), 0.25 mM 2-methoxyethanol, and 5 mM sodium cholate buffer. The reaction was stopped with a mixture of acetone:n-heptane (5:2), the extract was centrifuged (6080× *g*, 2 min at 4 °C), and the absorbance of the supernatant was read at room temperature at λ = 405 nm. Lipase activity was defined as the micromoles of substrate hydrolyzed per minute and milliliter of homogenate [40]. Trypsin (E.C. 3.4.21.4) activity was assayed at 27 °C and measured at λ = 407 nm using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) as substrate [41]. One U of trypsin per milliliter was defined as 1 µmol BAPNA hydrolyzed per minute and milliliter of enzyme. Chymotrypsin (EC. 3.4.21.1) activity was determined at 27 °C and measured at 256 nm using BTEE (N-benzoyl-L-tyrosine ethyl ester) as substrate in 80 mM Tris-HCl, 100 mM $CaCl_2$ buffer (pH 7.2). Chymotrypsin activity corresponded to 1 µmol BTEE hydrolyzed per minute and milliliter of homogenate [42]. Pepsin assays were performed at 27 °C and at λ = 280 nm using hemoglobin as substrate [42]. Pepsin activity was defined as 1 µmol of hemoglobin liberated per minute and milliliter of homogenate after 10 min of incubation. All spectrophotometric analyses were performed as recommended by Solovyev and Gisbert [43] in order to prevent sample deterioration. Enzymatic activities were read using a microplate scanning spectrophotometer (Synergy HT, Bio-Tek, Bad Friedrichshall, Germany) and expressed as specific (mU mg^{-1} protein) and total (mU $larva^{-1}$) enzyme activities. Soluble protein in enzyme extracts was quantified using the Bradford technique [44] using bovine serum albumin as a standard. All the assays were made in triplicate (methodological replicates).

2.7. Statistics

Results were expressed as mean ± S.D. (n = 3). All data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett's test). One-way ANOVA was performed to analyze differences in growth, proximate and fatty acid composition, histological measurements, gene expression, and enzyme activity during development and/or between dietary treatments. All pairwise multiple comparisons were performed using the Holm-Sidak method if significant differences were found at $p < 0.05$ to discrimi-

nate the significant differences. Statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, VA, USA).

3. Results

3.1. Growth and Survival

Although results of growth performance and survival were reported in Darias et al. [25], summary information is provided in Table 2. *P. punctifer* growth performance (TL and WW) and survival during the *Artemia* feeding period (4-12 dpf) did no show significant differences between experimental groups. However, differences in both WW and TL were observed among experimental groups at 20 and 26 dpf when fed the compound experimental diets; the groups fed the 45:15 and 30:10 diets showing the highest growth performance and the lowest WW values, respectively ($p < 0.05$). A similar trend was observed in terms of survival at the end of the experiment (26 dpf), with the groups fed the 45:15 and 30:10 P:L level diets presenting the highest and lowest values, respectively (Table 2, $p < 0.05$ [25]).

Table 2. Growth in terms of total length and wet weight at 12, 20, and 26 dpf, and survival at the end of the *Artemia* and compound diet feeding periods (12 and 26 dpf) of *P. punctifer* reared at 28 °C and in complete darkness. Data are expressed as means ± standard deviation (S.D.) ($n = 45$). Different superscript letters indicate statistically significant differences between dietary treatments (one-way ANOVA, $p < 0.05$). Dietary treatment codes correspond to the protein:lipid level included in the tested diets.

Parameters	Dietary Treatments			
	30:15	**30:10**	**45:15**	**45:10**
Total length (mm)				
12 dpf	11.92 ± 0.50	12.17 ± 0.47	12.19 ± 0.54	11.90 ± 0.70
20 dpf	22.45 ± 0.41 ^{b}	20.54 ± 1.08 ^{b}	25.78 ± 1.24 ^{a}	21.09 ± 0.94 ^{b}
26 dpf	38.35 ± 1.48 ^{b}	33.02 ± 0.77 ^{c}	46.90 ± 0.98 ^{a}	37.03 ± 0.94 ^{b}
Wet weight (mg)				
12 dpf	7.32 ± 0.74	7.19 ± 0.20	8.09 ± 0.35	7.49 ± 1.71
20 dpf	68.04 ± 4.90 ^{b}	55.31 ± 7.97 ^{b}	97.91 ± 19.48 ^{a}	60.63 ± 0.86 ^{b}
26 dpf	264.44 ± 19.80 ^{c}	220.85 ± 8.40 ^{d}	563.71 ± 8.75 ^{a}	306.76 ± 11.87 ^{b}
Survival (%)				
12 dpf	93.88 ± 6.77	85.62 ± 7.14	78.58 ± 5.88	85.21 ± 9.10
26 dpf	17.68 ± 1.52 ^{c}	12.66 ± 2.29 ^{d}	36.32 ± 1.23 ^{a}	23.44 ± 0.59 ^{b}

3.2. Proximate Composition and Fatty Acid of Diets and P. punctifer Early Juveniles

Proximate composition of diets and *P. punctifer* specimens are shown in Tables 3 and 4, respectively. The E:P ratios were similar between the diets with the same protein content ($p > 0.05$), whereas the lower protein diets presented a higher ratio (ca. 11 kcal g^{-1} protein) than that of the higher protein diets (ca. 7 kcal g^{-1} protein) ($p < 0.05$). At 26 dpf, feeding *P. punctifer* with these diets resulted in a similar proximate composition for individuals fed both the 30:15 and 30:10 diets (53% proteins, 13% lipids, and 6% carbohydrates) ($p > 0.05$), whereas individuals fed the 45:10 and 45:15 diets displayed different protein (57% vs. 47%, respectively), lipid (11% vs. 9%, respectively), and carbohydrate (3% vs. 2%, respectively) contents ($p < 0.05$).

Table 3. Composition of the experimental diets after manufacture. Data are expressed as mean ± S.D. (n = 3). Different superscript letters denote the presence of differences statistically significant between dietary treatments (one-way ANOVA, $p < 0.05$). Dietary treatment codes correspond to the protein:lipid level included in tested diets. DM, dry matter.

Analyses of the Diets (% DM)	Dietary Treatments			
	30:15	**30:10**	**45:15**	**45:10**
Proteins	30.07 ± 2.44 ^{b}	30.90 ± 4.01 ^{b}	43.13 ± 2.65 ^{a}	42.86 ± 2.03 ^{a}
Lipids	12.50 ± 1.21 ^{a}	7.43 ± 0.27 ^{b}	12.46 ± 0.51 ^{a}	10.36 ± 0.69 ^{b}
Carbohydrates	24.87 ± 1.22 ^{b}	31.31 ± 2.26 ^{a}	2.34 ± 0.17 ^{d}	7.58 ± 0.60 ^{c}
Moisture	18.89 ± 0.16 ^{b}	22.35 ± 0.02 ^{a}	17.48 ± 0.04 ^{c}	15.25 ± 0.07 ^{d}
Energy (KJ)	1387.50 ± 24.89	1316.13 ± 43.67	1203.68 ± 81.53	1291.56 ± 54.47
E:P ratio (kcal g^{-1} protein)	11.07 ± 1.10 ^{a}	10.19 ± 0.73 ^{a}	6.66 ± 0.04 ^{b}	7.20 ± 0.04 ^{b}

Table 4. Proximate composition (in % of dry matter) of *P. punctifer* individuals (26 dpf) fed the different diets. Data are expressed as mean ± S.D. (n = 3). Different superscript letters denote the presence of differences statistically significant between dietary treatments (one-way ANOVA, $p < 0.05$). Dietary treatment codes correspond to the protein:lipid level included in the experimental diets.

Proximate Composition (%)	Dietary Treatments			
	30:15	**30:10**	**45:15**	**45:10**
Proteins	52.70 ± 4.97	52.78 ± 1.79	47.06 ± 3.76	56.55 ± 3.65
Lipids	13.21 ± 0.23 ^{a}	13.02 ± 0.87 ^{a}	9.07 ± 0.45 ^{b}	11.21 ± 0.59 ^{a}
Carbohydrates	5.72 ± 0.02 ^{a}	5.95 ± 0.22 ^{a}	1.66 ± 0.12 ^{c}	3.31 ± 0.33 ^{b}
Ashes	1.65 ± 0.01	1.38 ± 0.03	1.80 ± 0.37	1.47 ± 0.09

Fatty acid composition of diets and *P. punctifer* specimens are shown in Tables 5 and 6, respectively. Total lipids and total fatty acids were highest in 30:15 and 45:15 diets and lowest in the 30:10 diet ($p < 0.05$). Regarding total n-6 PUFAs, the 30:15 diet contained the highest amount (34%), followed by the 45:15 diet (27%), whereas both the 30:10 and 45:10 diets contained the lowest amounts (ca. 3%) ($p < 0.05$). The 18:2n-6 (linoleic acid, LA) fatty acid was the one accounting for these differences among diets. Total n-3 PUFAs was highest in 30:10 and 45:10 diets (42%), followed by the 45:15 (23%) and the 30:15 diets (18%) ($p < 0.05$). The 18:3n-3 (linolenic acid, LLA), 20:5n-3 (eicosapentaenoic acid, EPA), and 22:6n-3 (docosahexaenoic acid, DHA) contents in the diets accounted for the abovementioned differences. The 18:3n-3 content was highest in the 30:15 and 45:15 diets (ca. 2.4%) and lowest in the 30:10 and 45:10 diets (ca. 0.8%) ($p < 0.05$). The EPA content was highest in the 30:10 and 45:10 diets (ca. 16.5%), followed by the 45:15 diet (9%) and the 30:15 diet (6%). The highest levels of DHA were found in the 30:10 and 45:10 diets (ca. 23%) and the lowest in the 30:15 and 45:15 diets (ca. 9%). Total PUFAs was highest in both 30:15 and 45:15 diets (ca. 50%) and lowest in both 30:10 and 45:10 diets (ca. 45%) ($p < 0.05$). The ratio n-3/n-6 PUFAs was highest in the 45:10 diet (14), followed by the 30:10 diet (11) and the 30:15 and 45:15 diets (ca. 0.7) ($p < 0.05$). The ratio DHA/EPA was highest in the 30:10 diet (1.5) and lowest in the 45:15 diet (1.1) ($p < 0.05$). Both ARA/DHA and ARA/EPA ratios were similar in the four experimental diets. The ratio LA/PUFA was highest in the 30:15 diet (0.7), followed by the 45:15 (0.5) and the 30:10 and 45:10 diets (ca. 0.05) ($p < 0.05$).

Concerning the total lipids and total fatty acid composition of *P. punctifer* specimens at 26 dpf, total lipids were similar in 30:15, 30:10, and 45:10 groups (ca. 122 mg g^{-1} DW), whereas the 45:15 group displayed a lower value (91 mg g^{-1} DW). Diets differing in the protein and lipid ratios significantly affected the fatty acid profile of fish ($p < 0.05$). Regarding total fatty acid content, individuals fed 30:15 and 30:10 diets showed the highest amount (ca. 85 mg g^{-1} DW), while those fed the 45:15 diet showed the lowest (59 mg g^{-1} DW) ($p < 0.05$). Total n-6 PUFAs was highest in fish from the 45:15 group (14%), followed by the 30:15 group (11.5%), whereas LA levels were lowest in the 30:10 and 45:10 groups

(ca. 5%) ($p < 0.05$). In particular, LA content accounted for such differences, being highest in specimens from the 45:15 group (13%), followed by the 30:15 group (10%), and was lowest in the 30:10 and 45:10 groups (3%). Total n-3 PUFAs levels were highest in both 30:10 and 45:10 groups (ca. 36%), followed by the 30:15 and 45:15 groups (ca. 31%) ($p < 0.05$). Several n-3 PUFAs accounted for these differences. In particular, the LLA content was highest in the 30:15 and 45:15 groups (ca. 1.1%) and lowest in the 30:10 and 45:10 groups (ca. 0.7%) ($p < 0.05$). The EPA content was highest in the 45:10 group (10%) and lowest in the 30:15 and 45:15 groups (ca. 8%) ($p < 0.05$). The 22:5n-3 (docosapentaenoic acid, DPA) content was highest in both the 30:10 and 45:10 groups (2.3%) and lowest in the 45:15 group (1.9%) ($p < 0.05$). The DHA content was highest on the 45:10 group (22%) and lowest in the 30:15 and 45:15 groups (ca. 18%) ($p < 0.05$).

Table 5. Total lipid and total fatty acids contents (in % of dry matter) and fatty acid composition (in % TFA) analyzed in the experimental diets. Data are expressed as mean ± S.D. ($n = 3$). Different superscript letters denote the presence of differences statistically significant between dietary treatments (one-way ANOVA, $p < 0.05$). Dietary treatment codes correspond to the protein:lipid level included in the tested diets.

Total Lipids and Fatty Acids	**Dietary Treatments**			
	30:15	**30:10**	**45:15**	**45:10**
Total lipid (mg g^{-1} DW)	124.99 ± 12.07 ^{a}	74.31 ± 2.75 ^{b}	124.58 ± 5.07 ^{a}	103.62 ± 11.64 ^{ab}
Total fatty acid (mg g^{-1} DW)	71.18 ± 7.85 ^{a}	38.91 ± 6.76 ^{b}	66.81 ± 3.38 ^{a}	52.16 ± 2.50 ^{ab}
14:0	0.82 ± 0.04 ^{c}	1.72 ± 0.00 ^{a}	1.04 ± 0.00 ^{c}	1.55 ± 0.25^{ab}
16:0	19.71 ± 1.86	20.99 ± 1.54	19.21 ± 1.21	19.94 ± 1.48
18:0	3.62 ± 0.52	3.37 ± 0.33	3.09 ± 0.18	3.15 ± 0.40
Total saturated	24.15 ± 1.30	26.08 ± 1.21	23.34 ± 1.02	24.64 ± 1.33
16:1	1.72 ± 0.79	3.60 ± 1.74	2.26 ± 0.90	3.09 ± 1.26
18:1n-9 (OA)	19.15 ± 2.90	16.71 ± 0.99	19.41 ± 1.26	19.16 ± 0.70
20:1	2.78 ± 0.04 ^{c}	6.61 ± 0.45 ^{a}	4.42 ± 0.46 ^{b}	7.06 ± 0.62 ^{a}
Total monounsaturated	23.66 ± 2.1 6 ^{b}	26.91 ± 0.30 ^{ab}	26.09 ± 0.82 ^{ab}	29.31 ± 0.06 ^{a}
18:2n-6 (LA)	33.88 ± 0.38 ^{a}	2.33 ± 0.17 ^{c}	26.99 ± 0.99 ^{b}	2.69 ± 0.46 ^{c}
18:3n-6	0.00 ± 0.00	0.21 ± 0.08	0.00 ± 0.00	0.08 ± 0.11
20:4n-6 (ARA)	0.16 ± 0.22	1.10 ± 0.35	0.16 ± 0.22	0.26 ± 0.37
22:5n-6	0.00 ± 0.00	0.10 ± 0.15	0.00 ± 0.00	0.00 ± 0.00
Total n-6 PUFA	34.03 ± 0.60 ^{a}	3.75 ± 0.45 ^{c}	27.15 ± 1.21 ^{b}	3.02 ± 0.02 ^{c}
18:3n-3 (LLA)	2.80 ± 0.37 ^{a}	0.73 ± 0.02 ^{b}	2.39 ± 0.16 ^{a}	0.83 ± 0.06 ^{b}
18:4n-3	0.29 ± 0.28	0.00 ± 0.00	0.31 ± 0.44	0.44 ± 0.62
20:4n-3	0.13 ± 0.18	0.30 ± 0.01	0.25 ± 0.05	0.37 ± 0.01
20:5n-3 (EPA)	6.11 ± 0.34 ^{c}	16.44 ± 0.21 ^{a}	9.12 ± 0.62 ^{b}	16.82 ± 0.69 ^{a}
21:5n-3	0.00 ± 0.00	0.06 ± 0.09	0.05 ± 0.06	0.14 ± 0.01
22:5n-3 (DPA)	0.30 ± 0.16	0.81 ± 0.20	0.48 ± 0.11	0.87 ± 0.24
22:6n-3 (DHA)	7.98 ± 0.20 ^{b}	24.00 ± 0.91 ^{a}	10.36 ± 0.98 ^{b}	22.43 ± 1.14 ^{a}
Total n-3 PUFA	17.62 ± 0.45 ^{c}	42.34 ± 0.59 ^{a}	22.96 ± 0.91 ^{b}	41.91 ± 0.92 ^{a}
Total PUFA	51.65 ± 1.04 ^{a}	46.09 ± 1.04 ^{b}	50.11 ± 0.31 ^{a}	44.93 ± 0.90 ^{b}
(n-3)/(n-6)	0.52 ± 0.00 ^{c}	11.37 ± 1.21 ^{b}	0.85 ± 0.07^{c}	13.86 ± 0.41^{a}
DHA/EPA	1.31 ± 0.04 ^{ab}	1.46 ± 0.07 ^{a}	1.14 ± 0.03 ^{b}	1.33 ± 0.01 ^{ab}
ARA/DHA	0.02 ± 0.03	0.05 ± 0.01	0.02 ± 0.02	0.01 ± 0.02
ARA/EPA	0.03 ± 0.04	0.07 ± 0.02	0.02 ± 0.03	0.02 ± 0.02
LA/PUFA	0.66 ± 0.01 ^{a}	0.05 ± 0.00^{c}	0.54 ± 0.02 ^{b}	0.06 ± 0.01 ^{c}
LLA/PUFA	0.05 ± 0.01 ^{a}	0.02 ± 0.00^{b}	0.05 ± 0.00 ^{a}	0.02 ± 0.00 ^{b}
OA/PUFA	0.37 ± 0.06	0.36 ± 0.01	0.39 ± 0.03	0.43 ± 0.01
PUFA/saturated	2.14 ± 0.07	1.77 ± 0.12	2.15 ± 0.08	1.83 ± 0.14

Table 6. Total lipid and total fatty acids contents (in % of dry matter) and fatty acid composition (in % TFA) analyzed in *P. punctifer* individuals of 26 dpf. Data are expressed as mean ± S.D. (n = 3). Different superscript letters denote differences statistically significant between dietary treatments (one-way ANOVA, $p < 0.05$). Dietary treatment codes correspond to the protein:lipid level included in the tested diets. TFA, total fatty acids.

Total Lipids and Fatty Acids	Dietary Treatments			
	30:15	30:10	45:15	45:10
Total lipid (mg g^{-1} DW)	132.11 ± 2.34 ^{a}	130.23 ± 8.69 ^{a}	90.74 ± 4.51 ^{b}	112.07 ± 5.94 ^{a}
Total fatty acid (mg g^{-1} DW)	88.99 ± 4.62 ^{a}	79.86 ± 2.03 ^{a}	59.33 ± 3.15 ^{b}	65.94 ± 2.40 ^{ab}
14:0	0.47 ± 0.07 ^{b}	1.07 ± 0.07 $^{a\,b}$	0.70 ± 0.13 ^{ab}	1.10 ± 0.21 ^{a}
16:0	23.34 ± 0.41	23.96 ± 0.78	23.09 ± 0.37	23.83 ± 1.73
18:0	8.06 ± 0.04	8.60 ± 0.36	7.79 ± 0.63	8.28 ± 0.63
Total saturated	31.88 ± 0.53	33.63 ± 1.24	31.58 ± 0.38	33.21 ± 2.61
16:1	1.49 ± 0.12 ^{b}	1.92 ± 0.09 ^{a}	1.47 ± 0.15 ^{b}	1.89 ± 0.09 ^{a}
18:1n-9 (OA)	14.21 ± 0.21	13.75 ± 0.10	14.36 ± 0.14	13.80 ± 0.30
20:1	4.27 ± 0.10	4.92 ± 0.51	4.00 ± 0.25	5.00 ± 0.33
Total monounsaturated	19.98 ± 0.15	20.59 ± 0.48	19.83 ± 0.22	20.69 ± 0.83
18:2n-6 (LA)	9.86 ± 0.38 ^{b}	3.10 ± 0.28 ^{c}	12.81 ± 0.93 ^{a}	3.00 ± 0.45 ^{c}
18:3n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:4n-6 (ARA)	1.35 ± 0.05	1.37 ± 0.04	1.25 ± 0.05	1.43 ± 0.12
22:5n-6	0.27 ± 0.01	0.26 ± 0.04	0.25 ± 0.03	0.28 ± 0.04
Total n-6 PUFA	11.48 ± 0.34 ^{b}	4.72 ± 0.32 ^{c}	14.31 ± 0.94 ^{a}	4.79 ± 0.31 ^{c}
18:3n-3 (LLA)	0.99 ± 0.12 ^{a}	0.70 ± 0.02 ^{b}	1.19 ± 0.12 ^{a}	0.68 ± 0.07 ^{b}
18:4n-3	0.59 ± 0.04 ^{b}	0.70 ± 0.05 ^{a}	0.56 ± 0.01 ^{b}	0.69 ± 0.02 ^{a}
20:4n-3	0.57 ± 0.00 ^{b}	0.67 ± 0.00 ^{a}	0.55 ± 0.00 ^{b}	0.67 ± 0.02 ^{a}
20:5n-3 (EPA)	8.44 ± 0.14 ^{b}	9.88 ± 0.14 $^{a\,b}$	7.57 ± 0.11 ^{b}	10.25 ± 0.73 ^{a}
21:5n-3	0.29 ± 0.00	0.51 ± 0.02	0.27 ± 0.00	0.45 ± 0.21
22:5n-3 (DPA)	2.08 ± 0.04 $^{a\,b}$	2.27 ± 0.09 ^{a}	1.90 ± 0.06 ^{b}	2.37 ± 0.34 ^{a}
22:6n-3 (DHA)	18.97 ± 0.06 ^{b}	21.19 ± 0.43 ^{ab}	17.22 ± 1.05 ^{b}	21.57 ± 0.98 ^{a}
Total n-3 PUFA	31.94 ± 0.33 ^{b}	35.92 ± 0.59 ^{a}	29.25 ± 1.02 ^{b}	36.68 ± 1.71 ^{a}
Total PUFA	43.42 ± 0.01 ^{ab}	40.64 ± 0.26 ^{b}	43.89 ± 0.68 ^{a}	41.47 ± 1.56 ^{b}
(n-3)/(n-6)	0.56 ± 0.02 ^{c}	7.61 ± 0.27 ^{a}	1.28 ± 0.11 ^{b}	7.68 ± 0.74 ^{a}
DHA/EPA	2.53 ± 0.08 ^{a}	2.50 ± 0.01 ^{a}	2.52 ± 0.15 ^{a}	2.11 ± 0.07 ^{b}
ARA/DHA	0.10 ± 0.01 ^{a}	0.06 ± 0.00 ^{b}	0.07 ± 0.00 ^{b}	0.07 ± 0.00 ^{b}
ARA/EPA	0.25 ± 0.02 ^{a}	0.15 ± 0.01 ^{b}	0.19 ± 0.01 $^{a\,b}$	0.14 ± 0.00 ^{b}
LA/PUFA	0.60 ± 0.01 ^{a}	0.14 ± 0.01 ^{c}	0.40 ± 0.02 ^{b}	0.07 ± 0.01^{d}
LLA/PUFA	0.04 ± 0.00 ^{a}	0.02 ± 0.00 ^{c}	0.03 ± 0.00 ^{b}	0.02 ± 0.00 ^{c}
OA/PUFA	0.33 ± 0.00	0.31 ± 0.00	0.32 ± 0.01	0.33 ± 0.01
PUFA/saturated	1.43 ± 0.02	1.25 ± 0.05	1.40 ± 0.04	1.24 ± 0.14

Total PUFA content in *P. punctifer* was highest in the 45:15 group (44%) and lowest in the 30:10 and 45:10 groups (ca. 41%) ($p < 0.05$). The ratio n-3/n-6 PUFA was highest in the 30:10 and 45:10 groups (ca. 8), followed by the 45:15 (1.3) and the 30:15 (0.6) groups ($p < 0.05$). The ratio DHA/EPA was highest in the 30:15, 30:10, and 45:15 groups (2.5) and lowest in the 45:10 group (2.1) ($p < 0.05$). The ratio ARA/DHA was highest in the 30:15 group (0.1) and the rest of the dietary groups showed lower values (ca. 0.07) ($p < 0.05$). The ARA/EPA ratio was highest in the 30:15 group (0.25) and lowest in the 30:10 and 45:10 groups (ca. 0.15) ($p < 0.05$). The ratio LA/PUFA was highest in the 30:15 group (0.7), followed by the 45:15, 30:10, and 45:10 groups (0.4, 0.1, and 0.07, respectively).

3.3. Histological Analyses

The level of lipid accumulation within hepatocytes was affected by the experimental diets (Table 7 and Figure 1).

Table 7. Hepatocyte number in the hepatic parenchyma and surface (S) of lipid vacuoles within hepatocytes of *P. punctifer* specimens (26 dpf) fed diets containing different protein:lipid levels (30:15, 30:10, 45:15, and 45:10). Data represent means ± S.D. of three replicate tests for each dietary treatment (n = 5 fish per replicate). Different letters within column denote statistically significant differences among dietary groups (one-way ANOVA, $p < 0.05$).

Dietary Treatments	Hepatocyte Number (in 100 μm^2)	S Hepatic Lipid Vacuoles (μm^2)
30:15	30 ± 0.9 ^{b}	140.99 ± 15.68 ^{a}
30:10	19 ± 1.2 ^{c}	117.89 ± 4.35 ^{a}
45:15	54 ± 3.2 ^{a}	19.97 ± 1.81 ^{c}
45:10	28 ± 2.1 ^{b}	26.53 ± 2.34 ^{b}

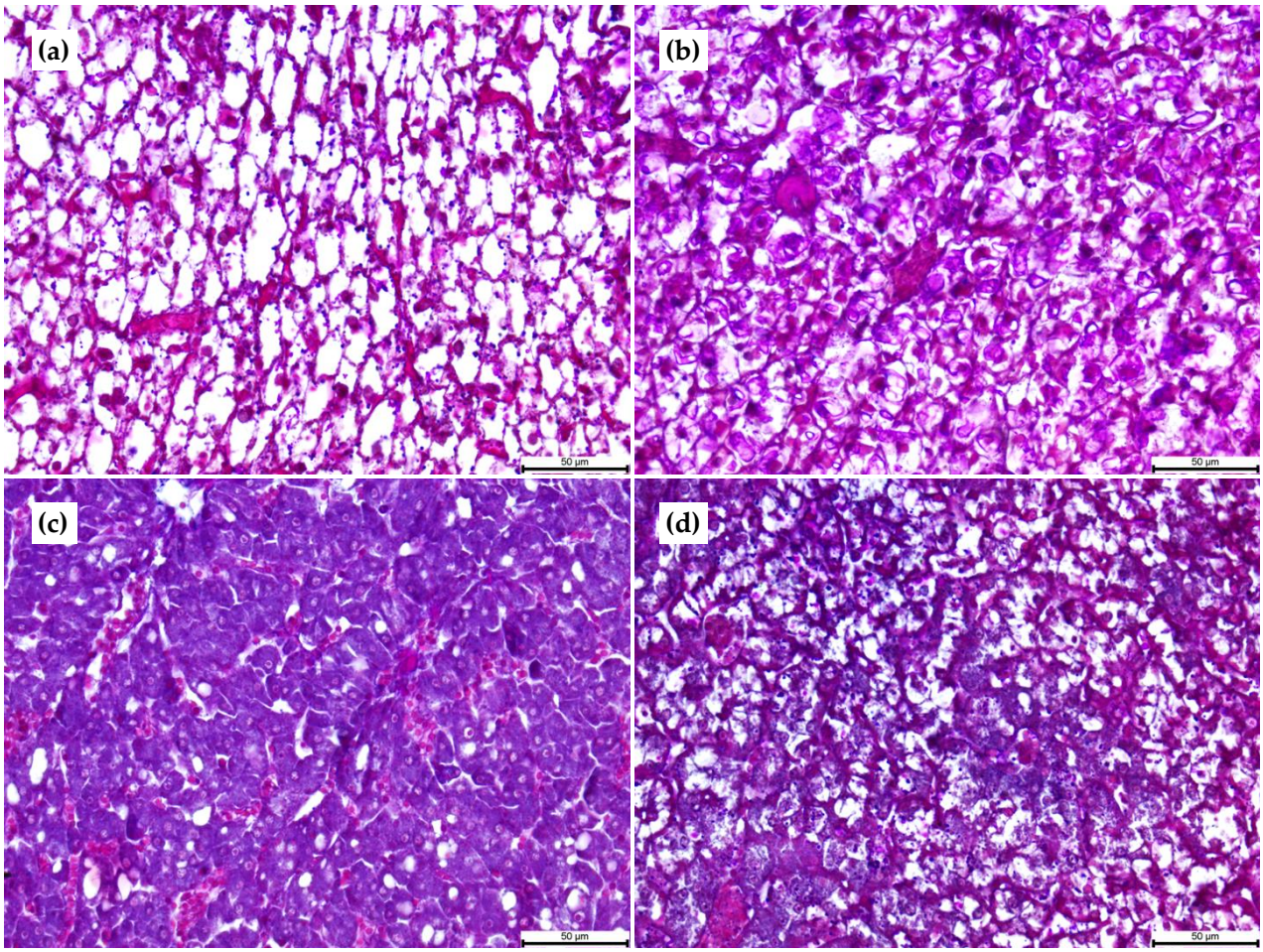

Figure 1. Longitudinal paraffin sections of the liver of *P. punctifer* (26 dpf) showing different levels of lipid accumulation. (**a**) Individuals fed the 30:15 diet, (**b**) individuals fed the 30:10 diet, (**c**) individuals fed the 45:15 diet, and (**d**) individuals fed the 45:10 diet. Magenta staining in the cytoplasm of hepatocytes indicates the presence of glycogen within cells. Staining: Periodic Acid Schiff (PAS)-Alcian Blue (AB) pH 2.5.

The surface size of the lipids vacuoles was highest in the 30:15 and 30:10 groups (ca. 130 μm^2) and lowest in the 45:15 group (20 μm^2) ($p < 0.05$). The PAS staining revealed a differential glycogen accumulation in the liver, which was associated with the different carbohydrate content of the diets. The number of enterocytes, intestinal folds, lipid inclusion, and goblet cells; the height of enterocytes; the length of intestinal folds; and the area of the lipid droplets in the intestine are shown in Table 8.

Table 8. Number and size of different components of the intestinal mucosa of *P. punctifer* specimens (26 dpf) fed the four different dietary treatments containing different protein:lipid levels (in % of dry matter). Data represent means ± S.D. of three replicate tanks for each dietary treatment (n = 5 fish per replicate). Different letters from "a" to "c" within columns denote statistically significant differences among dietary treatments within the same intestinal section (one-way ANOVA, $p < 0.05$). Different letters from "x" to "z" denote statistically significant differences between intestinal sections within the same dietary treatment (one-way ANOVA, $p < 0.05$).

Dietary Treatments	N° Enterocytes (in 100 μm)	Height Enterocytes (μm)	N° Folds (in 100 μm^2)	Length Folds (μm)	N° Lipid Droplets (in 100 μm)	S. Lipid Droplets (μm^2)	N° Goblet Cells (in 100 μm)
Anterior intestine							
30:15	30.0 ± 1.4^{ax}	16.9 ± 1.2^{by}	1.0 ± 0.0^{ay}	154.8 ± 122.6	0.0 ± 0.0^{y}	0.0 ± 0.0	3.8 ± 1.5^{b}
30:10	20.7 ± 1.2^{bx}	15.6 ± 1.2^{by}	0.0 ± 0.0^{b}	0.0 ± 0.0^{y}	0.0 ± 0.0^{y}	0.0 ± 0.0	3.9 ± 0.9^{bx}
45:15	26.0 ± 2.0^{ax}	14.4 ± 0.9^{bz}	1.0 ± 0.0^{a}	151.7 ± 54.9^{x}	0.0 ± 0.0^{y}	0.0 ± 0.0^{y}	11.0 ± 2.0^{ax}
45:10	18.0 ± 2.0^{bx}	22.4 ± 1.6^{ax}	1.0 ± 0.0^{ay}	282.8 ± 148.1	0.0 ± 0.0^{y}	0.0 ± 0.0^{y}	1.0 ± 0.9^{by}
Middle intestine							
30:15	22.3 ± 1.5^{ay}	23.0 ± 4.1^{axy}	2.2 ± 0.4^{bx}	75.4 ± 21.3^{a}	0.0 ± 0.0^{y}	0.0 ± 0.0	3.4 ± 1.9
30:10	16.3 ± 1.5^{by}	19.6 ± 1.5^{ax}	3.8 ± 0.3^{a}	10.9 ± 15.4^{cy}	0.0 ± 0.0^{y}	0.0 ± 0.0	5.3 ± 0.9^{x}
45:15	22.3 ± 1.5^{ay}	19.8 ± 0.9^{ay}	1.3 ± 0.6^{b}	37.3 ± 0.1^{bcy}	0.0 ± 0.0^{y}	0.0 ± 0.0^{y}	3.3 ± 1.3^{y}
45:10	18.0 ± 1.4^{bx}	11.5 ± 0.8^{bz}	2.0 ± 0.0^{bx}	56.71 ± 0.9^{ab}	0.0 ± 0.0^{y}	0.0 ± 0.0^{y}	4.1 ± 1.4^{x}
Posterior intestine							
30:15	11.3 ± 1.5^{bz}	27.8 ± 5.3^{bx}	0.7 ± 0.3^{y}	166.21 ± 9.1^{ab}	7.5 ± 1.9^{bx}	148.6 ± 107.5	3.0 ± 1.6
30:10	13.0 ± 0.7^{bz}	19.7 ± 0.5^{cx}	0.5 ± 0.7	111.5 ± 27.7^{bx}	7.0 ± 1.1^{bx}	48.4 ± 68.4	1.2 ± 0.5^{y}
45:15	18.3 ± 0.5^{az}	37.8 ± 4.5^{ax}	1.0 ± 0.0	227.9 ± 40.0^{ax}	11.5 ± 0.5^{ax}	301.5 ± 122.5^{x}	1.8 ± 1.3^{y}
45:10	11.7 ± 0.6^{by}	19.6 ± 1.1^{bcy}	0.7 ± 0.6^{y}	216.6 ± 52.3^{ax}	11.3 ± 1.2^{ax}	362.4 ± 141.4^{x}	0.3 ± 0.5^{y}

The number of enterocytes in the AI and MI was highest in the 30:15 and 45:15 groups and lowest in the 30:10 and 45:10 groups ($p < 0.05$). However, in the PI, the number of enterocytes was highest in the 45:15 group ($p < 0.05$) and similar in the rest of the dietary groups ($p > 0.05$). A decreasing number of enterocytes was observed from the AI to the PI in the 30:15, 30:10, and 45:15 groups ($p < 0.05$), whereas the number of enterocytes was highest and similar in the AI and MI ($p < 0.05$) and lowest in the PI in the group 45:10 ($p < 0.05$). The height of enterocytes of the AI was highest in the 45:10 group and lowest in the rest of the groups, whereas the opposite was found in the MI ($p < 0.05$). In the PI, enterocytes were taller in the 45:15 group followed by the 30:15 and 45:10 groups, with the 30:10 group displaying the shortest enterocytes. In the 30:15 group, the enterocytes of the PI were taller than those from the AI, with the height of the enterocytes of the MI being in between both sizes. In the 30:10 group, enterocytes were taller in the MI and PI than in the AI ($p < 0.05$). In the 45:15 group, the height of the enterocytes decreased from the PI to the AI. In the AI, no intestinal folds were observed within the surfaces analyzed in the 30:10 group, whereas it presented the highest number of intestinal folds in the MI ($p < 0.05$), followed by the rest of the dietary groups ($p > 0.05$). No differences in the number of intestinal folds were found in the PI between groups ($p > 0.05$). The number of intestinal folds in the groups 30:15 and 45:10 was higher in the MI than in the AI and PI ($p < 0.05$). No differences in the number of intestinal folds were found between the different intestinal regions in both the 30:10 and 45:15 groups ($p > 0.05$). The length of the intestinal folds was similar in all dietary groups (ca. 195 μm) ($p > 0.05$). In the MI, folds were longest in the 30:15 group and shortest in the 30:10 group ($p < 0.05$). However, in the PI, the 45:15 and 45:10 groups presented the longest folds and the 30:10 group presented the shortest folds ($p < 0.05$). The 30:15 and 45:10 groups presented similar intestinal fold sizes between the intestinal regions. However, the 30:10 group displayed longer intestinal folds in the PI than in the other two regions, whereas, in the 45:15 group, both the AI and PI had longer folds than the MI ($p < 0.05$). Lipid deposits were only found in the PI in all dietary groups. The 45:10 and 45:15 groups presented a similar number that was higher than that of the 30:10 and 30:15 groups ($p < 0.05$). However, the size of these lipid deposits within enterocytes was similar in all dietary groups ($p > 0.05$). The number of goblet cells in the AI was highest in the 45:15 group ($p < 0.05$), with the rest of the dietary groups presenting

similar values ($p > 0.05$). The number of goblet cells in the MI and PI was similar between the different dietary groups. The 30:15 group displayed a similar amount of goblet cells along the intestinal regions ($p > 0.05$), whereas the 30:10 group showed similar amount of goblet cells in the AI and MI ($p > 0.05$) that was higher than that of the PI ($p < 0.05$); the 45:15 group displayed higher number of goblet cells in the AI ($p > 0.05$) and similar amount in the MI and PI ($p > 0.05$); and the 45:10 group presented a higher number of goblet cells in the MI ($p < 0.05$) and similar amounts in the AI and PI ($p > 0.05$).

3.4. Gene Expression Analyses

The relative gene expression of *amy*, *lpl*, *phl*, *chy*, *try,* and *pep* at 12 and 26 dpf is shown in Figure 2. During the *Artemia* feeding phase (4 to 12 dpf), no significant differences were found in the expression of the analyzed digestive genes ($p > 0.05$).

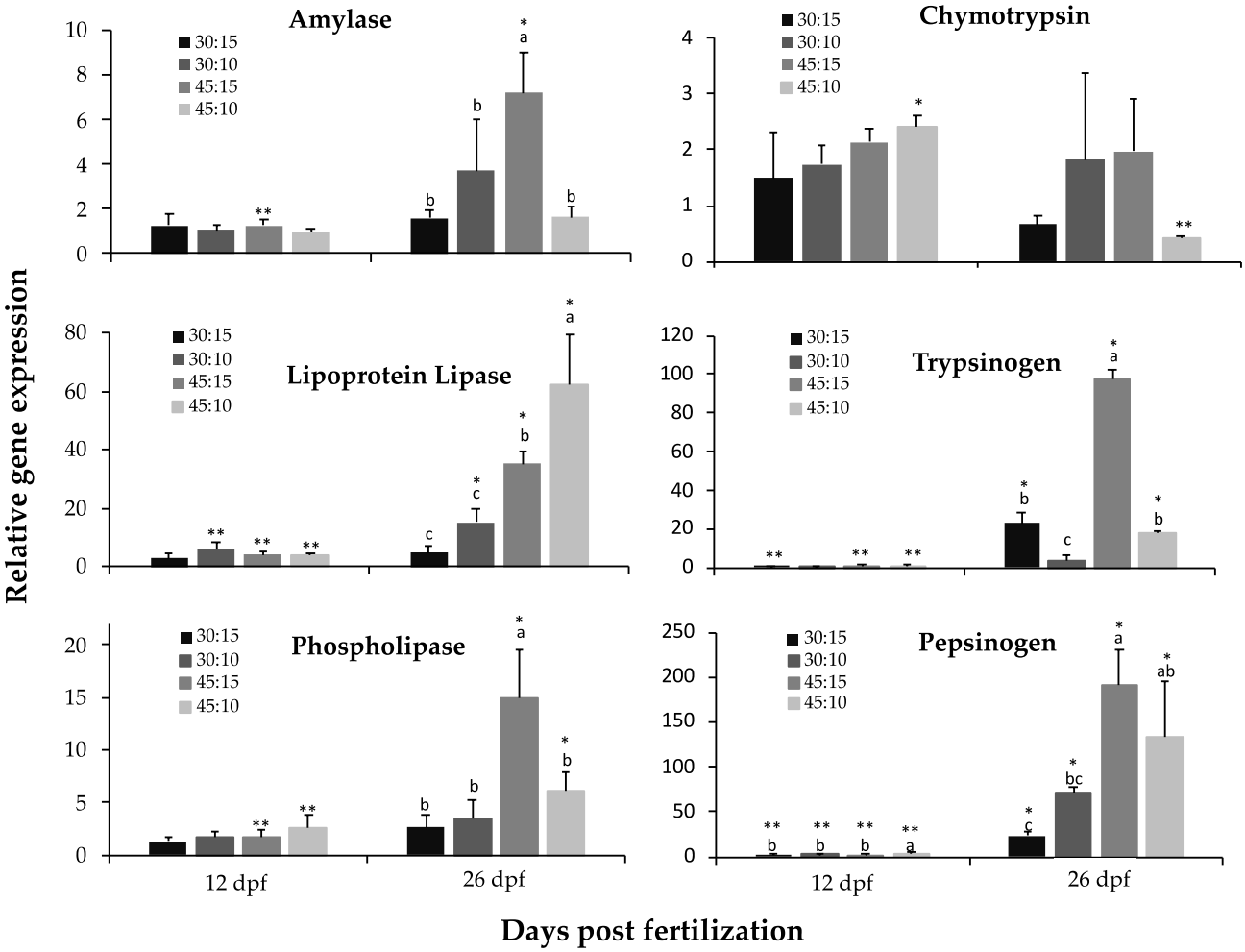

Figure 2. Relative expression of amylase, lipoprotein lipase, phospholipase, chymotrypsin, trypsinogen, and pepsinogen genes during the development of *P. punctifer* fed the different experimental diets containing different protein:lipid levels (in % of dry matter). Data are represented as means ± S.D. ($n = 3$). Values with a different superscript letter denote significant differences between dietary groups of the same age, and values with a different number of asterisks indicate significant differences between days for the same dietary treatment (one-way ANOVA, $p < 0.05$).

However, digestive genes were differentially modulated by the different compound diets at 26 dpf. The expressions of *try*, *amy*, *phl,* and *pep* were highest in the 45:15 group, whereas *lpl* expression was highest in the 45:10 group ($p < 0.05$). Similar levels of *amy* and *phl* expression were observed in groups 30:15, 30:10, and 45:10 ($p > 0.05$), whereas *lpl*, *try, and pep* expression were lowest in the 30:15 and 30:10 groups ($p < 0.05$). The lowest expression

for *try* was found in the 30:10 group followed by the 30:15 and 45:10 groups, whereas in the case of *pep*, the lowest expression was found in the 30:15 and 30:10 groups followed by the 45:10 group ($p < 0.05$). No significant differences were found in *chy* expression among the four dietary treatments ($p > 0.05$). *Try* and *lpl* expressions increased during development in all groups except in the 30:10 and 30:15 groups, respectively ($p < 0.05$). *Phl* expression increased during development in the 45:15 and 45:10 groups, whereas it remained stable in the others. *Chy* expression decreased during development in the 45:10 group, and *amy* expression increased during development in 45:15 group, whereas their expressions remained invariable in the rest of the groups ($p < 0.05$). *Pep* expression increased during development in all dietary groups (Figure 2; $p < 0.05$).

3.5. Digestive Enzyme Activity Analyses

The specific and total activities of the analyzed brush border (AP) and cytosolic (LAP) intestinal enzymes are shown in Figure 3. The specific and total AP activities were similar in all dietary treatments at 12 dpf ($p > 0.05$), whereas they were higher in the 45:15 group at 20 and 26 dpf ($p < 0.05$). The specific and total AP activities significantly increased during development in all dietary groups ($p < 0.05$). The specific and total LAP activities were similar in all dietary groups at 12 dpf ($p > 0.05$) and lowest in the 45:15 and 45:10 groups at 26 dpf ($p < 0.05$). The specific LAP activity decreased during development in all dietary groups ($p < 0.05$). In the 30:15 and 45:15 groups, total LAP activity increased from 12 to 20 dpf and then remained constant. In the 30:10 group, total LAP activity increased during the experimental period, and it remained invariable during development in the 45:10 group ($p > 0.05$). The high protein groups displayed higher specific AP/LAP ratio at 26 dpf and higher total AP/LAP ratio at both 20 and 26 dpf than the low protein groups ($p < 0.05$).

The specific and total activities of α-amylase, bile salt-activated lipase, chymotrypsin, trypsin, and pepsin are shown in Figure 4. The specific and total activities of α-amylase were similar in all dietary groups at 12 and 20 dpf ($p > 0.05$). However, at 26 dpf, specific and total α-amylase activities were highest in the 30:15 and 30:10 groups, and lowest in the 45:15 group ($p < 0.05$). Specific α-amylase activity decreased from 12 to 20 dpf in all dietary treatments to remain constant afterwards, with the exception of the 45:15 group, whose specific α-amylase activity continued to decrease at 26 dpf ($p < 0.05$). Total α-amylase activity remained constant during the experimental period with the exception of the 30:15 group, which showed an increase in activity from 20 to 26 dpf.

The specific and total activities of bile salt-activated lipase were similar in all dietary groups at 12 dpf ($p > 0.05$). At 20 dpf, specific activity continued to be similar in all dietary groups, whereas total activity was highest in the 45:15 group ($p < 0.05$). At 26 dpf, specific and total bile salt-activated lipase activities were highest in the 45:15 group and lowest in the 30:10 group ($p < 0.05$). Both specific and total bile salt-activated lipase activities increased during development in all dietary groups ($p < 0.05$).

The specific and total activities of chymotrypsin were similar in all dietary groups throughout the experimental period ($p > 0.05$). Chymotrypsin-specific activity remained invariable during development in the 30:15 group ($p > 0.05$), and total activity increased during development in the 30:10 group ($p < 0.05$). In the rest of dietary groups, chymotrypsin-specific and total activities increased from 12 to 20 dpf and remained constant thereafter ($p > 0.05$).

The specific and total activities of trypsin were similar in all dietary groups at 12 dpf ($p > 0.05$). At 20 dpf, specific and total trypsin activities were highest in the 45:10 and 45:15 groups, respectively, and lowest in both the 30:15 and 30:10 groups ($p < 0.05$). At 26 dpf, the level of specific and total trypsin activities was similar in all dietary groups ($p > 0.05$). The level of specific trypsin activity was constant during development in the 30:15 group ($p > 0.05$). In the 30:10 group, an increase in specific trypsin activity was observed between 12 and 26 dpf ($p < 0.05$). In the 45:15 and 45:10 groups, specific trypsin activity increased from 12 to 20 dpf and decreased afterwards ($p < 0.05$). Total trypsin activity increased

during development in the 30:10 group, whereas it increased from 12 to 20 dpf in both 45:15 and 45:10 groups to remain constant afterwards.

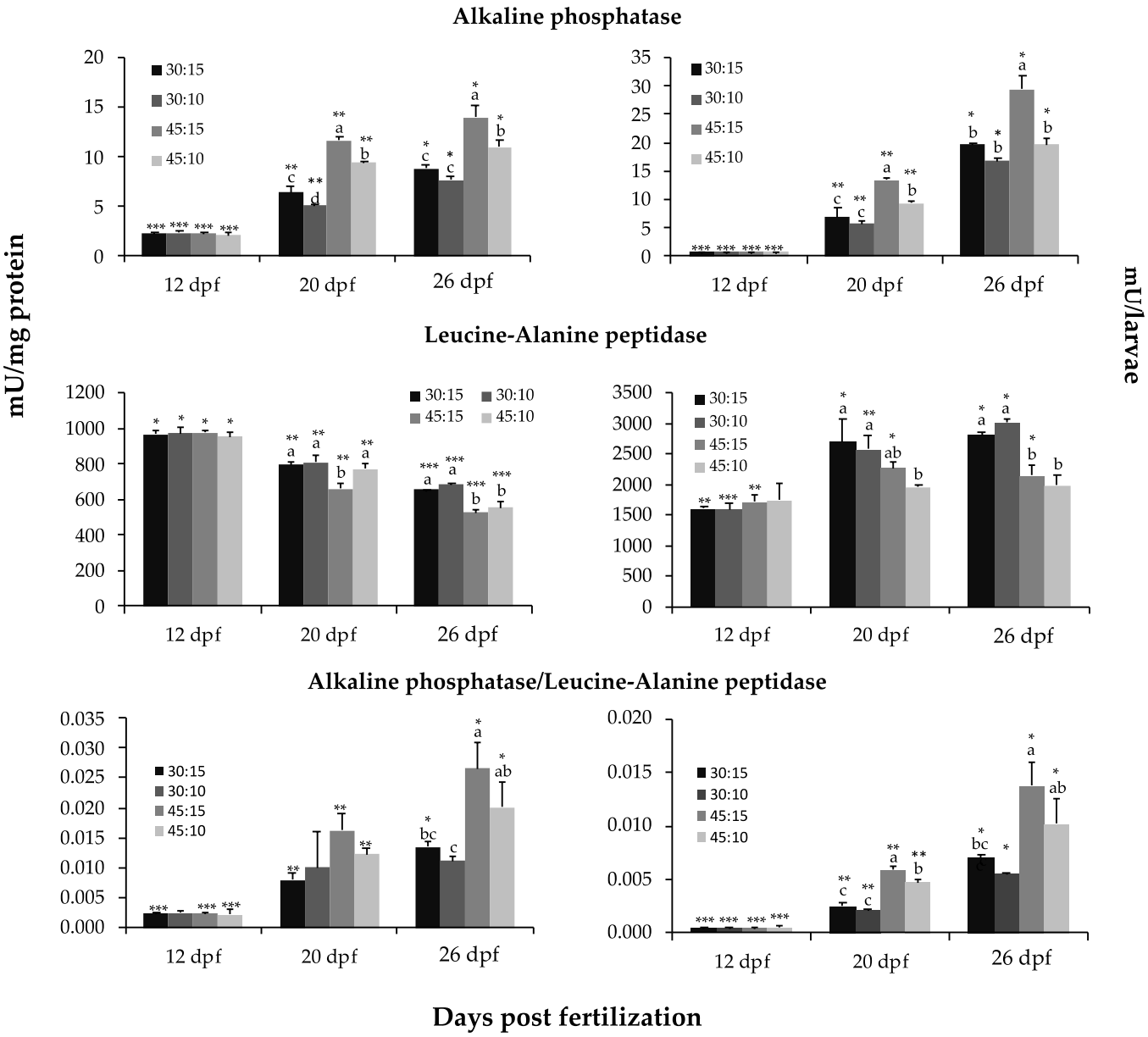

Figure 3. Specific (mU mg^{-1} protein, on the left) and total (mU $larva^{-1}$, on the right) activity of brush border (alkaline phosphatase) and cytosolic (leucine–alanine peptidase) intestinal enzymes and the ratio alkaline phosphatase to leucine–alanine peptidase ratio in *P. punctifer* (12, 20, and 26 dpf) fed the different experimental diets containing different protein:lipid levels (in % of dry matter). Data are represented as means ± S.D. (n = 3). Values with a different superscript letter denote significant differences between dietary groups of the same age, and values with a different number of asterisks indicate significant differences between days for the same dietary treatment (one-way ANOVA, $p < 0.05$).

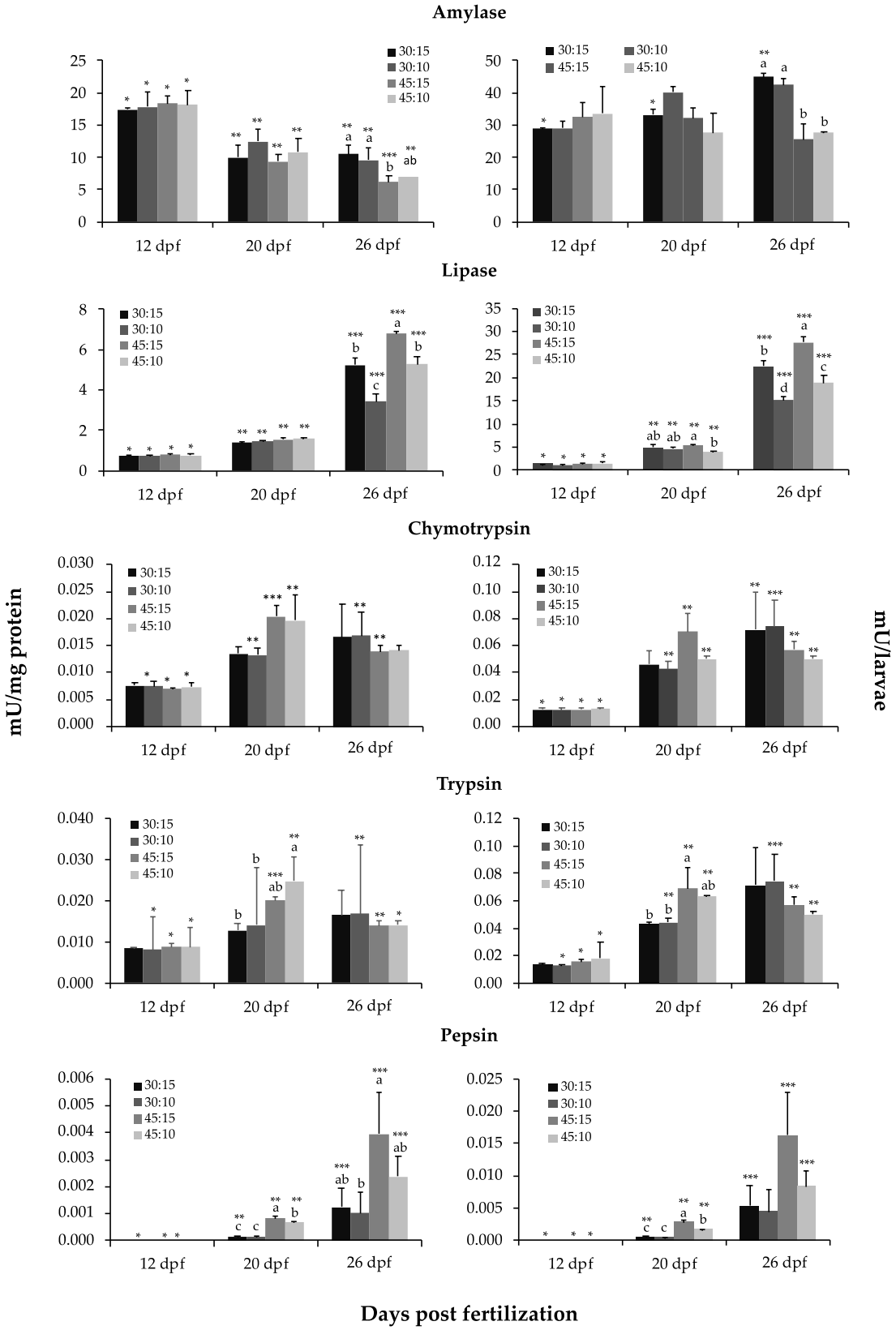

Figure 4. Specific (mU mg^{-1} protein, on the left) and total (mU $larva^{-1}$, on the right) activity of α-amylase, bile salt-activated lipase, chymotrypsin, trypsin, and pepsin in *P. punctifer* (12, 20, and 26 dpf) fed the different experimental diets containing different protein:lipid levels (in % of dry matter). Data are represented as means ± S.D. (n = 3). Values with a different superscript letter denote significant differences between dietary groups of the same age, and values with a different number of asterisks indicate significant differences between days for the same dietary treatment (one-way ANOVA, $p < 0.05$).

The specific and total activities of pepsin were similar in all dietary groups at 12 dpf ($p > 0.05$). At 20 dpf, specific and total pepsin activities were highest in the 45:15 group, and lowest in the 30:15 and 30:10 groups ($p < 0.05$). At 26 dpf, specific pepsin activity continued to be highest in the 45:15 group and lowest in the 30:10 group ($p < 0.05$), whereas total activity was similar in all dietary groups ($p > 0.05$). The specific and total pepsin activities were constant during development in the 30:10 group ($p > 0.05$) and increased during development in the rest of dietary groups ($p < 0.05$).

4. Discussion

The feeding protocol used in this study was adapted to the digestive capacities of developing *P. punctifer*. Thus, individuals were weaned at 13 dpf from live prey (*Artemia* nauplii) to the different experimental compound diets when the morphological and functional development of the digestive system was achieved [23,24], moment that we consider, from a digestive physiology point of view, the beginning of the juvenile stage in this species.

4.1. The Influence of Dietary Protein:Lipid:Carbohydrate Content and Ratios in P. punctifer Performance

This study aimed at understanding the physiological mechanisms underlying the different developmental responses observed in fish fed diets differing in their protein, lipid, and carbohydrate levels and ratios [25]. Four experimental diets were formulated with two different protein (30% vs. 45%) and lipid (10% vs. 15%) contents. Consequently, carbohydrates were used to complete the formula, with the low-protein diets having higher carbohydrate amounts (>25%) than the high-protein diets (<8%). The differences in growth and survival among experimental groups were primarily associated to the dietary protein content and, secondly, to the lipid level. The poorer growth performance in 30:10 and 30:15 groups indicated insufficient protein availability for maintaining proper growth and metabolism in *P. punctifer* early juveniles and insufficient and/or inadequate energy sources (lipids and carbohydrates) to compensate this protein deficiency.

As the carbohydrate content in the low-protein diets was significantly higher, their influence on the overall physiology of fish fed these diets cannot be neglected. In fact, both glycogen and lipid deposits in livers were correlated with the carbohydrate content of the diets. High carbohydrate levels (>30%) are known to increase lipid deposition in the liver of fish by enhancing lipogenesis and lipid uptake potential [12,17,45]. Moreover, fish feeds with an excess of carbohydrate content may lead to unbalanced fat depositions, suppressed immune function, and reduced health [10]. Excess carbohydrates are converted into simple sugars by digestion and excess glucose may be stored as glycogen (glycogenesis) or converted into lipids (lipogenesis) [46–49], playing a key role in nutrient retention. The increased glycogen and lipid deposits observed in the livers of individuals fed the 30:10 and 30:15 diets suggest that both glycogenesis and lipogenesis could have been occurred in the liver to regulate glucose homeostasis in *P. punctifer*. Further research on the regulation of glucose metabolism-related genes is needed to elucidate the carbohydrate metabolism in *P. punctifer* and to better determine the optimal dietary carbohydrate:lipid (C:L) ratio favoring protein sparing and overall physiological performance in this species. Indeed, although all diets were isoenergetic, high C:L ratio reduced growth, indicating that different energy sources influence the utilization of lipids and carbohydrates. This is because of the interconnection between these energy sources through the potential lipid conversion into glucose via gluconeogenesis and the potential glucose conversion into lipids via lipogenesis. Similar results have also been observed in other fish species [6,10,50]. Digestion and metabolism of dietary carbohydrates are closely associated to the feeding habits of the fish, with omnivorous and herbivorous fish being able to better utilize dietary carbohydrates [51–53] than carnivorous fish. This study showed that *P. punctifer* has a clear preference for lipids as a source of energy rather than carbohydrates. As observed in rainbow trout [17], dietary lipids had a better protein-sparing effect than carbohydrates at a similar level of digestible energy intake in *P. punctifer*, as shown by the better growth and survival of individuals fed the 45:15 diet (that contained 2% carbohydrates) compared to

the individuals fed the 45:10 diet (that contained 8% of carbohydrates). On the contrary, a preference of carbohydrates over lipids as a source of energy has been observed in some omnivorous freshwater fish [6,54]. Based on the overall results, the optimal dietary C:L ratio for *P. punctifer* was between 0.2 (45:15 diet) and 0.8 (45:10 diet).

The proximate composition of the whole body of *P. punctifer* was modulated by the different dietary protein:lipid:carbohydrate levels. The whole-body protein was the same in all dietary groups, which means that individuals fed the low-protein diets had to synthesize more proteins than those that had protein provided through the diet to enable them to reach the required amounts in their tissues (ca. 45%). On the contrary, the body protein content has been found to be positively correlated to the dietary protein content in other fish species [55–60]. The groups fed the low-protein diets presented higher carbohydrate content than the other dietary groups, and the fact that individuals fed the 45:15 diet presented the lowest carbohydrate and lipid content suggests that the E:P and/or C:L ratio promoted higher lipid accumulation in the other dietary groups. A dietary modulation of the body composition has also been observed in juveniles of tilapia *Oreochromis niloticus* × *O. aureus* [18], whereas no effect was observed in the Nile tilapia *O. niloticus* during the grow-out phase [4]. This could indicate differences in the regulation of nutrient metabolism depending on the species and developmental stages. Indeed, it has been suggested that anabolism and catabolism processes could take place in later stages of development to stabilize the proximate composition in whole body [4].

The E:P ratio can affect growth performance, feed efficiency, and body composition of fish. In this study, the growth performance and metabolism of *P. punctifer* were significantly affected by the dietary E:P ratio of the experimental diets. In particular, an E:P ratio of 11 kcal g^{-1} protein in the 30:15 and 30:10 diets induced lipid deposition in the liver and decreased somatic growth. Higher liver (or body) lipid content with increasing E:P ratio has been also observed in other fish species such as in grass carp *Ctenopharyngodon idella* [61], Chinese perch *Siniperca chuatsi* [3], *O. aureus* [1], and channel catfish *Ictalurus punctatus* [62]. A similar optimal E:P ratio for growth and overall performance to that found for *P. punctifer* (ca. 7 kcal g^{-1} protein) has been observed in Asian seabass *Lates calcarifer* [63], African sharptooth catfish *Clarias gariepinus* [64], and grass carp [61], whereas the higher E:P ratio that was suboptimal for *P. punctifer* (11 kcal g^{-1} protein) resulted to be optimal for channel catfish [62]. In *P. punctifer,* protein levels in the high E:P diets (30:10 and 30:15) were below the optimal level for this species and stage of development and were mostly used for body protein maintenance; and dietary lipids and carbohydrates were not used efficiently or were lacking in quantity for energetic purposes. In addition, a possible dietary starch interaction with protein digestibility cannot be neglected in these high-carbohydrate dietary groups, as a decrease in protein digestibility related to an increase in dietary starch content increase has been demonstrated in other fish [65]. The high carbohydrate content of these diets might have contributed to provide energy, as shown by the increased levels of α-amylase activity in these groups, although they did not contribute with sufficient efficiency to promote growth. Reduced growth as the result of overloading of carbohydrates in the fish diet has been observed in other fish species [17,45,66]. Therefore, low protein synthesis results in low growth performance and poor metabolism, whereas the opposite trend occurs when fish are fed with low E:P ratio diets [3].

If a diet is deficient in an essential nutrient, fish consume more feed to fulfill the demands for that specific nutrient [3]. In line with this, it has been shown that fish fed high carbohydrate diets need to increase their feed intake in order to gain adequate amino acids levels for promoting growth [4]. Assuming that this was also the case of *P. punctifer* fed the 30:10 and 30:15 diets, it can be hypothesized that energy supplied in these low-protein diets was insufficient in quantity and/or quality to compensate for the extra energy costs associated to deamination as well as to swimming and foraging behaviors; consequently, reducing the energy available for growth. Among the tested diets, the lower the E:P ratio the better growth and survival in *P. punctifer*. The opposite has been observed in the omnivorous Nile tilapia [4] and also in carnivorous fish such as in the black sea bream

Sparus macrocephalus [60]. This has been attributed to an excess of dietary protein that would result in the loss of energy for deamination of amino acids toward protein catabolism [4].

4.2. The Influence of Dietary Fatty Acid Composition in P. punctifer Body Fatty Acid Composition

Concerning the fatty acid composition of the different dietary groups, the DHA content of individuals from the 30:10 and 45:10 groups (ca. 23%) reflected that of the ingested diet (ca. 21%) and accounted for the majority of the total n-3 PUFA tissue content. However, fish fed the 30:15 and 45:15 diets, which contained lower DHA levels (ca. 9%), displayed considerably higher DHA content (ca. 18%). This suggests that *de novo* DHA synthesis occurred in those dietary groups, which is in accordance with the depletion of α-linolenic acid (LLA), the precursor of DHA biosynthesis, observed in these groups [67]. The DHA content in individuals from the 30:15 and 45:15 groups was significantly lower (ca. 18%) than that from the 30:10 and 45:10 groups (ca. 23%). These results suggest that the higher dietary DHA led to seemingly higher DHA levels in tissues than required.

As in the diets, LA accounted for the differences observed in the total n-6 PUFA content in fish. The total n-6 PUFA content was between 5 and 7 times higher in 30:15 and 45:15 diets compared to 30:10 and 45:10 diets. This profile in total n-6 PUFA content was also found in the dietary groups, although the differences were lower (ca. 2 times). When comparing the total n-6 PUFA content of the diet with that of the tissues of each dietary group, the total n-6 PUFA content (and so LA) remained the same in the 30:10 and 45:10 groups, whereas it was 2 and 3 times lower in the 45:15 and 30:15 groups, respectively. This indicates that higher dietary n-6 PUFA (meaning LA) content enabled the 30:15 and 45:15 groups to use some for physiological functioning and to stock a higher amount in their tissues than that allowed by the 30:10 and 45:10 diets. In addition, it appeared essential for 30:10 and 45:10 groups to retain the entirety of the low n-6 PUFA content (ca. 5%) of the 30:10 and 45:10 diets in their tissues. Since the 45:10 group displayed the second highest growth of the groups, it seems that a lower n-6 PUFA tissue content does not compromise the growth of specimens. The 30:15 and 45:15 groups displayed lower LA values than those of the diets, although it did not result in an increased ARA content. The different use of LA between both dietary groups resulted in the 45:15 group presenting the highest total n-6 PUFA content. The sum of total n-3 and n-6 PUFA contents of each dietary group led to a higher total PUFA content in 45:15 group and lower in the 30:10 and 45:10 groups, with the 30:15 group displaying intermediate values, although the differences in content were not very large (43% vs. 40%). The only clear differences between the group with the highest growth (45:15) and the rest of the dietary groups were the n-3/n-6 PUFA, the LA/PUFA, and the LLA/PUFA ratios for which the 45:15 displayed intermediate values. However, these profiles alone do not account for the differences in growth as the 45:10 group, which presented the second highest growth, displayed the highest n-3/n-6 ratio together with the lowest LA and LLA/PUFA ratios. The subtle balance between all the components in the 45:15 diet certainly accounted for the improved results observed in *P. punctifer* early juveniles in the present study, since the other diets that induced less efficient development had one or several compounds in a different proportion as compared to the composition of the 45:15 formula.

Contrary to marine fish [68], a dietary DHA:EPA ratio closer to 1 likely contributed to an improved performance in *P. punctifer*, as was the case of the 45:15 diet. Independently of the DHA:EPA ratio provided in the diets, *P. punctifer* early juveniles contained a similar DHA:EPA ratio in their tissues (ca. 2.5), suggesting that this is the proportion needed to maintain the correct physiological homeostasis. Similarly, the DHA:EPA ratio was consistently higher in the triacylglycerols deposited in farmed Atlantic salmon than in the fish oils used in salmon feeds [69]; and, in Atlantic salmon, the DHA:EPA ratio was also increased in the muscle compared to that of the diet, consistent with the selective oxidation of EPA, thereby resulting in a selective retention of DHA by the fish [67]. *P. punctifer* seemed to not depend on EPA or DHA, but rather on LLA to promote growth, as observed in the 30:15 and 45:15 groups, where the dietary LLA was used to synthesize DHA to meet the

tissue requirements vital for cell membrane structure and function (ca. 18% TFA). However, when dietary DHA levels were high enough for its tissue requirements, as it was the case of the 30:10 and 45:10 diets, then, the *de novo* DHA biosynthesis pathway from its precursor LLA seemed to be inhibited. Other freshwater fish such as the African sharptooth catfish and Tilapia zilli *Coptodon zillii* also convert C18 PUFA to HUFA and, in the case of the African catfish, high or low dietary n-3 HUFA levels have been shown not to influence growth [70,71]. The latter could not be confirmed in *P. punctifer* as all experimental diets contained high DHA levels (9–24% TFA). Due to the low protein and lipid content of the 30:10 diet, the lipids were mostly used to maintain the adequate lipid and fatty acid levels in tissues, which was similar to those of the other dietary groups, rather than for promoting somatic growth.

4.3. The Influence of Dietary Composition in the Digestive Function of P. punctifer

The number of hepatocytes in the liver was in line with the degree of physiological development of the individuals fed the different dietary treatments. Thus, individuals fed the 45:15 diet presented the highest number of hepatocytes and those from the 30:10 group the lowest. In terms of hepatic lipid accumulation, individuals fed the low-protein diets (30:15 and 30:10) showed the fattiest livers, likely due to an excess of dietary carbohydrate promoting lipogenesis in the liver [12,45,72,73]. As mentioned before, this pattern was also reflected in the total lipid and total fatty acid content of individuals. In particular, individuals from the 30:10 dietary group, although fed the lowest lipid (and protein) content, accumulated lipids and fatty acids similar to the levels observed in the 30:15 and 45:10 groups. A similar liver metabolic response was found in Nile tilapia, where lipogenesis increased in the liver when lipid intake was limited and *de novo* fatty acid synthesis increased the hepatic fatty acid content [73]. In Nile tilapia, the stimulated glycolysis by either dietary lipid deficiency or high dietary carbohydrate was suggested to provide the substrates for lipogenesis [73]. A similar situation could be happening in *P. punctifer* fed the 30:15 and 30:10 diets. The reduction in capacities for triglyceride and phospholipid hydrolysis in these individuals, as the reduced *lpl* and *phl* expression showed, could be indicators of the altered lipid metabolism observed in these individuals, hypothesis supported by the large accumulation of lipids in hepatocytes observed histologically. In addition, a decrease in lipase activity was observed in these dietary groups, which was correlated with the lipid inclusion levels in the posterior intestine. The reduced lipase activity observed in the 30:10 group together with the reduced number and area of lipid droplets in the posterior intestine suggests an alteration of the lipid digestion and absorption at the intestinal level in these individuals. The 30:10 diet seemed to have insufficient lipid and excessive carbohydrate content for *P. punctifer* early juveniles. The increase in α-amylase activity together with a decrease in bile salt-activated lipase activity and *lpl* expression suggests that both macronutrients are inducing an altered lipid metabolism. Similar findings have been observed in rats were increased levels of insulin produced by raised levels of glucose increased pancreatic α-amylase activity together with a decrease in chymotrypsinogen and lipase activities [74]. It has been shown in humans that *lpl* expression is more stimulated by carbohydrates than by lipids [75]. The *lpl* expression was particularly low in groups fed the 30:15 and 30:10 diets, which could be related to their higher dietary carbohydrate levels but also to their low protein content. Indeed, low dietary protein content has shown to decrease LPL activity and impair VLDL-TAG export from the liver in rats [76]. In line with this, the lower level of *lpl* gene expression observed in *P. punctifer* individuals fed the 30:10 and 30:15 diets could be indicating a limited VLDL export from the liver resulting in the development of fatty livers in these specimens. Moreover, protein deficiency can induce insulin resistance associated with reduced LPL activity, overproduction of TAG, and impaired VLDL catabolism [77]. In humans, insulin downregulates *lpl* expression in insulin-resistant individuals, which present an altered intracellular lipid metabolism [78]. Diabetics display a decrease in insulin following a high-carbohydrate diet [79]. In some fish, such as in rainbow trout, insufficient insulin secretion occurred when fed a high-carbohydrate

diet [48]. It is clear from this study that both carbohydrate and protein contents of the 30:10 and 30:15 diets were inadequate for *P. punctifer.* However, further research is needed to decipher the involvement and the interconnectivity of the mechanisms of action of these macronutrients in regards to the hepatic lipid metabolism in these specimens and whether these low-protein high-carbohydrate diets induce insulin resistance or not in *P. punctifer*. Concerning the 45:10 and 45:15 groups, the differences in the lipid:carbohydrate content ratio of the provided diets (1 vs. 5, respectively) could be responsible for the higher *lpl* expression in the 45:10 group compared to the 45:15 group. A higher dietary carbohydrate intake may have a stronger effect on fat storage in the liver, as observed in individuals from the 45:10 group in comparison to the 45:15 group. The higher *lpl* gene expression levels observed in the 45:10 and 45:15 groups reflect a better lipid transport from the vascular system to tissues, and the lowest lipid accumulation in the liver of these dietary groups indicates a more balanced lipid metabolism than the rest of the dietary groups. This was in line with the level of intestinal maturation of these groups compared to the others.

The activity of the pancreatic and intestinal enzymes are useful and reliable markers for assessing the development and state of the digestive function in fish [80]. As in many other fish, the specific activity of AP increases concomitantly with the decrease in the activity of LAP during the larval development of *P. punctifer* [24]. In this study, the ratio AP/LAP at 26 dpf measured in the four dietary groups indicated a lower degree of maturation of the intestine in the 30:10 and 30:15 groups, whereas the most mature intestine corresponded to individuals from the 45:15 group. These results indicate that the different dietary regimes were directly affecting the developing process of *P. punctifer*, which was also demonstrated at the histological level. Differences were particularly evident in regards to the number of enterocytes along the different parts of the intestine, the number and length of the intestinal folds, the degree of accumulation of lipids in the PI, and the number of goblet cells in the AI. In particular, the smaller the difference in the number of the enterocytes between the intestinal parts, the better the overall performance, as observed in the 45:15 group. In this sense, and considering the present results, a ratio of the number of enterocytes in the AI to the PI higher than 1.2 could be considered an indicator of a developmental delay in this species. These histological observations concerning the intestinal development were correlated with several indicators of the digestive function, such as the AP/LAP, bile salt-activated lipase and pepsin activities, and growth. In terms of lipid metabolism, the 30:15 and 30:10 groups presented the lowest number of lipid deposits in the PI, which is interpreted as an indicator of a delay in intestinal maturation in these groups [81]. In addition, both the 30:15 and 30:10 groups presented the fattiest livers of all groups, which, as mentioned before, seems to be associated to the high carbohydrate level contained in these diets. Further, the higher number of goblet cells found in the AI of the 45:15 group was correlated with the improved development and growth in these specimens, as one of the various roles of neutral mucins secreted by goblet cells is to contribute to the digestion and transformation of the food into chyme, as well as to the absorption of easily digestible molecules such as disaccharides and short-chain fatty acids [82].

The dietary proximate composition differently modulated the gene expression of pancreatic and gastric enzymes as well as the post-transcriptional regulation of the enzyme production. Thus, *amy* expression was higher in 45:15 group compared to the rest of the groups, whereas α-amylase activity displayed the opposite trend. These results suggest that α-amylase activity was indeed modulated at the post-transcriptional level in all dietary groups according to the carbohydrate content of the diets. Thus, the groups fed the 30:10 and 30:15 diets, which contained the highest carbohydrate levels (31% and 25%, respectively), showed the highest amylase activity. A similar response has been observed in European sea bass *Dicentrarchus labrax* [83]. From the data obtained on gene expression and enzyme activity regulation from the different dietary groups, it seems that *P. punctifer* juveniles have the capacity to respond to such high levels of carbohydrates by increasing α-amylase activity to a certain extent. Indeed, they accumulated higher carbohydrate content in their tissues than the other dietary groups. However, the excessive lipid accumulation

in livers of these individuals suggests that the carbohydrate content was excessive in these diets. Moreover, the low level of *amy* expression observed in the 30:10 and 30:15 groups, and even in the 45:15 group, could be indicating a negative regulation of gene expression in response to excessive dietary carbohydrate levels.

The diets with the higher lipid content promoted the development of the digestive system, as reflected by the higher number of enterocytes in the AI and MI found in the 30:15 and 45:15 groups. This could explain the better growth of the 30:15 group compared to the 30:10 group, despite the same protein content. This is also supported by the higher level of bile salt-activated lipase activity of the 30:15, which was similar to that of the 45:10 group, compared to the 30:10 group. The level of this lipolytic enzyme is generally modulated by the dietary lipid content [84]. However, in the present study, the 45:15 group displayed a higher level of lipase activity than the 30:15 group, which could be explained by the fact that, although the total lipid content was similar in both diets, the 45:15 diet contained higher amount of TAG than the 30:15 diet [25], as well as to a more mature digestive system. As observed in the low-protein groups, lipase activity seems to also be modulated by the protein content in the high-protein groups, since the 45:10 group presented higher lipase activity than the 30:10 group (which also presented similar fatty acid composition) and similar activity to the 45:15 and 30:15 groups.

The efficiency of utilization of dietary PUFAs depends on the dietary lipid class (neutral lipids or phospholipids). The lipid ingredients used to formulate the four diets were soybean and marine lecithin as sources of neutral lipids and phospholipids, respectively. The early stages of fish have high requirements for phospholipids [85], which are essential nutrients for growth, survival, and maturation of the intestinal functions [85,86]. The gene expression level of *phl* was correlated with the phospholipid content of the diets. The higher phospholipid content of the 45:15 diet compared to the 45:10 diet could also account for the better growth and performance of individuals fed the 45:15 diet, as well as to changes in hepatic lipid accumulation. Furthermore, the higher phospholipid content of the 30:15 diet could contribute to the similarity in measures of TL and WW (at 20 dpf) with the 45:10 group, despite the considerably lower amount of available dietary protein. Phospholipids are especially important in marine fish, as they use the dietary n-3 PUFAs from the phospholipid fraction more efficiently than those from the neutral lipid fraction [87]. Similarly, it has been shown in some freshwater fish, such as the African sharptooth catfish and goldfish *Carassius auratus*, that phospholipids are the main lipids catabolized [70,88]. Although the lipid class preference of *P. punctifer* has not been determined, the higher phospholipid content of the 30:15 diet compared to the rest of the diets could have accounted for promoting growth in the individuals of this group to the levels of the 45:10 group, despite the reduced amount of dietary protein.

The level of *try* expression was higher in the 45:15 group at 26 dpf, whereas trypsin activity was similar in all four dietary groups at that age. However, differences in trypsin activity were observed at 20 dpf; the groups fed the higher dietary protein content showing the highest level of activity. These results indicate that while *try* and *pep* were both contributing to protein digestion at 20 dpf, and, consequently, a modulation of the enzyme activity depending on the dietary regime is observed, pepsin activity increased at 26 dpf and trypsin activity levels remained stable in time and invariable among dietary regimes. At 26 dpf, *try* expression was highest in the 45:15 group and lowest in the 30:10 group. Unexpectedly, the level of *try* expression in the 45:10 group was lower than that of the 45:15 group, but similar to that of the 30:15 group. These findings suggest that, in *P. punctifer*, the modulation of *try* expression not only depends on the dietary protein level, but rather on the combination of nutrients. Indeed, it has been shown in rats that *try* expression increases when a protein-free carbohydrate-rich diet is offered [89]. Moreover, in cod *Gadus morhua* (fed to satiation), trypsin was the only enzyme among those examined by Lemieux and colleagues [90] that could potentially limit food conversion efficiency, therefore, limiting growth rate. Overall, in cod, trypsin showed a stronger correlation with growth rate than with food intake, and in *P. punctifer,* the high protein diet gave the best results for growth

and highest *try* expression at 26 dpf [90]. The levels of trypsin activity were similar in all dietary groups indicating a post-transcriptional regulation [83]. The similar level of trypsin activity among the four dietary groups indicates that similar levels are used to digest protein content ranging from 30% to 43% and/or that there are other nutrients inducing the activity of this enzyme in the low-protein groups. Further research on the influence of carbohydrates in the regulation of trypsin activity as well as on the involvement of the digestive hormone cholecystokinin in the regulation of *try* secretion [91] will contribute to better understand the regulation of trypsin activity in this species.

Pep expression was found to be modulated by the dietary protein content. Thus, high-protein dietary groups displayed higher levels of *pep* expression than the low-protein dietary groups. At the enzyme level, however, the 45:15 group showed the highest pepsin activity and the 30:10 group the lowest, with the 45:10 and 30:15 group displaying intermediate values. These results could be related to the high interindividual variability obtained in the enzymatic assays that resulted in a nonsignificant differentiation of pepsin activity between those dietary groups, although it was noted that pepsin activity tended to be higher in the 45:10 group than in the 30:15. Alternatively, these results could show a post-transcriptional regulation of this gene to adapt to a specific nutrient combination, although some authors support that pepsin activity is poorly modulated by dietary protein [92]. In this hypothetical case, in addition to protein content, the different energy content of the 45:10 and 30:15 diets could be accounting for a similar level of pepsin activity. However, further knowledge on the nutrient's interaction and communication with the different enzymatic machinery is needed to confirm this and to better understand the complex mechanisms underlying nutrition in fish.

5. Conclusions

This study showed that protein followed by lipid content are the main drivers for successful growth and survival in *P. punctifer* early juveniles. The dietary protein:lipid:carbohydrate levels and ratios influenced the whole body proximate composition, the digestive physiology and development, and hence growth, as well as survival. Individuals fed the 45:15 diet grew and survived best. This diet promoted the most rapid development of the digestive system as demonstrated by histological and functional indicators: higher number of hepatocytes, higher number of goblet cells in the anterior intestine, higher number of enterocytes in all intestinal parts, and longer folds in the posterior intestine; highest *amy*, *lpl*, *phl*, *try*, and *pep* expression; and highest lipase and pepsin activity and higher AP:LAP ratio. Altogether, this enabled individuals to better digest, absorb and metabolize nutrients. In addition to the protein content, phospholipids seemed to be key for the improved performance of the 45:15 group. It appears that a better use of the phospholipid fraction enabled this group to take advantage of all the benefits that these lipids have for growth, development, and behavior [25].

This study also found that growth performance and metabolism of *P. punctifer* were significantly affected by the dietary E:P ratio. At high E:P ratios (e.g., 11), energy was used to assure body protein requirements and the remaining energy did not contribute efficiently enough to promote growth. In addition, results indicated that *P. punctifer* has a clear preference for lipids as source of energy rather than carbohydrates, with lipids even promoting a protein-sparing effect at adequate E:P ratio. Moreover, dietary carbohydrate content higher than 25% appeared to be excessive for this species, leading to unbalanced lipid metabolism and fat deposition in livers. According to the present study, a 0.2–0.8 C:L ratio is optimal for *P. punctifer*. In view of the current findings and of the large gap in carbohydrate content between the best and the worst tested diets (2% vs. 31%) in terms of growth and survival, further research on the regulation of glucose metabolism-related genes is needed to elucidate the carbohydrate metabolism in *P. punctifer* and to better determine the optimal dietary C:L ratio that favors protein sparing and overall physiological performance in this species.

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Data Availability Statement: Data presented in this study are available from the corresponding authors upon reasonable request.

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2.2

DHA-enrichment of live and compound feeds influences the incidence of cannibalism, digestive function, and growth in the Neotropical catfish *Pseudoplatystoma punctifer* (Castelnau, 1855) during early life stages

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Aquaculture (2021), en revisión

Resumen

El objetivo de este estudio fue evaluar la influencia del enriquecimiento de DHA en la dieta en la incidencia del canibalismo, la fisiología digestiva y el crecimiento durante las primeras etapas de la vida de *Pseudoplatystoma punctifer*, y si la necesidad de DHA en la dieta varía a lo largo del desarrollo. Las larvas y los juveniles tempranos se alimentaron con *Artemia* de 4 a 18 días después de la fertilización (dpf) y se destetaron a una dieta compuesta entre los 15 y 26 dpf. Se utilizó un enriquecimiento comercial con alto contenido de DHA (4% del total de ácidos grasos en *Artemia* y 10% en la dieta compuesta) para generar cuatro tratamientos dietéticos: *Artemia* y dieta compuesta no enriquecidas (C, grupo control), *Artemia* enriquecida y dieta compuesta no enriquecida (T1), *Artemia* no enriquecida y dieta compuesta enriquecida (T2), y *Artemia* y dieta compuesta enriquecidas (T3). A partir de los 26 dpf, todos los grupos fueron alimentados con la misma dieta compuesta no enriquecida hasta los 36 dpf. Se realizaron mediciones de crecimiento y supervivencia, incidencia de canibalismo, análisis de composición de ácidos grasos y proximales, análisis histológicos de intestino e hígado, y expresión génica cuantitativa de las principales enzimas digestivas (amilasa, tripsina, quimotripsina, pepsina, fosfolipasa A2 y lipoproteína lipasa) en los diferentes grupos dietéticos al final de cada período de alimentación. Los resultados mostraron que tanto el período como la duración de la suplementación con DHA en la dieta tuvieron un impacto en la incidencia del canibalismo, la función digestiva y el metabolismo y, en última instancia, en la supervivencia y el crecimiento de *P. punctifer*. La *Artemia* y/o la dieta compuesta enriquecida (T1, T2, T3) redujo la incidencia de canibalismo y aumentó la supervivencia, mientras que la dieta compuesta enriquecida promovió el crecimiento (T2, T3). El cambio observado en la incidencia de canibalismo durante el destete en los diferentes grupos dietéticos mostró que el canibalismo se vio fuertemente afectado por la nutrición en esta especie. Además, la nutrición larvaria condicionó la función digestiva y el metabolismo durante la etapa juvenil temprana.

DHA-enrichment of live and compound feeds influences the incidence of cannibalism, digestive function, and growth in the Neotropical catfish *Pseudoplatystoma punctifer* (Castelnau, 1855) during early life stages

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Abstract (400 words)

The aim of this study was to evaluate the influence of dietary DHA enrichment in the incidence of cannibalism, digestive physiology, and growth performance during the early life stages of *Pseudoplatystoma punctifer*, and whether the need of dietary DHA would vary throughout development. Larvae and early juveniles were fed *Artemia* from 4 to 18 days post fertilization (dpf) and weaned onto a compound diet from 15 to 26 dpf. A commercial

enrichment with high DHA content (4% of total fatty acids in *Artemia* and 10% in compound diet) was used to generate four dietary treatments: non-enriched *Artemia* and compound diet (C, control group), enriched *Artemia* and non-enriched compound diet (T1), non-enriched *Artemia* and enriched compound diet (T2), and enriched *Artemia* and compound diet (T3). From 26 dpf, all groups were fed the same non-enriched compound diet until 36 dpf. Growth and survival measurements, incidence of cannibalism, proximal and fatty acid composition analyses, histological analyses of the intestine and liver, and quantitative gene expression of the main digestive enzymes (amylase, trypsin, chymotrypsin, pepsin, phospholipase A2 and lipoprotein lipase) were performed on the different dietary groups at the end of each feeding period. Results showed that both the period and duration of dietary DHA supplementation had an impact on the incidence of cannibalism, the digestive function and metabolism, and ultimately on survival and growth in *P. punctifer*. Enriched *Artemia* and/or compound diet (T1, T2, T3) reduced the incidence of cannibalism and increased survival, whereas the enriched compound diet promoted growth (T2, T3). The shift observed in the incidence of cannibalism at weaning in the different dietary groups showed that cannibalism was strongly affected by nutrition in this species. Moreover, larval nutrition conditioned the digestive function and metabolism during the early juvenile stage.

Keywords

DHA; cannibalism; nutrition; catfish larvae; digestive system

1. Introduction

The most cultivated catfish species in South America belong to the genus *Pseudoplatystoma* Bleeker, 1862, which are piscivorous migratory species native to the major river basins of South America and have total lengths of up to 140 cm (Buitrago–Suárez and Burr, 2007;

Gisbert et al., 2021). Current aquaculture production mostly relies on interspecific (e.g., *Pseudoplatystoma reticulatum* x *Pseudoplatystoma corruscans*) and intergeneric hybrids with omnivorous pimelodid species (*Leiarius marmoratus* or *Phractocephalus hemioliopterus*), since the latter present less cannibalism during early life stages and readily accept compound diets than the *Pseudoplatystoma* spp. parent species (Gisbert et al., 2021; Hashimoto et al., 2012). However, given the risks associated to the culture of hybrid species (Hashimoto et al., 2015, 2013), research efforts are being made to develop more efficient culture practices for pure *Pseudoplatystoma* species, as is the case for *Pseudoplatystoma punctifer* (Castelnau, 1855), a species native to the Amazon basin (e.g., Castro-Ruiz et al., 2021a, 2021b, 2019; Darias et al., 2015; Gisbert et al., 2014). The commercial farming of this highly appreciated species has been hampered by low survival during early life stages due to the high incidence of cannibalism and the low acceptability of compound diets at weaning (Baras et al., 2011; Gisbert et al., 2014). However, recent studies have found a strong correlation between nutrition and the cannibalistic behavior in this species; in particular, we have shown that by means of a feeding protocol adapted to digestive capacities and nutritional needs during early life stages, growth and survival are substantially improved (Castro-Ruiz et al., 2021a, 2021b, 2019; Darias et al., 2015).

During the early life stages of fish, an optimal dietary fatty acid composition, especially polyunsaturated fatty acids (PUFA), is essential to promote adequate development and growth (Lund et al., 2012; Mourente, 2003; Watanabe, 1993). The absence of dietary PUFA leads to deficiency symptoms, including reduced growth and increased mortality (Glencross, 2009; Tocher, 2010). Freshwater fish are able to maintain a certain degree of D6 and D5 desaturase and elongase activities to synthesize docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and arachidonic acid (ARA, 20:4n-6) from their precursors, linoleic (LA, 18:2n-6) and linolenic (ALA, 18:3n-3) acids, present in the diet.

Thus, LA and ALA are considered the essential fatty acids (EFA) in freshwater species (Izquierdo et al., 2008; Satoh et al., 1989; Watanabe, 1982). For that reason, freshwater species have lower requirements for n-3 highly unsaturated fatty acids (HUFA) than marine fish larvae (Verreth et al., 1994). However, fatty acid composition in *Artemia*, naturally lacking n-3 HUFAs (Sargent et al., 1999), may also affect growth in freshwater species (Bengtson et al., 1991). DHA is particularly important during the larval development due to its structural role in biomembranes, especially in neural tissues, such as the retina and the brain (Bell et al., 1996; Mourente, 2003; Wassall and Stillwell, 2008). In *P. punctifer*, previous studies have shown that *Artemia* nauplii did not satisfy the nutritional needs of this species from 12 dpf onwards, coinciding with the beginning of the juvenile stage, leading to decreased growth and increased incidence of cannibalism (Castro-Ruiz et al., 2019; Darias et al., 2015; Gisbert et al., 2014). Providing sufficient amounts of dietary HUFAs is key to ensure successful survival, growth, and metamorphosis in fish larvae (Izquierdo et al., 2000; Sargent et al., 1999). Dietary DHA and EPA provided in adequate quantities promote digestive system development and maturation, growth, survival, and adequate morphogenesis (Cahu et al., 2003; Takeuchi, 2014; Villeneuve et al., 2005; Zambonino Infante and Cahu, 1999), whereas dietary DHA deficiency promotes physiological stress (Lund et al., 2012). Considering that stress can induce cannibalistic behavior (Naumowicz et al., 2017) and that inadequate nutrition has shown to influence the incidence of cannibalism in *P. punctifer* (Castro-Ruiz et al., 2021b; Darias et al., 2015), the aim of this study was to evaluate the influence of dietary DHA supplementation on the incidence of cannibalism, digestive physiology, and growth performance during the early life stages in *P. punctifer*. A feeding trial in which *Artemia* metanauplii and compound diets were either enriched or not with DHA was used to elucidate whether the dietary requirements in DHA varied throughout development. The effects of these feeding regimes on the development

and function of the digestive system of early juveniles of *P. punctifer* were analyzed at histological and molecular levels. Regarding the latter, the study focused on the expression of the main digestive enzyme precursors α-amylase (*amy*), phospholipase A2 (*pla2*), lipoprotein lipase (*lpl*), trypsinogen (*try*), chymotrypsinogen (*ctr*), and pepsinogen (*pga*). The outcomes of this study will contribute to an improved understanding of the nutritional needs of this species during early development, and to ameliorate the deficiencies in nutritional composition of feeds as well as to optimize the feeding protocols used in order to promote adequate growth and health in this Amazonian species.

2. Materials and Methods

2.1. Rearing protocol

Spawning of a sexually mature couple of *P. punctifer* (♀: 4.15 kg; ♂: 1.15 kg) from a broodstock maintained in captivity at the Instituto de Investigaciones de la Amazonia Peruana (IIAP, Iquitos, Peru) was hormonally induced. The female and male were injected intramuscularly with carp pituitary extract (Argent Chemical Laboratories, Inc., Redmond, WA, USA) at 5 mg kg^{-1} and 1 mg kg^{-1} of body weight, respectively. Hormone injections were administered in two doses 12 h apart: the first at 10% and 50% of the total dose, and the second at 90% and 50% of the total dose for the female and the male, respectively. Stripping of the female, sperm collection and fertilization procedure were performed according to Nuñez et al. (2008). Fertilized eggs (fertilization rate ~99.9%) were incubated at 28 °C in 60-L tanks connected to a freshwater recirculating system; hatching took place 18 ± 2 h later (hatching rate ~87%). Larvae were transferred at 4 days post fertilization, dpf (5.1 ± 0.7 mm total length, TL), into 40 L tanks (30-L water volume, 1000 larvae per tank) connected to a clear water recirculation system with mechanical and biological filters. Water conditions throughout the experiment were: temperature, 27.1 ± 0.6 °C; pH, 7.0 ± 0.6;

126 dissolved oxygen, 8.9 ± 1.5 mg L^{-1}; NO_2^-, 0.04 ± 0.02 mg L^{-1}, NH_4^+, 0.14 ± 0.05 mg L^{-1}.
127 Water temperature, pH, and dissolved oxygen were measured daily and NO_2^- and NH_4^+
128 weekly in each tank. Water supply was adjusted in each tank to assure a water flow rate of
129 0.2 L min^{-1}. Larvae were reared from 4 to 36 dpf under a photoperiod of 0L:24D.
130

131 **2.2. Experimental design and feeding protocol**

132 In order to evaluate whether there were different DHA requirements during the early
133 development of *P. punctifer* and the impact of this nutrient on the incidence of cannibalism,
134 the digestive function and growth, a commercial enriching product containing high levels of
135 DHA (Algamac 3050, Pacific Trading Aquaculture Ltd., Dublin, Ireland) was used in
136 different feeding periods as shown in Figure 1. Four experimental feeding protocols were
137 tested in triplicate: the control group C was exclusively fed 5 times a day from 4 to 14 dpf
138 with *Artemia* spp. metanauplii in slight excess (0.6-12 nauplii ml^{-1}), considering larval
139 density, weight increase, and the daily food ration. As previous studies have shown *P.*
140 *punctifer* early juveniles prefer moist over dry feeds at weaning (Fernández-Méndez et al.,
141 2015), individuals were weaned from 15 dpf onto a compound moist diet within 3 days. The
142 moist diet was elaborated using a commercial compound diet (Purina®, Cargill Incorporated,
143 Lima, Peru) containing 45% protein, 10% lipids, 2% fiber, 12% moisture and 12% ash, to
144 which water and neutral gelatin was added (Fernández-Méndez et al., 2015). Juveniles were
145 solely fed the compound diet from 18 dpf onwards 5 times a day (5% fish wet weight, WW)
146 until 26 dpf, when the nutritional trial ended. The experimental treatment 1 (T1) consisted
147 in the same feeding regime described above, but *Artemia* metanauplii were enriched with
148 Algamac 3050. One-day-old *Artemia* nauplii (100 nauplii ml^{-1}) were enriched for 16 h at 28
149 °C with 0.2 g L^{-1} of Algamac 3050, following manufacturer's instructions. After enrichment,
150 *Artemia* metanauplii were washed with sterile, filtered slightly salted water to reduce the

bacterial load and remove residues of the enrichment emulsions, and kept at 4 °C in sterile, filtered slightly salted water with aeration until administered to the larvae. In the second experimental treatment (T2), only the compound moist diet was enriched with Algamac 3050 at 10%. Finally, in the experimental treatment 3 (T3), both *Artemia* and compound diet were enriched as described above (Figure 1). From 27 dpf, all groups were fed the commercial compound diet (Purina®) 3 times a day (5% fish wet weight, WW) until an additional control of survival was made at 36 dpf.

2.3. Fish performance

Individuals of *P. punctifer* were sampled from each tank at 14 and 26 dpf and euthanized with an overdose of Eugenol (0.05 μL mL^{-1}; Moyco®, Moyco, Lima, Peru). In order to monitor growth, 15 individuals per tank were placed in a Petri dish, photographed using a scale bar and TL was measured on the digital images (300 dpi) using ImageJ software (Schneider et al., 2012). WW was determined at 14 and 26 dpf using an analytic microbalance (Sartorius BP 211 D, Data Weighing Systems, Inc., Elk Grove, IL, USA, ± 0.01 mg). The number of cannibals was counted in each tank twice a day (08:00 h and 17:00 h) and the incidence of cannibalism expressed as the percentage of fish displaying cannibalistic behavior at each feeding period. Two types of cannibalism were recorded: type I, when larvae were partially damaged (pectoral fins and/or stomach bitten), and type II, when individuals were completely ingested by their siblings. Survival was evaluated by counting the individuals surviving at 14, 26, and 36 dpf with respect to the number of individuals at the beginning of each feeding period, and calculated considering the number of individuals sampled at each sampling point.

In the absence of an *ad hoc* ethical committee at the IIAP where this trial was conducted, the animal experimental procedures were conducted in compliance with the Guidelines of the

European Union Council (2010/63/EU) on the protection of animals used for scientific purposes.

2.4. Proximate composition and fatty acid analyses

The experimental feeds (enriched and non-enriched *Artemia* and compound diets) were sampled in triplicate (*ca*. 1 g per replicate) and stored at −80 °C until analysis. Sampled *P. punctifer* individuals at 4, 14 and 26 dpf (*ca*. 1 g per tank) were washed with distilled water and kept at −80 °C after removing the excess of water. Total lipids of the feeds and *P. punctifer* individuals were extracted in chloroform:methanol (2:1, v/v) using the method of Folch et al. (1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow followed by overnight vacuum desiccation. Total lipids were stored in chloroform:methanol (2:1, 20 mg mL^{-1}) containing 0.01% butylated hydroxytoluene (BHT) at −20 °C until analysis. Acid-catalyzed transmethylation was carried out using the method of Christie (1982). Methyl esters were extracted twice using isohexane:diethyl ether (1:1, v/v), purified on thin-layer chromatography plates (Silica gel 60, VWR, Lutterworth, UK), and analyzed by gas–liquid chromatography on a Thermo Electron Trace GC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 × 0.25 mm id; SGE, Milton Keynes, UK), using a two-stage thermal gradient from 50 °C (injection temperature) to 150 °C after ramping at 40 °C min^{-1} and holding at 250 °C after ramping at 2 °C min^{-1}, helium (1.2 mL min^{-1} constant flow rate) as the carrier gas and on-column injection, and flame ionization detection at 250 °C. Peaks of each fatty acid were identified by comparison with known standards (Supelco Inc., Bellefonte, PA, USA) and a well characterized fish oil, and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chrom-Card for Windows (Trace GC, Thermo Finnigan, Milan, Italy). Results of fatty acid content were expressed as a percentage of total fatty acids

(TFA). Protein and carbohydrate contents were determined following the Lowry et al. (1951) and the DuBois et al. (1956) methods, respectively.

2.5. Histological analyses

Individuals of *P. punctifer* (n = 10) were sampled at 14 and 26 dpf from each tank and fixed in buffered formaldehyde (pH = 7.2) at 4 °C overnight. The day after, individuals were dehydrated with graded series of ethanol and stored in 70% ethanol at 4 °C until further processing. After the dehydration process, individuals were embedded in paraffin with an automatic tissue processor Histolab ZX-60 Myr (Especialidades Médicas MYR SL, Tarragona, Spain). Then, paraffin blocks were prepared in an AP280-2Myr station and cut into serial sagittal sections (3 μm thick) with an automatic microtome Microm HM (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Paraffin cuts were kept at 40 °C overnight. After that, samples were deparaffinized with a graded series of xylene substitute and stained by means of hematoxylin and eosin for general micromorphological observations. Histological preparations were observed under a Leica DM2000 LED microscope equipped with a camera Leica MC170 HD (Leica Microsystems Nussloch GmbH, Nussloch, Germany) as described in Gisbert et al. (2008).

2.6. RNA extraction and gene expression analyses

Fifteen individuals per tank were used for total RNA extraction. RNA from 100 mg of whole fish homogenates at 14 and 26 dpf was extracted using TRIzol™ (Invitrogen, San Diego, CA, USA) according to manufacturer's protocol. RNA concentration and quality were determined by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Madrid, Spain) measuring the absorbance at λ = 260 and 280 nm and by denaturing electrophoresis in TAE agarose gel (1.5 %), respectively. Total RNA was treated with DNase I

Amplification Grade (Invitrogen, San Diego, CA, USA) according to manufacturer's protocol and then reverse transcribed in 10 μL reaction volume containing 3 μg total RNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, San Diego, CA, USA) with oligo (dT) (12-18) (0.5 μg/ul) and random hexamers primers (50 ng μL^{-1}), 10X RT buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCL), 25 mM MgCl2, 0.1 M DTT, 10 mM dNTP mix, SuperScript™ II RT (50 U μL^{-1}), RNaseOUT™ (40 U μL^{-1}), followed by RNase H (2 U μL^{-1}) (Invitrogen, San Diego, CA, USA) treatment. Reverse transcription reactions were carried out in a thermocycler (Mastercycle R nexus GSX1, Eppendorf AG, Hamburg, Germany) and run according to manufacturer's protocol. The samples were diluted 1:20 in molecular biology grade water and stored at −20 °C until further analyses. The expression of *amy* (AC MT006358), *ctr* (AC MT006344), *try* (AC MT006359), *lpl* (AC MT006346), *pla2* (AC MT006345), and *pga* (AC MT006343) (Castro-Ruiz et al., 2021a) was analyzed in individuals from the four experimental groups at 14 and 26 dpf. Quantitative PCR analyses were carried out in triplicate in a 7300 Real-Time PCR System (Applied Biosystems, Roche, Barcelona, Spain). The amplification mix contained 1 μL cDNA, 0.5 μL primers (20 μM), and 10 μL SYBR Green Supermix (Life Technologies, Carlsbad, CA, USA) in a total volume of 20 μL. A negative control was included (no template control) for each set of reactions on each 96-well plate. The amplification conditions were as follows: 10 min at 95 °C, 40 cycles of 20 s at 95 °C, and 1 min at 65 °C, followed by 15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C, and finally, 15 s at 60 °C. A standard curve was obtained by amplification of a dilution series of cDNA for calculation of the efficiency (E) for each set of primers. Real-time PCR efficiencies were determined for each gene from the slopes obtained with Applied Biosystems software, applying the equation E=10[−1/slope], where E is PCR efficiency. The relative gene expression ratio (R) for each gene was calculated according to Pfaffl's (2001) formula: $R = (E_{\text{target gene}})^{\Delta Cq \text{ target gene (mean sample } - \text{ mean reference}}$

$^{\text{sample}}$)/($E_{\text{reference gene}}$)$^{\Delta Cq \text{ reference gene (mean sample} - \text{mean reference sample)}}$, where ΔCq is the deviation of the target sample minus the reference sample. The relative expression of the genes was normalized using glyceraldehyde-3-phosphate dehydrogenase (*gapdh*, AC MT006341) (Castro-Ruiz et al., 2021a) as the reference gene, since it did not exhibit any significant variation in expression between samples.

2.7. Statistics

Results were expressed as mean ± standard deviation (n = 3). All data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett's test). One-way ANOVA was performed to analyze differences in growth, proximate and fatty acid composition, and gene expression during development and/or between dietary treatments. All pairwise multiple comparisons were performed using the Holm-Sidak method if significant differences were found at $P < 0.05$ to discriminate the significant differences. Statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, VA, USA).

3. Results

3.1. Growth performance

Growth in terms of WW and TL is shown in Figure 2. At 14 dpf, individuals presented similar WW among dietary groups, whereas individuals from T2 and T3 groups displayed higher WW than those from C and T1 groups at 26 dpf (Figure 2; $P < 0.05$). There were no differences in TL between the different dietary treatments at both sampling times (14 and 26 dpf) ($P > 0.5$).

3.2. Incidence of cannibalism and temporal occurrence

The incidence of cannibalism was reduced by half in all enriched groups (T1, T2 and T3) (Fig. 4). In addition, a 3-fold increase in survival was observed in T1, T2 and T3 groups (Figure 3). Figure 4 shows the incidence of cannibalism for each feeding period. In general, the total incidence of cannibalism was lowest during the *Artemia* feeding period (ca. 0.5 %), increased at weaning (ca. 2 %), and peaked during the compound diet feeding period (ca. 8 %). The incidence of total cannibalism during the *Artemia* feeding period was higher in the C and T2 groups and lower in the T1 and T3 groups ($P < 0.05$). However, during the weaning period, the above-mentioned patterns in cannibalism changed. In particular, groups C and T1 showed higher incidence of cannibalism than groups T2 and T3 ($P < 0.05$). The overall percentage of cannibalism at the end of the nutritional trial was lowest in the groups T1, T2, and T3 ($P < 0.05$). The incidence of type I cannibalism was higher than type II cannibalism during the *Artemia* feeding phase. The incidence of type I cannibalism was highest in C group and lowest in T3 group ($P < 0.05$); however, no differences were found in the incidence of type II cannibalism between dietary groups ($P > 0.05$). The incidence of both types of cannibalism was similar during the weaning period with the exception of the group T3, which presented a higher incidence of type I cannibalism than type II. The C, T1, and T3 groups presented a similar incidence of type I cannibalism that was higher than that of the T2 group ($P < 0.05$). In contrast, type II cannibalism was lowest in the T3 group. The incidence of both types of cannibalism was highest in the C group during the compound diet feeding period, and the incidence of type I was higher than that of type II ($P < 0.05$).

When comparing the temporal incidence of cannibalism at morning and evening time, during the *Artemia* feeding phase, type I cannibalism was only detected in the morning and the incidence was similar in all dietary groups ($P < 0.05$). However, this pattern changed at weaning, where cannibalism was detected both in the morning and evening. Type I cannibalism in the morning was higher in groups T1 and T3 ($P < 0.05$), whereas it was

similar in all dietary groups at evening ($P > 0.05$). In groups T1 and T3, cannibalism in the morning was higher than at evening, whereas the opposite was found in the T2 group ($P < 0.05$). During the compound diet feeding period, the incidence of type I cannibalism was similar in all dietary groups in the morning ($P > 0.05$), whereas at evening it was higher in the C group ($P < 0.05$). The T2 group showed a higher incidence of type I cannibalism at the morning time ($P < 0.05$).

Type II cannibalism was only detected in the morning in groups C and T1 during the *Artemia* feeding period, and the incidence was similar in both groups. No incidence of cannibalism was detected in any group at evening. At weaning, type II cannibalism appeared both in the morning and in the evening. The highest incidence in the morning was detected in group C and the lowest in group T3 ($P < 0.05$). No differences were found in the incidence of type II cannibalism in the evening between the dietary groups ($P > 0.05$). Only the C group showed a higher incidence of type II cannibalism in the morning than in the evening ($P < 0.05$). Finally, during the compound diet feeding period, the C group presented the highest incidence of cannibalism both in the morning and in the evening. Similar to what was observed at weaning, the T2 group showed a higher incidence of cannibalism in the morning ($P < 0.05$).

3.3. Proximate composition, lipids classes and total fatty acids composition of live preys and experimental diets

Proximate composition and lipid classes of the experimental diets are presented in Tables 1 and 2, respectively. Protein content was similar in all experimental diets (*ca*. 37 %). Lipid content was similar (*ca*. 14 %) in all diets, except for the non-enriched compound diet, which contained a lower amount of dietary lipids (10 %). Carbohydrate content was higher in

compound diets than in *Artemia* (25 % *vs* 10 %, respectively). Moisture also varied between experimental diets, with *Artemia* being moister than the compound diets (Table 1).

Total lipid and total fatty acid contents and composition of the experimental diets are presented in Table 2. Total lipid and total fatty acid contents were similar between the experimental diets (*ca*. 141 mg g^{-1} total lipid and *ca*. 97 mg g^{-1} TFA, DW), with the exception of the non-enriched compound diet, which presented lower values. In terms of TFA, the compound diets presented higher contents of saturated fatty acids than *Artemia.* Total monounsaturated fatty acids were more abundant in non-enriched *Artemia* and in the non-enriched compound diet compared to the respective enriched forms. Total n-6 PUFAs were higher in compound diets than in *Artemia*; LA (18:2n-6) and docosapentaenoic acid (DPA, 22:5n-6) accounted for such differences in non-enriched and enriched compound diets, respectively. Total n-3 PUFAs content were higher in *Artemia* than in compound diets, with ALA accounting for such a difference. Among compound diets, the DHA-enriched one contained higher total n-3 PUFA levels, as expected, due to the DHA content of the enriching product. With the exception of the non-enriched compound diet, which showed lower total PUFAs content, the rest of the experimental diets presented similar total PUFA levels.

All experimental diets had a similar ratio of total n-3/n-6 PUFA. The DHA/EPA ratio was higher in the enriched compound diet, followed by enriched *Artemia*. Enriched *Artemia* and the enriched compound diet had the highest and the lowest ARA/DHA ratio, respectively. The LA/PUFA and OA/PUFA ratios were lower in enriched than in non-enriched compound diets. The PUFA/saturated ratio was higher in *Artemia* than in the compound diets.

Concerning the lipid classes, total phospholipid content was higher in *Artemia* (18 %) than in compound diets (*ca*. 9 %), where phosphatidylcholine (PC) and phosphatidylethanolamine (PE) accounted for such differences. On the contrary, total neutral

lipids, were higher in compound diets (91 %) than in *Artemia* (82 %), with triglycerides (TAG) accounting for such differences (Table 3).

3.4. Proximate and total fatty acids content and composition of *P. punctifer*

Proximate composition and total lipids and fatty acids of *P. punctifer* individuals fed the different experimental diets are presented in Tables 4 and 5, respectively. The protein content of *P. punctifer* remained invariable during development, and regardless of the experimental feeding protocol used ($P > 0.05$). Lipid content was similar between dietary treatments at 14 dpf ($P > 0.05$), whereas differences were found at 26 dpf ($P < 0.05$), where the group T3 presented a higher lipid content than the rest of the dietary groups. In addition, the lipid content of the individuals from the T1 group was lower at 26 dpf than at 14 dpf ($P < 0.05$). Carbohydrate content was also similar between dietary treatments at 14 dpf ($P > 0.05$), whereas it was higher in the T3 group compared to the C group. Besides, carbohydrate content of the individuals from the T3 group was lower at 26 dpf than at 14 dpf ($P < 0.05$). The total fatty acid content of *P. punctifer* specimens generally reflected that of the experimental diets. Individuals from groups C and T2 presented higher content of total monounsaturated fatty acids ($P < 0.05$), whereas this difference disappeared at 26 dpf ($P > 0.05$). Besides, the content of total monounsaturated fatty acids decreased from 14 to 26 dpf in all dietary groups ($P < 0.05$). The total n-6 PUFAs content was higher in groups T1 and T3 at 14 dpf, and in T2 and T3 at 26 dpf ($P < 0.05$). The main fatty acids accounting for such differences were DPA and ARA. In addition, the total n-6 PUFAs content increased between 14 and 26 dpf in groups T2 and T3 ($P < 0.05$). The total n-3 PUFA content was similar in all dietary groups both at 14 and 26 dpf ($P > 0.05$); however, differences were found among several n-3 PUFAs. For instance, EPA content was higher in the C group and lower in the T3 group at 26 dpf; higher DPA and lower DHA contents were detected in the groups C and

374 T2 at 14 dpf ($P < 0.05$), whereas at 26 dpf, DHA was similar in all dietary groups ($P > 0.05$).
375 Total PUFA was higher in groups T1 and T3 at 14 dpf ($P < 0.05$), a difference that was not
376 maintained at 26 dpf ($P > 0.05$). At 14 dpf, the groups C and T2 presented a higher ratio of
377 total n-3/n-6 PUFA than the groups T1 and T3, whereas at 26 dpf, the groups C and T1
378 showed the highest ratio, and the T2 and T3 the lowest ($P < 0.05$). At 14 dpf, the DHA/EPA,
379 ARA/EPA, and PUFA/saturated ratios were lower in the groups C and T2 than in the groups
380 T1 and T3, whereas the opposite was found for the ratios LA/PUFA and OA/PUFA ($P <$
381 0.05). At 26 dpf, however, the DHA/EPA and PUFA/saturated ratios were higher, and the
382 ARA/DHA and OA/PUFA ratios lower in the T2 and T3 groups than in the C and T1 groups
383 ($P < 0.05$).
384
385 **3.5. Histological analyses**
386 The main histological changes in lipid deposition in the liver and intestine of *P. punctifer*
387 individuals reared under different dietary treatments are shown in Figure 5. Histological
388 analyses at 14 dpf revealed the presence of fattier livers in groups fed non-enriched *Artemia*
389 (C, T2) in comparison to those fed enriched *Artemia* (T1, T3). Lipid deposits in the intestine
390 were similar in all dietary treatments, mainly corresponding to very low-density lipoproteins
391 (Figure 5). At the end of the nutritional trial (26 dpf), groups fed non-enriched compound
392 diets (C, T1) showed low-moderate lipid deposits in the hepatic parenchyma, whereas groups
393 fed enriched compound diets (T2, T3) presented a moderate-high liver lipid accumulation.
394 Hepatic diverticula were visible and spaced in groups with low lipid accumulation, whereas
395 this space disappeared in fish displaying significant lipid accumulation. Lipid deposits in the
396 intestine in fish from the C and T1 groups where located throughout the folds of the posterior
397 part of the intestine. The totality of the enterocytes in group C contained lipid deposits and
398 the size (diameter) and surface of the lipid inclusions varied from 7 to 14 μm and from 210

399 to 450 μm^2, respectively. In the T1 group, the size of lipid inclusions varied between 7 and
400 10 μm in diameter and between 140 and 400 μm^2 in surface, and the level of accumulation
401 was low. Group T2 presented a low-moderate lipid accumulation in the intestine, with a
402 diameter and surface of lipid inclusions that ranged from 3 to 13 μm, and from 70 to 140
403 μm^2, respectively. The lipid accumulation did not affect the shape or the organization of
404 intestinal folds, nor were there signs of epithelial damage observed. Group T3 presented
405 moderate levels of intestinal lipid deposits, with sizes and surfaces ranging from 3 to 14 μm
406 and from 250 to 350 μm^2, respectively. Contrary to groups C and T1, most lipids were
407 observed in the apical zone of the villi in group T3 (Figure 5). The C group showed an
408 significant accumulation of lipids in the posterior intestine (steatosis) compared to the liver,
409 contrary to the other groups that presented similar amounts of lipids in both tissues,
410 indicating a more balanced lipid metabolism.
411

412 **3.6. Gene expression of digestive enzymes**

413 Gene expression of the analyzed digestive enzymes was differentially modulated between
414 both dietary treatments and feeding periods (Figure 6). At the end of the *Artemia* feeding
415 period (14 dpf), *amy* expression was higher in the T3 group than in the C group ($P < 0.05$).
416 The expression of *try* and *pga* was not influenced by the dietary treatments ($P > 0.05$),
417 whereas the expression of *ctr* was higher in the enriched groups (T1 and T3) than in the C
418 group ($P < 0.05$). The *pla2* expression was highest in the T2 and lowest in the T3 groups (P
419 < 0.05). The *lpl* expression was highest in the T2 group and lowest in the T1 group ($P >$
420 0.05).
421 At the end of the nutritional trial (26 dpf), the expression of all analyzed genes, except for
422 *lpl*, was highest in the T1 group ($P < 0.05$). Unexpectedly, the expression of these genes

423 decreased in the T3 group to the levels of the rest of the dietary treatments ($P < 0.05$), despite
424 having also been fed enriched *Artemia* as in the T1 group.
425 The expression of *amy* increased in the groups C, T1, and T2 and remained stable in the T3
426 group ($P < 0.05$). The expression of *try* increased in the T1 and T2 groups and remained
427 stable in the C and T3 groups ($P < 0.05$); whereas *ctr* expression increased in the C and T1
428 groups, decreased in the T3 group ($P < 0.05$), and remained invariable in the T2 group ($P >$
429 0.05). The expression of *pla2* increased during development in the groups T1, T2, and T3 (P
430 < 0.05) and remained invariable in the C group ($P > 0.05$). The expression of *lpl* and *pga*
431 decreased in the C and T2 groups ($P < 0.05$) and remained stable in the groups T1 and T3
432 for *lpl* ($P > 0.05$), whereas for *pga*, the gene expression decreased in T1 ($P < 0.05$), whereas
433 it remained invariable in T3 ($P > 0.05$).
434
435 **4. Discussion**
436 This study aimed to evaluate the influence of dietary DHA enrichment in the incidence of
437 cannibalism, digestive physiology, and growth performance during the early life stages of
438 *P. punctifer*, and whether DHA requirements would vary throughout development.
439 Present results showed that the DHA enrichment during the *Artemia* feeding period (larval
440 period) did not have any effect on growth performance. This could be related to the fact that
441 freshwater species are able to synthesize DHA de novo from 18:3 precursors and thus often
442 have lower n-3 HUFA requirements than marine fish larvae (Bell and Sargent, 2003).
443 Similarly, a nutritional study performed in *Clarias gariepinus* showed that changes in dietary
444 fatty acid composition did not affect larval growth (Verreth et al., 1994). However, under
445 current experimental condition we have shown that DHA-enriched *Artemia* metanauplii
446 reduced the incidence of cannibalism and likely contributed to favor the survival observed
447 at the end of the experimental period. Nutritional deficiencies can favor size dispersion and

promote cannibalism (Baras and Jobling, 2002). In this study, however, size dispersion was similar between all dietary treatments throughout the trial. The incidence of cannibalism in *P. punctifer* increased at weaning, peaked two days after the end of the weaning period, and disappeared five days later. Therefore, the cannibalistic behavior at weaning seemed to rather be a response to the change of the diet than to size dispersion.

DHA is essential for the development of the brain and the ontogeny of behavior in fish (Benítez-Santana et al., 2012, 2007; Ishizaki et al., 2001; Masuda et al., 1999). Dietary reduction in n-3 PUFAs, and hence in fish tissues, has been shown to reduce larval escaping behavior (Benítez-Santana et al., 2012). In mammals, inadequate intake of DHA is associated with elevated behavioral indices of anxiety, aggression, and depression (Fedorova and Salem, 2006) and, both in mammals and fish, DHA is known to reduce stress (Lund et al., 2012; Lund and Steenfeldt, 2011; Takeuchi et al., 2003; Xu et al., 2016). Considering that groups fed enriched *Artemia* contained higher DHA levels, as well as higher PUFA/saturated and lower LA/PUFA and OA/PUFA ratios in their tissues than groups fed non-enriched *Artemia*, one could speculate that the reduced incidence of cannibalism in the first groups could be associated with a better developed nervous system and escaping behavior, as well as reduced stress in those specimens. This hypothesis is based on the results of cannibalism observed in the juveniles from the group T1 (fed enriched *Artemia*, but non-enriched compound diet), where the incidence of cannibalism was reduced to the levels of the groups T2 and T3 after weaning despite being fed a non-enriched compound diet. Further research will be necessary to confirm this hypothesis.

At weaning, a transition period in the cannibalistic behavior could be observed, in which the introduction of the enriched compound diet reduced the incidence of cannibalism (groups T2 and T3), whereas the transition from enriched *Artemia* to a non-enriched compound diet temporarily increased the cannibalistic behavior (T1 group). The fact that the cannibalistic

behavior of the T1 group at weaning (14 dpf) did not last and decreased at 26 dpf to similar levels of cannibalism to those of groups T2 and T3 suggested that the change of diet induced transitory stress; and that the dietary DHA provided during the larval phase had a conditioning effect towards the development of less cannibalistic behaviors in juveniles. In line with this, the long-term effect of early nutritional history of DHA and EPA deficiencies on anxiolytic behavior has been observed in fish (Lund et al., 2012). In particular, first feeding pikeperch (*Sander lucioperca*) larvae fed a DHA-deficient diet increased stress sensitivity in the future juveniles, whereas a DHA-rich diet increased stress tolerance (Lund et al., 2012). In *P. punctifer*, the hypothesis that a low dietary DHA content early in the development promoted stress in juveniles was further supported by the fact that total cannibalism was higher in the C group at the end of the trial. Also, there was a higher incidence of type II cannibalism in the C group compared to the T1 group during the compound diet feeding phase, both in the morning and in the evening. Another hint about the dietary conditioning during the larval stage was the higher levels of expression of the majority of the digestive genes analyzed in the T1 group compared to the C group, as a similar gene expression profile at 26 dpf would have been expected in these groups fed a non-enriched compound diet. These higher levels of gene expression in the T1 group at 26 dpf were not reflected in a better performance in terms of growth, as this group presented similar wet weight as the C group, which was lower than that of the groups fed the enriched compound diet.

The fatty acid biosynthesis and metabolism differed in groups fed non-enriched and enriched *Artemia*. Despite having offered them the same amount of dietary LA, the groups fed enriched *Artemia* showed higher ARA content than the groups fed non-enriched *Artemia*. The groups fed enriched *Artemia* biosynthesized DPA and ARA from LA, resulting in a lower LA tissue accumulation than in the groups fed non-enriched *Artemia*. On the contrary,

groups fed non-enriched *Artemia* showed some ARA synthesis and lipid accumulation in tissues, but not DPA. As a consequence, the contents of ARA and LA were lower and higher, respectively, in the C and T2 groups than in those fed enriched *Artemia* (T1, T3). The differences in the DPA n-6 biosynthesis between both dietary groups could be due to a desaturation process (delta 4 desaturase) from 22:4n-6 contained in enriched *Artemia*, which was absent in non-enriched *Artemia*. This would indicate that delta 4 desaturase also exists in this species (Monroig et al., 2018). The levels of OA were higher in non-enriched than in enriched *Artemia* and this same pattern was reflected in the fish tissue. The 20:4n-3 and 22:5n-3 contents, both substrates for the elongase Elovl2 (Monroig et al., 2018), was similar in both enriched and non-enriched *Artemia.* However, groups fed enriched *Artemia* presented lower 20:4n-3 and 22:5n-3 contents than those fed non-enriched *Artemia*, suggesting that elongation of these PUFAs was promoted in these dietary groups to produce DHA. The higher EPA content in enriched (3% TFA) versus non-enriched (1% TFA) *Artemia* accounted for this biosynthetic pathway, as all dietary groups presented similar EPA content in their tissues (3% TFA). While groups fed non-enriched *Artemia* had to synthesize EPA *de novo* to accumulate 3% TFA in their tissues, the groups fed enriched *Artemia* had covered their EPA requirements through the diet.

During the *Artemia* feeding phase, groups fed with non-enriched *Artemia* showed higher lipid deposition in the liver than individuals fed enriched *Artemia,* which could be associated with the lower PUFA/saturated and higher OA/PUFA ratios observed in these groups compared to those fed enriched *Artemia* (Boglino et al., 2012). The higher *lpl* expression observed in non-enriched *Artemia* groups might have helped to prevent hepatic steatosis in these groups, as LPL is essential in buffering the circulatory TAG load, which protects against ectopic TAG accumulation (Frayn, 2002). The similar lipid accumulation in the intestine in all dietary groups indicated a differential regulation in the liver lipid uptake

between the enriched and non-enriched dietary groups. Indeed, differential regulation in lipid-related gene expression was observed between them, where *pla2* and *lpl* expression was higher in non-enriched than in enriched groups. This could be associated to the higher OA, LA, and ALA contents of the groups fed non-enriched *Artemia* (C, T2). Therefore, despite that dietary DHA did not have a significant effect on growth during the larval phase of *P. punctifer*, it seemed to be important for preserving larval quality and tissue health. Fatty acids have a role in controlling LPL activity to assure that fatty acids are not formed more rapidly than they can be taken up by the peripheral tissue (Saxena et al., 1989). In the present study, there was an inverse correlation between lipid accumulation in the liver and *lpl* gene expression in all dietary groups. For instance, groups fed non-enriched *Artemia* showed higher levels of *lpl* expression together with lower liver lipid accumulation than their congeners fed enriched *Artemia*. In the absence of differences in lipid classes (including PL and TAG) between enriched and non-enriched *Artemia*, the most important difference in fatty acid composition between them is their DHA content. These results may indicate that DHA modulated *lpl* gene expression. Specifically, DHA decreased *lpl* gene expression, which likely controlled the lipid uptake in the liver and this might explain the lower levels of lipid accumulation in the liver observed in the enriched groups (T1, T3). A similar trend was also observed for *pla2* expression among dietary groups. However, at the early juvenile stage, the C group showed a significant accumulation of lipids in the posterior intestine (steatosis) compared to the liver, contrary to the other groups that presented similar amounts of lipids in both tissues. The accumulation of large lipid droplets (mainly consisting of TAG) in the intestine of fish is generally due to a reduced lipid export from the intestinal mucosa to the circulatory system (Fontagné et al., 1998). In this sense, although the differences were not statistically significant between the dietary groups, *lpl* expression in the C group tended

to be lower, which might be indicating its action in modulating TGA deposition in the liver in these individuals.

The dietary switch from enriched *Artemia* to the non-enriched compound diet (T1 group) had a marked impact on the expression of the digestive enzymes analyzed. Despite having a similar carbohydrate content in both enriched and non-enriched compound diets that was higher than in the enriched *Artemia*, an increase in *amy* expression was only observed in the T1 group, but not in juveniles from the T3 group. In addition, even though all the enriched and non-enriched diets had similar protein contents, *pga*, *try*, and *ctr* gene expression was up-regulated in the T1 group. These results suggest a clear interaction between dietary fatty acids and genes involved in carbohydrate and protein digestion. These findings are in line with previous nutritional studies with this species (Castro-Ruiz et al., 2021b; Darias et al., 2015). When comparing the fatty acid composition of enriched *Artemia* and the non-enriched compound diet, LA and EPA were found in higher amounts in the non-enriched compound diet as compared to the enriched *Artemia*. Therefore, these two fatty acids likely accounted for the increased gene expression of *amy*, *pga*, *try*, *pla2*, and *ctr* observed in the T1 group. In fact, dietary LA has been shown to induce *amy*, *try*, and *ctr* gene expression in the hepatopancreas of juvenile grass carp (*Ctenopharyngodon idellus*) (Zeng et al., 2016). These authors also demonstrated that an optimal dietary LA:ALA ratio of 1.03 was necessary to promote growth in this species, which they considered was partly attributed to the enhancement of trypsin, chymotrypsin, lipase, and amylase activities. In the present study, the T1 group presented, together with the C group, the lowest growth at 26 dpf. This suggests either a post-transcriptional negative regulation of these enzymes and/or the influence of other fatty acids in the nutrient metabolism in the T1 group, leading to a lower weight gain. For instance, similar to the C group, the n-3/n-6 PUFA ratio was higher in juveniles from the T1 group than from the T2 and T3 groups. These results suggest that a diet rich in n-6

572 PUFA promoted weight gain in *P. punctifer*. Although growth at the end of the experiment
573 was similar in T2 and T3, when considering all the variables analyzed, the feeding protocol
574 used in the T3 group favored a more balanced digestive physiology compared to the other
575 dietary treatments, including a more balanced lipid metabolism and fat storage in the hepatic
576 parenchyma and intestinal epithelium. In addition, enriched *Artemia* in the T3 group
577 contributed to reduce the incidence of cannibalism, which led to a better survival rate at the
578 end of the trial.
579
580 **5. Conclusions**
581 The present results showed that dietary DHA levels influenced the physiology and
582 cannibalistic behavior of *P. punctifer* in a stage-dependent manner. In particular, DHA-
583 enriched *Artemia* during the larval stage contributed to reduce the incidence of cannibalism
584 and improved survival, whereas the DHA-enriched compound diet during the early juvenile
585 stage improved growth. Altogether, the group fed both DHA-enriched *Artemia* and
586 compound diet (T3) provided the best results in terms of growth, survival, incidence of
587 cannibalism, and digestive physiology. This study also showed that the nutritional history
588 during the larval period affected fish nutrition and behavior during the early juvenile stage.
589 For example, the dietary switch from DHA-enriched *Artemia* to the non-enriched compound
590 diet (T1 group) strongly influenced the expression of the digestive enzymes analyzed at the
591 early juvenile stage. In addition, in this same dietary group, DHA provided during the larval
592 stage contributed to reduce the cannibalistic behavior of the early juveniles. The shift
593 observed in the incidence of cannibalism from the *Artemia* feeding period to weaning
594 between dietary treatments demonstrated that cannibalism is strongly affected by nutrition
595 in *P. punctifer*. Moreover, the incidence of both types of cannibalism was modulated by the
596 nutritional composition of the diet. These results also suggested that more research is needed

to better understand the effects of early dietary fatty acid composition on the digestive metabolism at the early juvenile stage and the mechanisms linking the anti-stress effects of DHA and the cannibalistic behavior in this species.

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805

Tables

Table 1. Proximal composition (in % of dry matter) of the different experimental diets. Data are expressed as mean ± S.D. (n = 3). Different superscript letters denote differences statistically significant between the experimental diets (one-way ANOVA, $P < 0.05$).

Proximate Composition (%)	**Non-enriched *Artemia***	**Enriched *Artemia***	**Non-enriched compound diet**	**Enriched compound diet**
Proteins	36.77±0.67	35.20±0.46	38.79±6.22	37.74±0.81
Lipids	13.56±0.03^{a}	14.61±0.64^{a}	9.92±0.29^{b}	13.67±0.92^{a}
Carbohydrates	10.73±0.48^{b}	9.01±0.25^{b}	24.76±0.91^{a}	24.95±0.98^{a}
Moisture	79.52±0.45^{a}	81.03±1.37^{a}	43.99±0.26^{b}	33.06±0.42^{c}

812 **Table 2.** Lipid classes (in % of dry matter) analyzed in the experimental diets. Data expressed as mean ± SD
813 (n = 3). Different superscript letters denote differences statistically significant between diets (one-way
814 ANOVA, $P < 0.05$).

	Non-enriched Artemia	**Enriched Artemia**	**Non-enriched compound diet**	**Enriched compound diet**
SM	0.261±0.09^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
Lyso PC	1.08±0.10^{ab}	0.86±0.09^{b}	1.39±0.09^{a}	1.03±0.17^{ab}
PC	7.85±0.57^{a}	7.57±1.02^{a}	5.75±0.34^{ab}	4.90±0.20^{b}
PS	0.98±0.45	1.07±0.49	0.91±0.10	0.88±0.12
PI	1.60±0.59	1.51±0.66	0.00±0.00	0.00±0.00
Lyso PE	1.14±0.50	1.14±0.31	0.00±0.00	0.00±0.00
PE	5.78±0.75^{a}	5.83±0.76^{a}	1.63±0.22^{b}	1.19±0.02^{b}
Total PL	*18.55±2.09^{a}*	*17.98±1.92^{a}*	*9.68±0.75^{b}*	*8.00±0.24^{b}*
MAG	5.67±1.25^{a}	3.79±0.65^{a}	0.00±0.00^{b}	0.00±0.00^{b}
CHOL	4.66±1.55	6.14±2.05	7.44±0.70	5.39±0.36
FFA	14.63±1.38^{a}	12.08±2.27^{ab}	9.27±1.41^{ab}	6.98±1.21^{b}
TAG	48.35±3.69^{b}	53.43±3.75^{b}	69.71±0.56^{a}	75.77±1.62^{a}
SE+W	8.14±0.90^{a}	6.59±1.08^{ab}	3.90±0.61^{b}	3.87±0.29^{b}
Total NL	*81.45*±2.09^{b}	*82.02*±1.92^{b}	*90.32*±0.75^{a}	*92.00*±0.24^{a}

815 CHOL, Cholesterol; FFA, Free fatty acids; Lyso PC, Lysophosphatidylcholine; NL, Neutral lipids; PC,
816 Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; PL, Phospholipids; PS,
817 Phosphatidylserine; SE, Sterolesters; SM, Sphingomieline; TAG, Triacylglycerides; W, wax.

818

819 **Table 3.** Total lipid and total fatty acids contents (in % of dry matter) and fatty acid composition (in % TFA)
820 analyzed in the experimental diets. Data are expressed as mean ± S.D. (n = 3). Different superscript letters
821 denote differences statistically significant between diets (one-way ANOVA, $P < 0.05$).

	Non enriched Artemia	Enriched Artemia	Non enriched compound diet	Enriched compound diet
Total lipid (mg g^{-1} DW)	138.77±2.78^{a}	146.87±5.54^{a}	99.16±2.90^{b}	136.68±0.92^{a}
Total fatty acid (mg g^{-1} DW)	93.22±8.59^{a}	101.31±2.97^{a}	63.15±6.96^{b}	96.55±0.73^{a}
14:0	0.53±0.05^{c}	0.69±0.20^{c}	2.65±0.04^{b}	4.77±0.61^{a}
16:0	11.82±1.06^{b}	12.25±1.88^{b}	23.33±1.88^{a}	24.27±1.67^{a}
18:0	3.79±0.58	4.65±0.95	5.05±0.28	3.43±0.29
Total saturated	16.14±0.63^{b}	17.59±1.13^{b}	31.04±1.64^{a}	32.47±1.99^{a}
16:1	2.49±0.90	1.54±0.88	5.55±2.68	2.54±0.72
18:1n-9 (OA)	30.24±0.45^{a}	27.72±0.42^{b}	21.63±0.96^{c}	14.01±0.53^{d}
20:1	0.25±0.14^{b}	0.20±0.28^{b}	1.21±0.21^{a}	0.90±0.10^{a}
Total monounsaturated	32.98±0.39^{a}	29.47±0.18^{b}	28.39±1.51^{b}	17.45±0.29^{c}
18:2n-6 (LA)	6.72±0.11^{b}	6.44±0.11^{b}	9.20±0.72^{a}	6.48±0.00^{b}
18:3n-6	0.00±0.00	0.35±0.50	0.08±0.11	0.23±0.22
20:4n-6 (ARA)	0.06±0.11^{b}	0.71±0.16^{ab}	1.21±0.31^{a}	0.57±0.21^{ab}
22:4n-6	0.00±0.00^{b}	1.39±0.14^{a}	0.32±0.28^{b}	0.00±0.00^{b}
22:5n-6	0.00±0.00^{b}	0.00±0.00^{b}	0.18±0.25^{b}	6.20±0.16^{a}
Total n-6 PUFA	6.78±0.21^{b}	8.89±0.89^{b}	10.99±1.45^{a}	13.49±0.15^{a}
18:3n-3 (ALA)	35.66±0.56^{a}	30.56±0.30^{b}	1.57±0.04^{c}	1.04±0.02^{c}
18:4n-3	5.08±0.32^{a}	3.88±0.07^{b}	1.31±0.05^{c}	1.18±0.23^{c}
20:4n-3	1.13±0.14^{a}	1.05±0.13^{a}	0.44±0.04^{b}	0.65±0.07^{b}
20:5n-3 (EPA)	1.19±0.15^{d}	3.06±0.20^{c}	10.88±0.30^{a}	7.34±0.40^{b}
21:5n-3	0.00±0.00	0.00±0.00	0.13±0.18	0.06±0.08
22:5n-3 (DPA)	0.00±0.00^{b}	0.03±0.04^{b}	1.59±0.54^{a}	0.97±0.26^{a}
22:6n-3 (DHA)	0.00±0.00^{d}	4.39±0.06^{c}	12.95±0.68^{b}	25.05±1.28^{a}
Total n-3 PUFA	43.05±0.72^{a}	42.97±0.12^{a}	28.87±1.39^{c}	36.30±1.71^{b}
Total PUFA	49.83±0.92^{a}	51.85±1.02^{a}	39.87±2.84^{b}	49.78±1.86^{a}
(n-3)/(n-6)	6.35±0.10	4.86±0.47	2.64±0.22	2.69±0.10
DHA/EPA	0.00±0.00^{d}	1.44±0.07^{b}	1.19±0.03^{c}	3.41±0.01^{a}
ARA/DHA	0.00±0.00^{c}	0.16±0.03^{a}	0.09±0.02^{b}	0.02±0.01^{c}
ARA/EPA	0.06±0.10	0.23±0.04	0.11±0.03	0.08±0.02
LA/PUFA	0.13±0.00^{b}	0.12±0.00^{b}	0.23±0.00^{a}	0.13±0.00^{b}
OA/PUFA	0.61±0.01^{a}	0.53±0.00^{b}	0.54±0.01^{b}	0.28±0.00^{c}
PUFA/Saturated	3.09±0.18^{a}	2.96±0.25^{a}	1.29±0.16^{b}	1.54±0.15^{b}

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824 **Table 4.** Proximate composition (in % of dry matter) of *P. punctifer* individuals at 4 dpf (yolk sac larvae) and
825 at 14 and 26 dpf fed the different dietary treatments. Data are expressed as mean ± S.D. (n = 3). Different
826 superscript letters denote differences statistically significant between dietary treatments (one-way ANOVA, P
827 < 0.05). Different asterisks denote differences statistically significant between age within a dietary treatment.
828 Dietary treatment codes: C, non-enriched *Artemia* and compound diet; T1, enriched *Artemia* and non-enriched
829 compound diet; T2, non-enriched *Artemia* and enriched compound diet; T3, enriched both *Artemia* and
830 compound diet.

	4 dpf	**14 dpf**				**26 dpf**			
		C	**T1**	**T2**	**T3**	**C**	**T1**	**T2**	**T3**
Proteins	53.63±4.62	57.09±1.26	48.89±3.28	54.92±4.65	51.25±1.45	52.66±2.39	55.32±4.81	45.86±1.48	51.22±0.90
Lipids	22.98±0.65	13.05±2.68	12.08±0.86*	14.09±1.79	12.24±1.82	9.08±1.08^{b}	9.92±0.28^{b**}	11.22±0.13^{b}	13.67±0.13^{a}
Carbohydrates	2.90±0.44	3.55±0.40	3.57±0.25	3.70±0.28	3.29±0.32**	3.14±0.13^{b}	3.61±0.24^{ab}	3.67±0.25^{ab}	5.49±0.76^{a*}
Moisture	0.95±0.05	1.32±0.25	1.42±0.07**	1.51±0.09**	1.43±0.03	1.84±0.08	1.76±0.09*	1.82±0.14*	1.58±0.09

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Table 5. Total lipid and total fatty acids contents (in % of dry matter) and fatty acid composition (in % TFA) analyzed in *P. punctifer* individuals at 4 dpf (yolk sac larvae) and at 14 and 26 dpf fed the different dietary treatments. Data are expressed as mean ± S.D. (n = 3). Different superscript letters denote differences statistically significant between dietary treatments (one-way ANOVA, P < 0.05). Different asterisks denote differences statistically significant between ages within a dietary treatment (one-way ANOVA, *P* < 0.05). Dietary treatment codes: C, non-enriched *Artemia* and compound diet; T1, enriched *Artemia* and non-enriched compound diet; T2, non-enriched *Artemia* and enriched compound diet; T3, enriched both *Artemia* and compound diet.

	4 dpf	14 dpf				26 dpf			
		C	T1	T2	T3	C	T1	T2	T3
Total lipid (mg g^{-1} DW)	229.76±6.54	130.54±26.83	120.77±8.59	140.91±17.94	122.41±18.23	90.75±3.52b	99.22±10.78b	112.18±1.30ab	136.75±1.31a
Total fatty acid (mg g^{-1} DW)	130.93±7.86	72.60±17.75	64.77±6.58	78.29±8.23	68.72±13.28	40.49±3.04d	50.20±4.26c	63.68±1.13b	77.70±2.83a
14:0	0.28±0.03	$0.24±0.03^{a**}$	$0.29±0.02^{a**}$	$0.20±0.01^{b**}$	$0.30±0.03^{a**}$	$0.42±0.03^{d*}$	$0.76±0.04^{c*}$	$1.37±0.06^{b*}$	$1.73±0.11^{a*}$
16:0	22.50±0.53	$15.46±0.26^{ab**}$	$15.86±0.60^{a**}$	$14.85±0.02^{b**}$	$15.63±0.21^{ab**}$	$20.55±0.76^{c*}$	$21.99±0.30^{b*}$	$23.96±0.52^{a*}$	$25.64±0.20^{a*}$
18:0	21.09±0.78	$14.02±0.22^{**}$	14.76±0.46	$13.95±0.49^{*}$	$14.40±0.34^{*}$	$15.88±0.45^{a*}$	$14.29±0.08^{b}$	$10.80±0.15^{c**}$	$9.87±0.24^{d**}$
Total saturated	43.99±1.28	$29.82±0.34^{ab**}$	$31.02±1.02^{a**}$	$29.08±0.49^{b**}$	$30.43±0.18^{ab**}$	$37.09±1.17^{*}$	$37.33±0.32^{*}$	$36.43±0.73^{*}$	$37.56±0.13^{*}$
16:1	1.03±0.08	$1.06±0.01^{a}$	$0.85±0.09^{b**}$	$1.07±0.08^{a**}$	$0.85±0.05^{b**}$	$1.23±0.13^{c}$	$1.76±0.15^{b*}$	$1.98±0.00^{ab*}$	$2.34±0.06^{a*}$
18:1n-9 (OA)	16.43±0.29	$17.70±0.26^{a*}$	$15.38±0.25^{b*}$	$17.79±0.23^{a*}$	$15.49±0.47^{b*}$	$14.16±0.30^{ab**}$	$14.57±0.00^{a**}$	$12.66±0.16^{c**}$	$13.59±0.12^{b**}$
20:1	0.60±0.03	$0.45±0.01^{**}$	$0.44±0.02^{**}$	0.52±0.09	0.45±0.01	$0.54±0.03^{*}$	$0.61±0.01^{*}$	0.62±0.05	0.33±0.47
Total monounsaturated	20.28±0.40	$26.48±0.68^{a*}$	$23.49±0.53^{b*}$	$26.88±0.67^{a*}$	$23.78±0.79^{b*}$	$19.64±0.75^{**}$	$20.46±0.15^{**}$	$18.62±0.33^{**}$	$19.24±0.41^{**}$
18:2n-6 (LA)	1.91±0.05	$4.67±0.03^{a*}$	$3.56±0.26^{b}$	$4.63±0.08^{a*}$	$3.59±0.18^{b**}$	$3.24±0.27^{b**}$	$4.14±0.04^{a}$	$4.32±0.10^{a**}$	$4.79±0.22^{a*}$
18:3n-6	0.35±0.08	0.91±0.13	$0.65±0.16^{*}$	0.76±0.20	$0.86±0.06^{*}$	$0.66±0.07^{a}$	$0.00±0.00^{b**}$	$0.53±0.02^{ab}$	$0.51±0.14^{ab**}$
20:4n-6 (ARA)	3.12±0.12	$1.26±0.10^{b**}$	$2.69±0.09^{a}$	$1.21±0.08^{b**}$	$2.78±0.06^{a*}$	$2.81±0.03^{a}$	$2.86±0.11^{a*}$	$1.94±0.15^{b*}$	$1.71±0.04^{b**}$
22:4n-6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
22:5n-6	0.35±0.01	$0.05±0.08^{b**}$	$2.23±0.14^{a}$	$0.00±0.00^{b**}$	$2.29±0.17^{a**}$	$1.21±0.04^{d*}$	$2.09±0.02^{c}$	$4.66±0.03^{b*}$	$4.76±0.04^{a*}$
Total n-6 PUFA	7.12±0.15	$7.73±0.37^{b}$	$9.80±0.41^{a}$	$7.49±0.44^{b**}$	$10.17±0.12^{a**}$	$8.67±0.34^{c}$	$9.61±0.11^{b}$	$11.93±0.17^{a*}$	$12.16±0.11^{a*}$
18:3n-3 (ALA)	0.45±0.15	$14.69±0.41^{a*}$	$11.42±1.01^{b*}$	$15.27±0.68^{a*}$	$11.59±0.27^{b*}$	$1.38±1.21^{**}$	$0.85±0.10^{**}$	$1.05±0.03^{**}$	$0.89±0.05^{**}$
18:4n-3	0.00±0.00	$3.23±0.14^{a*}$	$2.26±0.09^{b*}$	$3.32±0.08^{a*}$	$2.23±0.02^{b*}$	$0.70±0.10^{**}$	$0.64±0.04^{**}$	$0.78±0.02^{**}$	$0.84±0.04^{**}$
20:4n-3	0.34±0.01	$3.06±0.19^{a*}$	$2.33±0.09^{b*}$	$3.14±0.12^{a*}$	$2.36±0.11^{b*}$	$1.04±0.02^{**}$	$0.75±0.11^{**}$	$0.78±0.13^{**}$	$0.67±0.08^{**}$
20:5n-3 (EPA)	4.08±0.07	$3.08±0.08^{**}$	$3.49±0.21^{**}$	$3.03±0.37^{**}$	$3.26±0.11^{**}$	$5.12±0.24^{a*}$	$5.02±0.18^{ab*}$	$4.11±0.36^{ab*}$	$3.97±0.03^{b*}$
21:5n-3	0.25±0.02	$0.16±0.02^{**}$	$0.19±0.05^{**}$	0.30±0.15	$0.15±0.01^{**}$	$0.36±0.06^{*}$	$0.30±0.02^{*}$	0.31±0.00	$0.35±0.02^{*}$
22:5n-3 (DPA)	1.84±0.08	$2.11±0.09^{a**}$	$1.55±0.09^{b**}$	$1.98±0.06^{a}$	$1.67±0.10^{b}$	$2.67±0.10^{a*}$	$2.09±0.02^{b*}$	$1.85±0.10^{c}$	$1.55±0.03^{d}$
22:6n-3 (DHA)	17.13±1.28	$5.49±0.39^{b**}$	$10.83±0.67^{a**}$	$5.57±0.44^{b**}$	$11.00±0.71^{a**}$	$20.19±0.89^{*}$	$20.54±0.59^{*}$	$22.21±0.53^{*}$	$21.65±0.35^{*}$

Total n-3 PUFA	24.08±0.98	31.82±0.52	32.07±0.38*	32.61±0.17*	32.27±0.48*	31.47±0.03	30.19±0.82**	31.10±0.86**	29.92±0.24**
Total PUFA	31.20±1.13	39.55±0.75b	41.86±0.76a	40.09±0.27b**	42.45±0.60a	40.14±0.37ab	39.80±0.71b	43.03±1.03a*	42.08±0.13ab
(n-3)/(n-6)	3.38±0.07	4.12±0.18a*	3.28±0.11b	4.37±0.29a*	3.17±0.01b*	3.63±0.11a**	3.15±0.14b	2.61±0.03c**	2.46±0.04c**
DHA/EPA	4.20±0.38	1.78±0.13b**	3.10±0.17a**	1.85±0.14b**	3.38±0.27a**	3.95±0.03b*	4.09±0.02b*	5.42±0.35a*	5.45±0.12a*
ARA/DHA	0.18±0.01	0.23±0.01ab*	0.25±0.02a*	0.22±0.00b*	0.25±0.01a*	0.14±0.02a**	0.14±0.00a**	0.09±0.00b**	0.08±0.00b**
ARA/EPA	0.76±0.04	0.41±0.04b	0.77±0.07a*	0.40±0.03b**	0.85±0.03a*	0.55±0.06	0.57±0.00**	0.47±0.01*	0.43±0.01**
LA/PUFA	0.06±0.00	0.12±0.00a*	0.08±0.00b**	0.12±0.00a*	0.08±0.01b**	0.08±0.01b**	0.10±0.00a*	0.10±0.00a**	0.11±0.01a*
OA/PUFA	0.53±0.03	0.45±0.01a*	0.37±0.00b	0.44±0.01a*	0.36±0.02b*	0.35±0.00a**	0.37±0.01a	0.29±0.01c**	0.32±0.00b**
PUFA/Saturated	75.19±0.14	69.37±0.44b**	72.89±0.58a**	69.17±0.33b**	72.88±0.72a**	77.23±0.80b*	77.13±0.39b*	79.45±0.30a*	79.63±0.26a*

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Figure legends

Figure 1. Experimental design of the nutritional trial. Different feeding windows coinciding with different developmental stages were used to create four experimental groups: the C (control) group was fed non-enriched *Artemia* during the larval period (4-15 dpf) and non-enriched compound diet during the early juvenile stage (16-26 dpf); the group T1 was fed enriched *Artemia* but non-enriched compound diet; the group T2 was fed non-enriched *Artemia* but enriched compound diet; and the group T3 was fed both enriched *Artemia* and compound diet. All groups were exclusively fed a non-enriched compound diet from 27 to 36 dpf. Enrichment was done with a reference commercial product, Algamac 3050. dpf, days post-fertilization.

Figure 2. Growth in terms of wet weight (mg) and total length (mm) of *P. punctifer* at 14 dpf (end of the *Artemia* feeding phase, larval stage) and 26 dpf (end of the experimental compound diet feeding phase, juvenile stage). Data are presented as means ± S.D. (n = 45). Values with a different letter denote significant differences between dietary treatments within a developmental stage and asterisks denote significant differences during development within a dietary treatment (one-way ANOVA, $P < 0.05$).

Figure 3. Incidence of total cannibalism at 26 dpf (end of the experimental compound diet feeding phase, juvenile stage), and survival at 14 dpf (end of the *Artemia* feeding phase, larval stage), 26 dpf (end of the nutritional trial, juvenile stage) and 36 dpf (end of the experimental trial, juvenile stage) of *P. punctifer* fed the different experimental feeding protocols. Data are presented as means ± S.D. (n = 45). Values with a different letter denote significant differences between dietary treatments (one-way ANOVA, $P < 0.05$).

Figure 4. Incidence of cannibalism of *P. punctifer* at different developmental and feeding stages. The incidence of cannibalism is shown by total cannibalism, by type of cannibalism (I or II), and by time of the day for each type of cannibalism. In figures of cannibalism by type, full bars indicate type I cannibalism and lined bars indicate type II cannibalism. In figures of type I and II cannibalism by time of the day, full bars indicate day time, while lined bars indicate night time. Data are presented as means ± S.D. (n = 45). Values with a different letter denote significant differences between dietary treatments within a developmental stage and asterisks denote significant differences between the type of cannibalism within a dietary treatment or between the time of the day for a type of cannibalism within a dietary treatment (one-way ANOVA, $P < 0.05$).

Figure 5. Longitudinal paraffin sections of the liver and anterior intestine of *P. punctifer* at 14 and 26 dpf fed the different experimental treatments. Staining, hematoxylin-eosin. While lipid deposits in the intestine are similar in all dietary groups at 14 dpf, note the fattier livers of groups C and T2. At 26 dpf, groups T2 and T3 showed the fattiest livers, and lipid accumulation in the intestine was concentrated in the apical zone, whereas in groups C and T1 lipid was accumulated along the intestinal folds. Contrary to the rest of the dietary groups, note the important accumulation of lipids in the intestine (steatosis) compared to the liver in the C group.

Figure 6. Relative expression of α-amylase (*amy*), trypsinogen (*try*), chymotrypsin (*ctr*), pepsinogen (*pga*), phospholipase A2 (*pla2*) and lipoprotein lipase (*lpl*) genes in *P. punctifer* at 14 and 26 dpf. Data are presented as means ± S.D. (n = 9). Values with a different letter denote significant differences between dietary treatments within a developmental stage and

887 asterisks denote significant differences during development within a dietary treatment (one-
888 way ANOVA, $P < 0.05$).

Figure 1

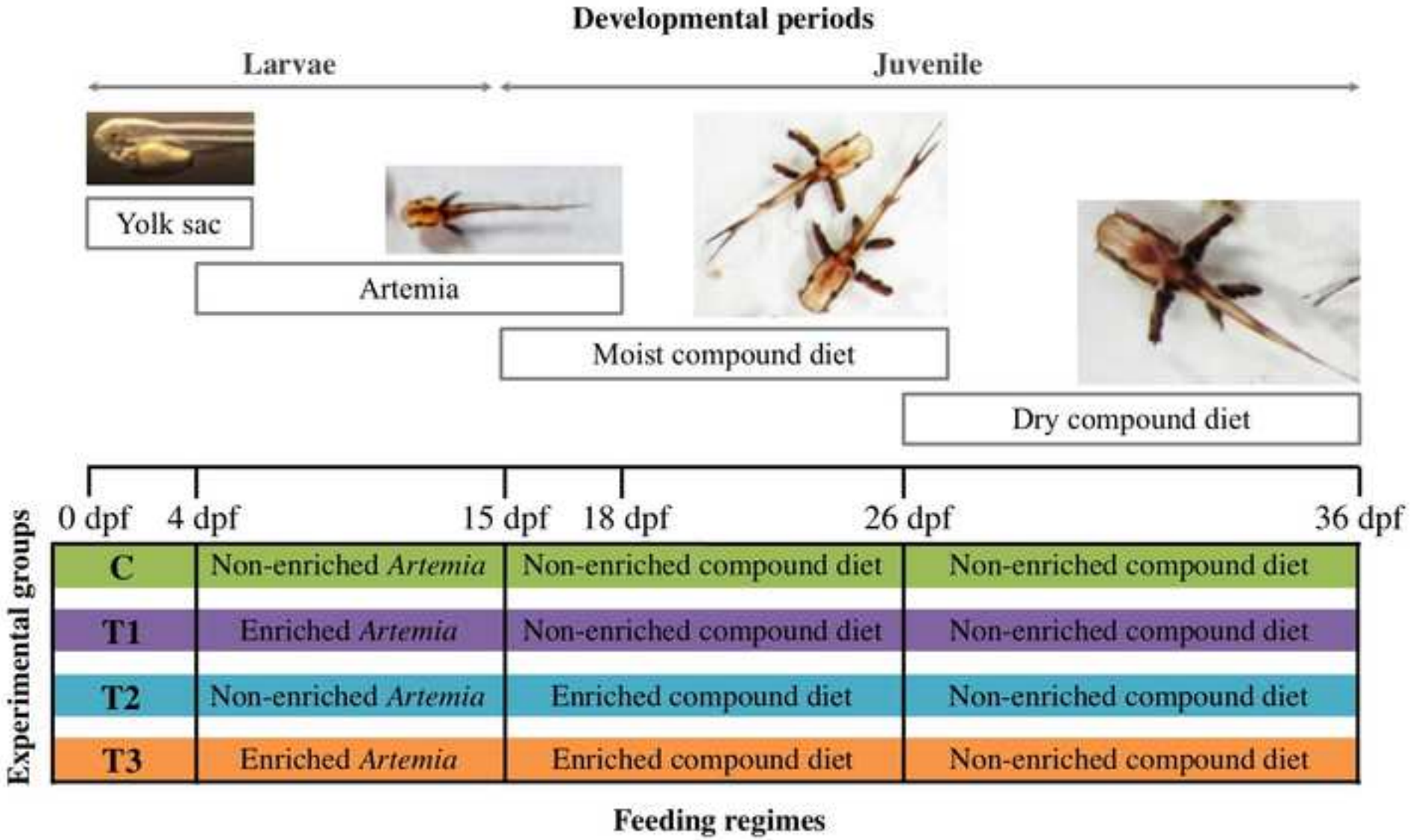

Developmental periods
Larvae
Juvenile
Yolk sac
Artemia
Moist compound diet
Dry compound diet
0 dpf
4 dpf
15 dpf
18 dpf
26 dpf
36 dpf
Experimental groups
C
Non-enriched Artemia
Non-enriched compound diet
Non-enriched compound diet
T1
Enriched Artemia
Non-enriched compound diet
Non-enriched compound diet
T2
Non-enriched Artemia
Enriched compound diet
Non-enriched compound diet
T3
Enriched Artemia
Enriched compound diet
Non-enriched compound diet
Feeding regimes

Figure 2

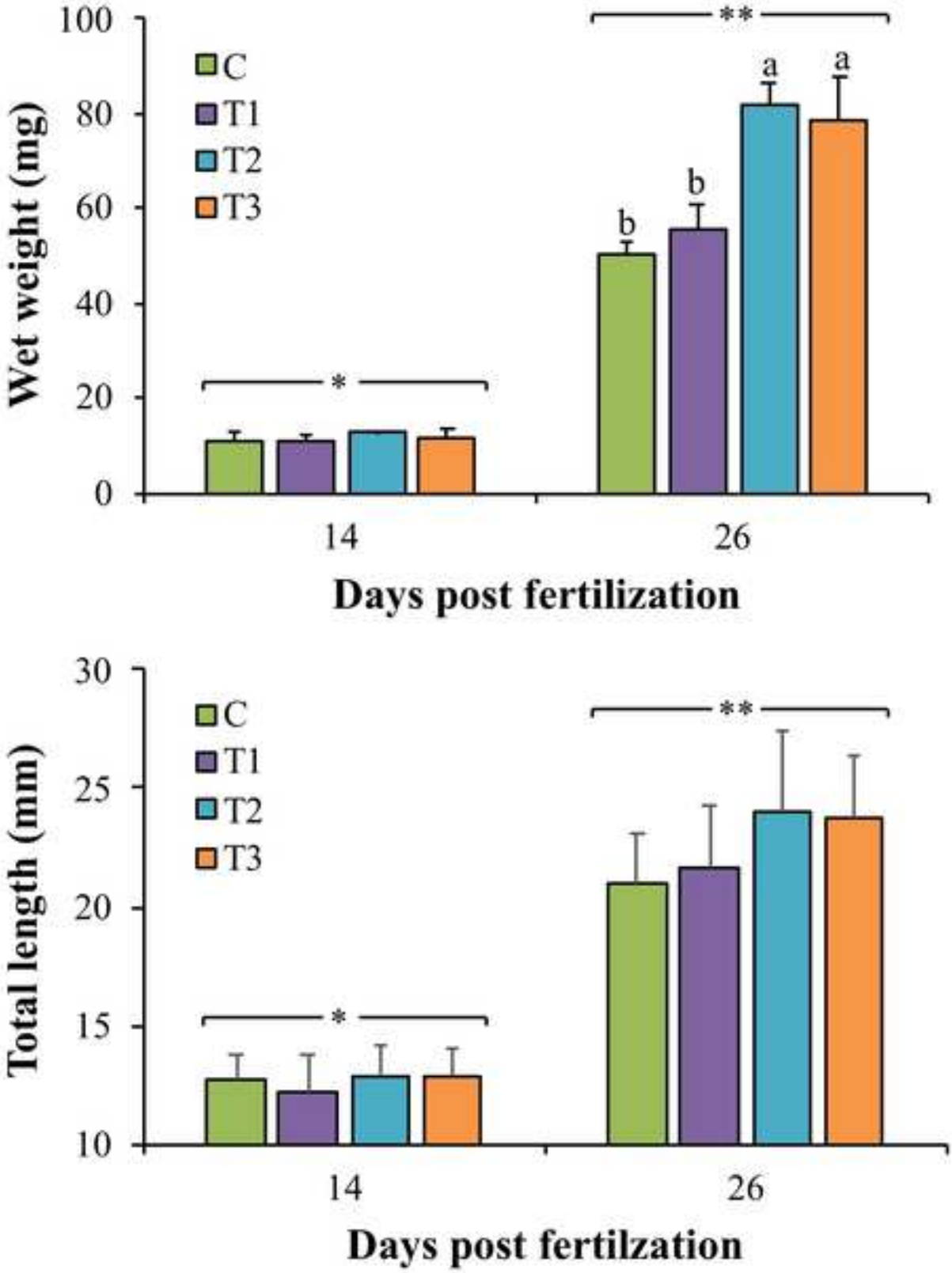
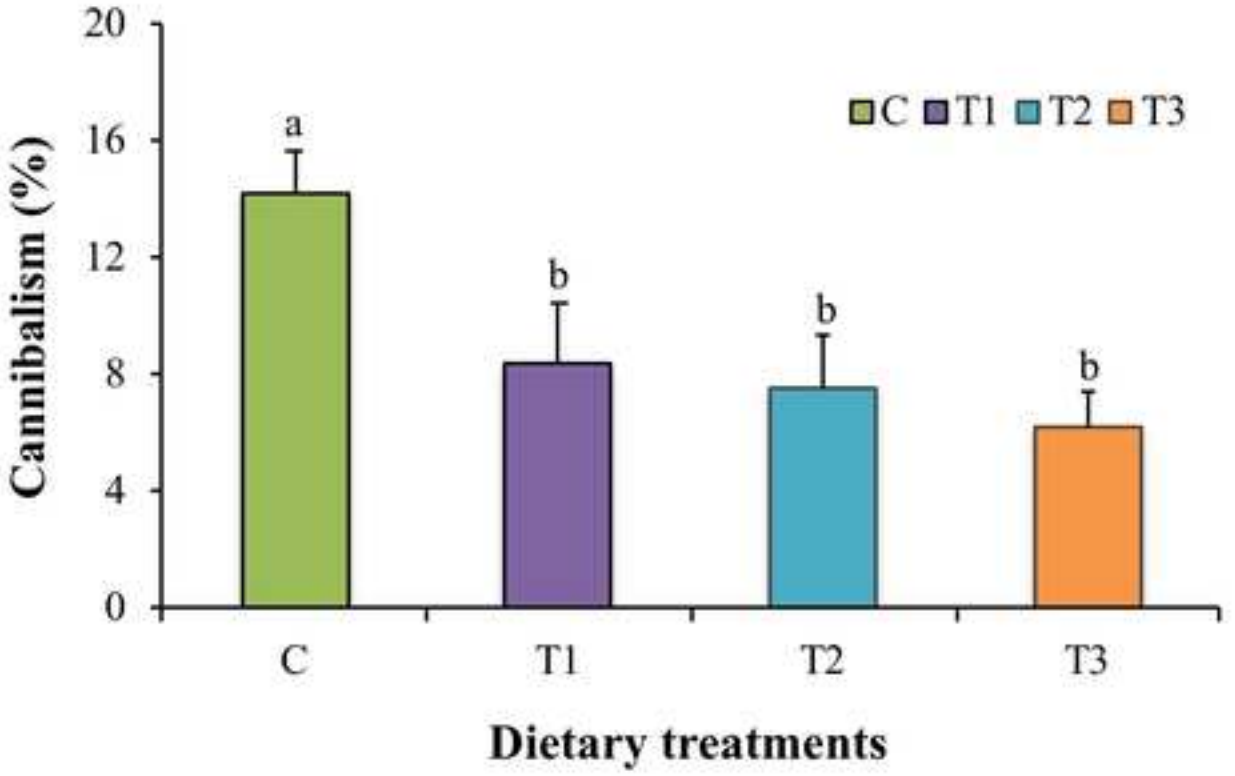

Wet weight (mg)
100
80
60
40
20
0
C
T1
T2
T3
*
**
a
a
b
b
14
26
Days post fertilization
Total length (mm)
30
25
20
15
10
C
T1
T2
T3
*
**
14
26
Days post fertilzation

Figure 3


Cannibalism (%)
20
16
12
8
4
0
a
b
b
b
C
T1
T2
T3
C T1 T2 T3
Dietary treatments

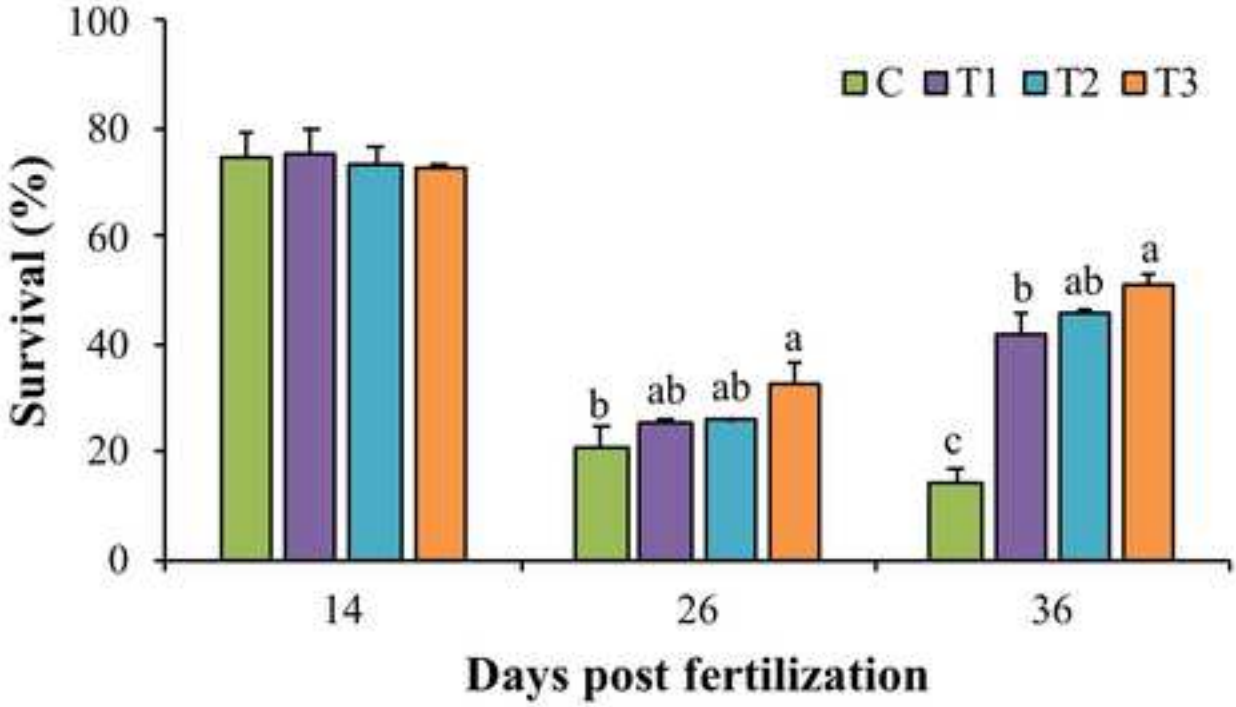

Survival (%)
100
80
60
40
20
0
b
ab
ab
a
c
b
ab
a
14
26
36
C T1 T2 T3
Days post fertilization

Figure 4

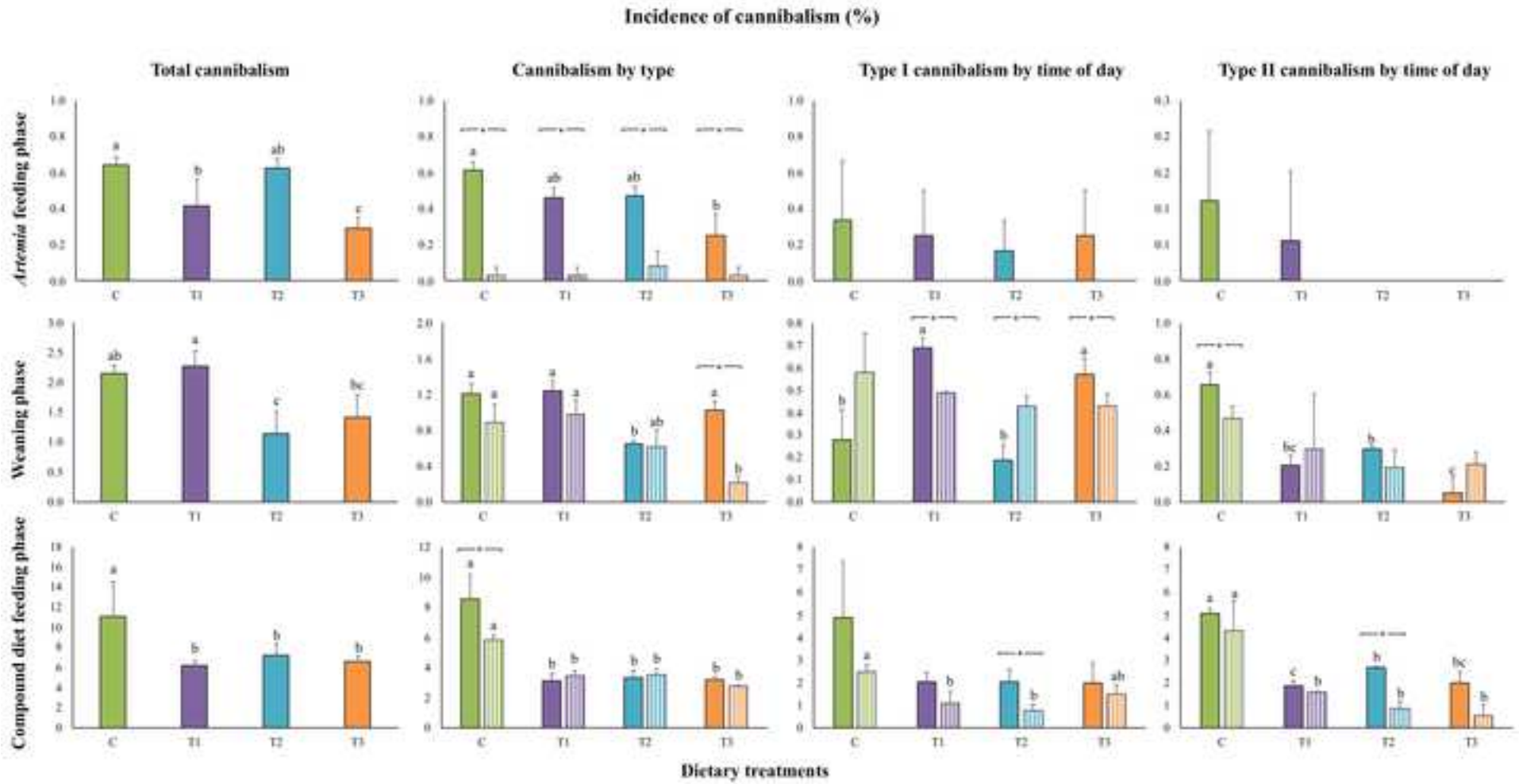

Incidence of cannibalism (%)
Total cannibalism
Cannibalism by type
Type I cannibalism by time of day
Type II cannibalism by time of day
Artemia feeding phase
Weaning phase
Compound diet feeding phase
C
T1
T2
T3
Dietary treatments

Figure 5

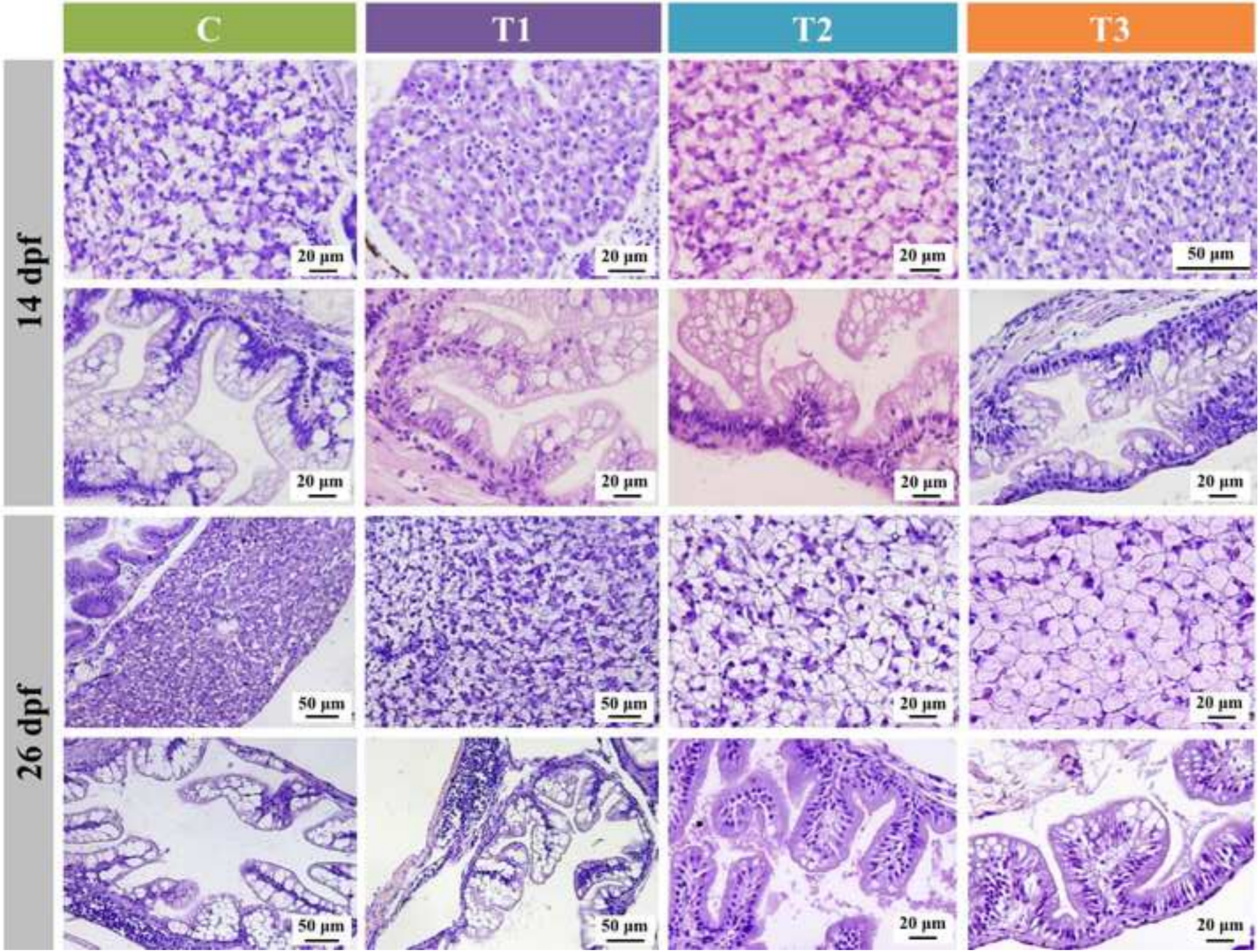
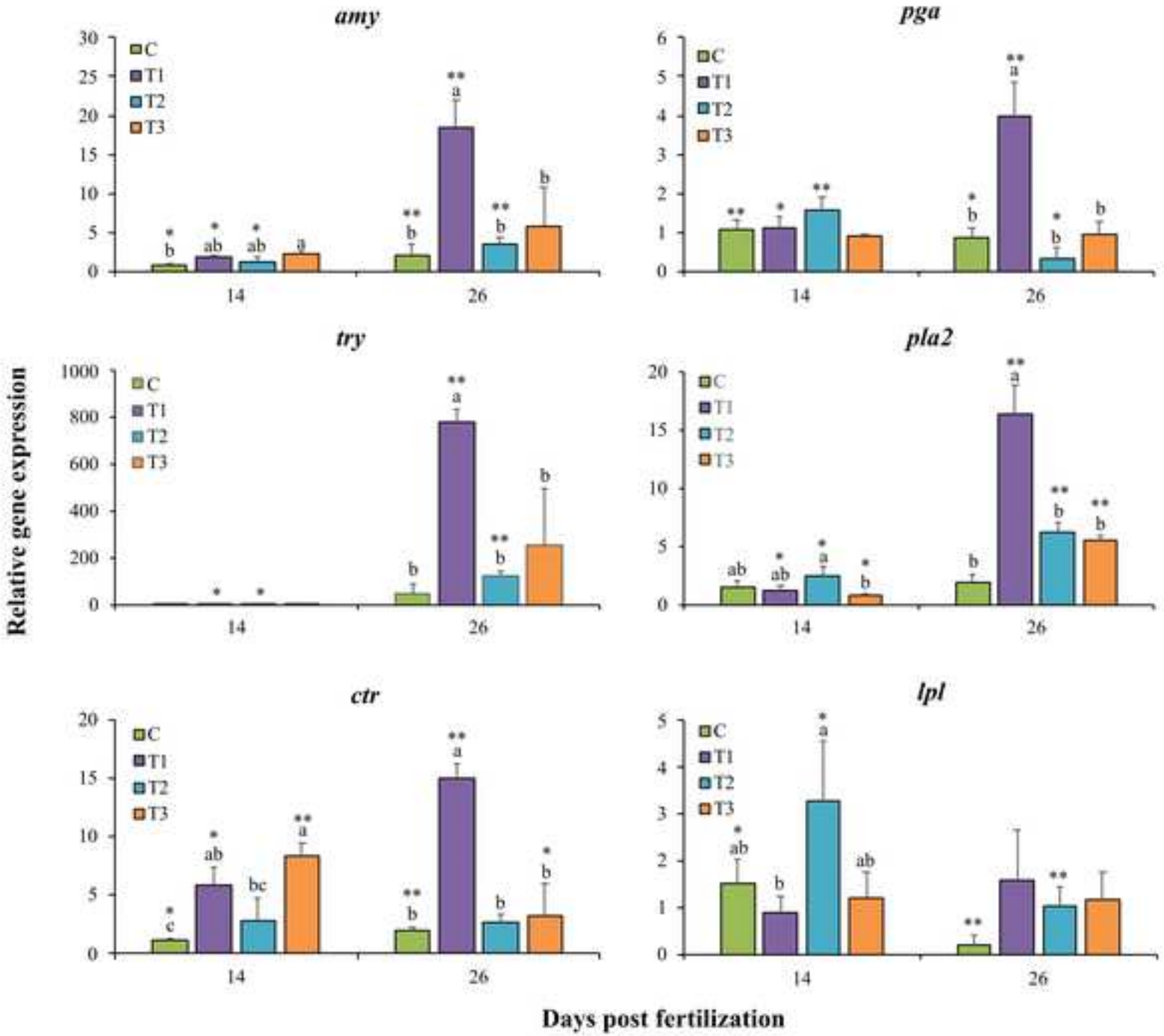


Figure 6


amy
pga
try
pla2
ctr
lpl
C
T1
T2
T3
Relative gene expression
Days post fertilization
14
26

DISCUSIÓN GENERAL

La acuicultura de agua dulce representa la mayor parte de la producción acuícola mundial (FAO, 2020) y su crecimiento ha permitido una mayor accesibilidad a fuentes de proteína acuática, especialmente en países de ingresos bajos y medios, haciendo una importante contribución a la seguridad alimentaria y nutricional de la población (Belton et al., 2020). El cultivo de peces de agua dulce tiene en general un costo más bajo que los peces marinos y está basado en tecnologías relativamente más básicas, por lo que suelen ser más accesibles a los consumidores y productores de países en desarrollo (Belton et al., 2020). Entre las distintas familias de peces de agua dulce cultivados, los bagres poseen un amplio repertorio de características que los hacen especialmente adecuados para fines acuícolas, tales como el alto potencial de adaptación a condiciones de cultivo intensivo, alta fecundidad, hábitos alimenticios nocturnos, capacidad para vivir en aguas turbias, resistencia relativamente alta a enfermedades infecciosas, una conversión alimenticia eficiente y la ausencia de espinas intramusculares, lo que facilita enormemente el procesamiento del filete (Gisbert et al., 2022). En la Amazonía, que alberga la mayor diversidad de peces del mundo (Jézéquel et al., 2020), los siluriformes representan el segundo orden más diverso con 956 especies descritas (Dagosta y Pinna, 2019), de las cuales las especies del género *Pseudoplatystoma*, y en concreto *Pseudoplatystoma punctifer*, la especie de este género más extendida en la cuenca amazónica, han despertado un gran interés como especies de cultivo desde hace décadas. En el Perú, el desarrollo del cultivo de *P. punctifer* es considerado una prioridad, si bien las dificultades de su cultivo, especialmente durante las etapas tempranas de vida, han impedido hasta ahora su cultivo comercial. Aunque las larvas de peces pueden ser morfológicamente capaces de capturar diferentes alimentos, su sistema digestivo sufre una serie de cambios antes de ser completamente funcional, por lo que el conocimiento de la ontogenia del sistema digestivo puede contribuir al desarrollo de protocolos de alimentación larvaria más eficientes (Gisbert et al., 2022). Por otro lado, los conocimientos sobre los requerimientos nutricionales de las diferentes especies de bagres durante el desarrollo temprano son muy limitados a pesar de ser esenciales para optimizar las dietas y los protocolos de alimentación y, por lo tanto, mejorar la calidad de las larvas y los juveniles, promoviendo su crecimiento, salud y bienestar (Gisbert et al., 2022; Hamre et al., 2013). Esta tesis ha buscado, por tanto, contribuir a mejorar el cultivo temprano de esta especie, estudiando los patrones de su desarrollo digestivo y sus requisitos nutricionales específicos.

En la presente discusión se han considerado dos artículos relacionados con la tesis publicados antes del comienzo de la misma con el fin de discutir los resultados de manera más completa e integrativa. Estos son el estudio de la ontogenia histológica del sistema digestivo (anexo 1, A1) y la primera parte del experimento del capítulo 2.1 en el que se estudió el efecto de las dietas en el crecimiento, la supervivencia y la incidencia del canibalismo (anexo 2, A2).

Ontogenia del aparato digestivo de *Pseudoplatystoma punctifer*

En comparación con los peces de aguas templadas, los peces tropicales poseen un desarrollo más rápido debido al efecto de la temperatura sobre la tasa metabólica (Gillooly et al., 2002). El patrón de crecimiento de las larvas de *P. punctifer* y de los juveniles tempranos en términos de peso mostró una fase inicial de crecimiento lento (SGR 0.19 ± 0.00) hasta los 12 días post fertilización–dpf (*ca.* 12 mm longitud total–LT), correspondiente a la etapa larvaria, seguida de una tasa mayor de crecimiento exponencial (SGR 0.53 ± 0.10) coincidiendo con el inicio de la etapa juvenil (*cf.* 1.2). Este tipo de patrón de crecimiento también se ha reportado en otras especies de peces tropicales marinos y de agua dulce como *Pelteobagrus fulvidraco* (Yang et al., 2010), *Mystus nemurus* (Srichanun et al., 2012), *Centropomus undecimalis* (Jimenez-Martinez et al., 2012), *Petenia splendida* (Uscanga-Martínez et al., 2011) o *Lutjanus guttatus* (Moguel-Hernández et al., 2014), entre otros. La baja tasa de crecimiento observada durante la fase larvaria de *P. punctifer* puede interpretarse como una estrategia evolutiva para asignar la energía obtenida de las reservas vitelinas y de las presas a promover cambios fisiológicos larvarios tales como el desarrollo de los diferentes sistemas, en lugar del crecimiento somático, como también se ha reportado en *Ompok bimaculatus* (Pradhan et al., 2013), *Pangasianodon hypophthalmus* (Rangsin et al., 2012) y *Atractosteus tropicus* (Frías-Quintana et al., 2015). Sin embargo, también podría deberse a que la *Artemia*, ofrecida durante este período del desarrollo, no satisface completamente las necesidades nutricionales de las larvas, ni les permiten explotar completamente su potencial de crecimiento, como mostraron diferentes estudios de la presente tesis (*cf.* 1.2, A1, A2). De hecho, en el estudio presentado en el anexo 2, se demostró que la composición nutricional de la dieta modula el SGR de *P. punctifer* durante la etapa juvenil temprana de 12 a 26 dpf, por lo que también cabría esperar una tasa de crecimiento mayor durante la fase larvaria si se ofreciera un régimen de alimentación adecuado a las necesidades nutricionales específicas de las larvas. En este sentido, investigar el desarrollo y la funcionalidad del sistema digestivo, así como las estrategias de destete, se hace necesario para establecer un protocolo de alimentación óptimo, ya que la fisiología y morfogénesis del tracto digestivo larvario podrían verse estimuladas o deterioradas dependiendo de cómo se realice el proceso de co-alimentación (Cahu y Zambonino Infante, 2001; Pradhan et al., 2013).

Ontogenia histológica del aparato digestivo de P. punctifer

Los bagres son uno de los grupos de especies de agua dulce que más atención han recibido en cuanto al desarrollo histológico de su sistema digestivo (Gisbert et al., 2022, 2013), lo que probablemente esté relacionado con su rápido potencial de crecimiento, la calidad de su filete y su adaptabilidad a las condiciones de cultivo, además de su importancia en términos acuícolas a nivel mundial (Gisbert et al., 2022). El estudio histológico del desarrollo del sistema digestivo de *P. punctifer* es el primero en representar a la familia Pimelodidae. Aunque la morfogénesis del tracto digestivo y de las glándulas accesorias en *P. punctifer* resultó ser similar a la de otras especies de bagres de agua dulce, se observaron varias diferencias en el tiempo de diferenciación y el desarrollo de los órganos entre esta

especie y otras del orden Siluriformes (*cf.* A1). En este sentido, en el estudio presentado en el anexo 1 nos centramos en analizar los cambios ontogenéticos de la organización histomorfológica de los órganos digestivos y su vínculo con las prácticas de cultivo que podrían ser útiles para mejorar el cultivo temprano de *P. punctifer*, así como la comparación interespecífica de la organización histológica y el desarrollo con otras especies de bagres.

El desarrollo del sistema digestivo de *P. punctifer* siguió el patrón típico de una especie altricial y, tal y como se esperaba de una especie de bagre tropical de crecimiento rápido, presentó un rápido desarrollo larvario y transformación en juvenil. Así, en sólo 2 semanas (temperatura de cultivo a 28 °C) tuvo lugar la morfogénesis del tracto digestivo a partir de un canal indiferenciado en la eclosión a un tracto digestivo complejo similar al de un juvenil. Durante este período de 2 semanas, las larvas fueron alimentadas con *Artemia* y mostraron un crecimiento y una tasa de supervivencia (95%) correctos. Por tanto, la ontogenia del tracto digestivo presentado en esta tesis puede considerarse como el desarrollo histológico estándar del sistema digestivo de *P. punctifer* usando presas vivas. La eclosión en *P. punctifer* ocurrió alrededor de 18 h post fertilización y, durante las primeras horas después de la eclosión (0 a 1 dpf), se abrió el tubo digestivo y se formaron las glándulas anejas (hígado y páncreas). Los gránulos de zimógeno (precursores de las enzimas digestivas pancreáticas) fueron detectados en el páncreas exocrino (1 a 2 dpf, *ca.* 4 mm LT) antes del inicio de la alimentación exógena (4 dpf, *ca.* 6 mm LT). Al inicio de la alimentación exógena, los peces tenían una boca bien diferenciada con estructuras para la captura y degustación de las presas, una mucosa intestinal desarrollada y glándulas digestivas accesorias diferenciadas y funcionales para la producción de enzimas (páncreas) y el almacenamiento de nutrientes (hígado). En esta etapa, los datos histológicos indicaron que el sistema digestivo era funcional y capaz de digerir los alimentos, como fueron, por ejemplo, los primeros signos de acumulación de lípidos observados en la región anterior e intermedias del intestino y en el hígado poco después del inicio de la alimentación exógena y al final de la fase de alimentación mixta.

La transición de la alimentación endógena a la exógena es una etapa crítica del desarrollo larvario y puede resultar en altas tasas de mortalidad si los alimentos no se administran adecuadamente a las larvas una vez que se agotan sus reservas vitelinas (Sarasquete et al., 1995). En la mayoría de las especies de bagre estudiadas existe una fase de alimentación mixta durante la cual coocurre la alimentación endógena y exógena. La duración de este período varía entre especies, desde solo 5 gd en *C. gariepinus* (Verreth et al., 1992), 54 y 56 gd en *O. bimaculatus* (Pradhan et al., 2012) y *P. punctifer* (presente tesis), hasta 69 y 79 gd en *Silurus glanis* (Kozarić et al., 2008) y *Clarias nieuhofii* (Saelee et al., 2011). Estas diferencias pueden estar relacionadas con diferentes tamaños de larvas y de sacos vitelinos (Gisbert et al., 2000; Kamler, 1992), así como también con diferentes condiciones de cultivo, las cuales pueden afectar la tasa de consumo del vitelo y el comienzo de la alimentación exógena. Este período de alimentación mixta tiene gran importancia para el desempeño larvario, ya que puede contrarrestar carencias potenciales en el suministro de nutrientes antes de que se agoten las reservas vitelinas, así como servir de reserva temporal de nutrientes que permita a la larva resistir breves períodos

de escasez de alimentos (Treviño et al., 2011). En este sentido, la presencia de una etapa de nutrición mixta podría constituir una ventaja para el cultivo larvario de *P. punctifer* en estanques, ya que garantizaría una transición exitosa a la alimentación exógena o minimizaría los posibles efectos negativos de un retraso en el comienzo de la primera alimentación (Gisbert y Williot, 1997). La transición a alimentos exógenos en presencia de reservas vitelinas generalmente implica que el tubo digestivo es funcional, aunque continúa el desarrollo estructural y funcional desde las formas larvaria a juvenil y adulta (Jaroszewska y Dabrowski, 2011).

Los tejidos y órganos digestivos son particularmente sensibles a las condiciones de alimentación no óptimas o al estrés nutricional durante el desarrollo larvario porque se encuentran bajo una morfogénesis progresiva e intensiva y, en consecuencia, responden con rapidez y sensibilidad a los trastornos nutricionales (Gisbert et al., 2008). En este sentido, los cambios en la organización histológica del hígado y del intestino se han utilizado como marcadores histológicos para analizar el estado nutricional de las larvas de peces y dilucidar los efectos de diferentes regímenes alimenticios o nutrientes en el estado y condición nutricional de las larvas (Boglino et al., 2012b; Papadakis et al., 2009; Pradhan et al., 2014). En este estudio, la acumulación de lípidos en la mucosa intestinal poco después del inicio de la alimentación exógena podría interpretarse como un indicador de la digestión y absorción luminal, así como del almacenamiento temporal de lípidos, lo que refleja el desarrollo funcional del intestino (Gisbert et al., 2008). Además, la acumulación moderada de lípidos en el intestino se correlacionó positivamente con cambios en el nivel de depósitos de lípidos en el hígado durante la mayor parte del período estudiado, lo que indicó que el contenido de lípidos del alimento no excedió la capacidad de absorción y de exportación de ácidos grasos de los enterocitos. Sin embargo, la gran acumulación de depósitos de lípidos en el intestino y el hígado a 41 dpf (*ca.* 58 mm LT) podría atribuirse a un cambio en la capacidad juvenil temprana para absorber y exportar lípidos a través del sistema circulatorio hacia el hígado para ser almacenados y movilizados para el crecimiento cuando sea necesario (Tso, 1994) y/o a un desequilibrio nutricional con respecto al contenido de proteínas y lípidos de la dieta compuesta administrada. En cualquier caso, la acumulación de lípidos en el intestino o el hígado no resultó en una situación patológica potencial (esteatosis) que pudiera afectar la funcionalidad celular, y finalmente el desempeño larvario, ya que no se detectaron signos de abrasión epitelial, necrosis celular y/o reacciones inflamatorias como consecuencia de estos importantes depósitos lipídicos (Gisbert et al., 2008). Sin embargo, estos resultados parecieron indicar que las dietas compuestas para los juveniles tempranos de *P. punctifer* podrían refinarse con el fin de satisfacer los requisitos nutricionales específicos de la especie y mejorar el rendimiento de los alevines.

En los peces, el estómago es uno de los últimos órganos digestivos en desarrollarse durante la ontogenia. El inicio de la digestión ácida muestra el cambio del modo de digestión larval al juvenil, caracterizado por una mejora notable de la digestión de proteínas complejas. Este momento se suele considerar la transición de la etapa larvaria a la juvenil desde una perspectiva de fisiología digestiva y un momento adecuado para el destete a alimentos compuestos en condiciones de cultivo (Lazo et al.,

2011; Rønnestad et al., 2013; Zambonino Infante et al., 2008). Aunque el comienzo de la diferenciación morfológica del estómago en *P. punctifer* apareció como una dilatación entre el esófago y el intestino anterior antes del inicio de la alimentación exógena (2 a 3 dpf, *ca.* 5 mm LT), las glándulas gástricas no aparecieron hasta los 8 dpf (7,31 ± 0,48 mm LT) y la diferenciación morfoanatómica completa (regiones cardíaca, fúndica y pilórica) se completó alrededor de los 15 dpf (*ca.* 16 mm LT) (*cf.* A1). Las propiedades histoquímicas de las células mucosas (PAS positivo: secreción de mucinas neutras) que recubren la región gástrica fúndica observadas a 8 dpf pueden considerarse un signo indirecto de la funcionalidad del estómago (Gisbert et al., 2013), ya que las mucosustancias neutras protegen al estómago de procesos de autodigestión causados por el HCl y las enzimas producidas por glándulas gástricas (Chen et al., 2006). La aparición de las glándulas gástricas normalmente indica la formación de un estómago funcional (Stroband y Kroon, 1981), que también se considera un criterio histológico para diferenciar a las larvas de los juveniles (Sarasquete et al., 1995). Resultados similares se han reportado para distintos Siluriformes (Kozarić et al., 2008; Pradhan et al., 2012; Saelee et al., 2011; Verreth et al., 1992; Yang et al., 2010), aunque existen diferencias específicas para cada especie en términos de morfogénesis del estómago y de la transición de la digestión alcalina a la ácida (*cf.* A1). Por otro lado, *P. punctifer* presenta un comportamiento caníbal que aparece en torno a la transición de la etapa larvaria a la juvenil, lo que sugiere que las necesidades nutricionales podrían estar cambiando y que los protocolos de alimentación podrían influir en la incidencia de tal comportamiento. De hecho, el comportamiento caníbal comenzó a aparecer coincidiendo con la formación de las válvulas orales y las glándulas gástricas del estómago (a partir de los 11 dpf). Esto podría indicar que la *Artemia* podría no cubrir completamente las necesidades nutricionales de las larvas de *P. punctifer*. En ese momento, las válvulas orales ya están equipadas con papilas gustativas que se cree sirven para evaluar la calidad de los alimentos antes de que pasen a la cavidad bucal (Gamal et al., 2012; Yashpal et al., 2006). Además, la incidencia de canibalismo aumentó claramente en el destete, especialmente al final de la co-alimentación (21 dpf), cuando la cantidad de *Artemia* ofrecida disminuyó hasta en un 75% de la ración. Este comportamiento caníbal persistió hasta que las larvas comenzaron a ser alimentadas con la segunda dieta compuesta. Aunque la dieta compuesta utilizada para el destete de las larvas les permitió crecer con normalidad y los resultados histológicos mostraron que el sistema digestivo a 18 dpf estaba listo para procesar dietas compuestas, la correlación observada entre el canibalismo y el protocolo de alimentación sugirió que las larvas alimentadas con la primera dieta compuesta no estaban explotando plenamente su potencial de crecimiento. Por tanto, consideramos necesario evaluar si la calidad y la composición de la dieta podrían reducir tal comportamiento. Además, la tasa de supervivencia al final de la tercera fase de alimentación con dietas compuestas (33 a 41 dpf) continuó disminuyendo, aunque el canibalismo pareció reducirse. Sin embargo, no se observaron animales muertos en los tanques, sino que sólo desaparecieron. El motivo de la menor incidencia de canibalismo en este período de cultivo podría deberse a que los juveniles de *P. punctifer* pudieron canibalizar especímenes más grandes y, junto con la maquinaria digestiva más eficiente, sus necesidades nutricionales podrían cubrirse con una tasa

más baja de canibalismo. Esto podría indicar nuevamente que la dieta compuesta utilizada para alimentar a los juveniles de *P. punctifer* durante ese período también podría ser inapropiada.

***Ontogenia funcional del aparato digestivo de* P. punctifer**

El análisis de la ontogenia funcional se realizó a través del estudio de la expresión de los principales precursores enzimáticos digestivos, así como de la actividad enzimática intestinal, pancreática y estomacal.

El capítulo 1.1 representa la primera descripción de la ontogenia transcripcional de algunas de las enzimas digestivas más importantes de una especie de pez amazónico del género *Pseudoplatystoma* y brinda información sobre la filogenia molecular de las enzimas digestivas y el desarrollo de las capacidades digestivas y las preferencias alimentarias durante la etapa temprana de vida de *P. punctifer*. Las relaciones filogenéticas de las secuencias proteicas de los genes digestivos estudiados (*α-amilasa* (*amy*), *fosfolipasa A2* (*plA2)*, *lipoproteína lipasa* (*lpl*), *tripsinógeno* (*try*), *quimotripsinógeno* (*ctr*) y *pepsinógeno* (*pga*)) en *P. punctifer* (*cf.* 1.1) correspondieron a la clasificación filogenética de los peces óseos (Betancur-R et al., 2017). Como era de esperar, las secuencias de proteínas de *P. punctifer* se agruparon junto con las de otras especies de bagres en todos los árboles filogenéticos, y en su mayoría se agruparon con las secuencias de proteínas de especies pertenecientes al clado principalmente de agua dulce de los Otophysi (Siluriformes, Cypriniformes, Characiformes y Gymnotiformes) (Betancur-R et al., 2017; Nelson et al., 2016), excepto en aquellos árboles con mayor número de nodos menos soportados (*try*, *ctr*). La mayoría de las secuencias de proteínas estudiadas de *P. punctifer* (*amy*, *plA2*, *try* y *pga*) también mostraron máxima homología con las de otras especies de bagre, con la excepción de *lpl* y *ctr*, probablemente debido al uso de secuencias parciales y/o a problemas en la anotación de las isoformas. Sin embargo, las relaciones filogenéticas permitieron identificar algunas isoformas de las secuencias de proteínas. Las secuencias *ctr* y *pga* aisladas de *P. punctifer* se localizaron en clados con secuencias de las isoformas *ctrA* y *pgaA1*, respectivamente. Por el contrario, fue difícil identificar la isoforma de *try*, ya que las secuencias de las otras especies de bagres eran todas secuencias de codificación predichas.

La expresión de todos los genes analizados en *P. punctifer*, así como de la actividad enzimática correspondiente, fue detectada antes del inicio de la alimentación exógena, lo que denota que se trata de un proceso programado genéticamente (*cf.* 1.1), tal y como se ha reportado en otras especies de peces cultivadas (Cahu et al., 2004; Darias et al., 2007a, 2007b, 2006; Galaviz et al., 2015; Mata-Sotres et al., 2016; Péres et al., 1998; Zambonino Infante et al., 2008).

La expresión de *amy* no siguió el patrón ontogenético habitual observado en especies carnívoras, caracterizado por un alto nivel de expresión y de actividad enzimática inicial que disminuye gradualmente hasta el final del estadio larvario (Cahu et al., 2004; Darias et al., 2006; Galaviz et al., 2015; Moguel-Hernández et al., 2016; Péres et al., 1998; Srichanun et al., 2013; Zambonino Infante y Cahu, 1994). Al contrario, la expresión de *amy* en *P. punctifer* aumentó gradualmente hasta el final del

desarrollo larvario (10 dpf, *ca.* 11 mm LT) y permaneció relativamente alta después (*cf.* 1.1). Sin embargo, a nivel enzimático, se detectaron valores altos de α-amilasa desde la eclosión (*cf.* 1.2), lo que puede deberse a la presencia de depósitos de glucógeno acumulados en el saco vitelino (Gisbert y Doroshov, 2006). Además, la presencia de actividad de α-amilasa antes de la alimentación exógena puede ser provocada por mecanismos intrínsecos más que por la estimulación de la dieta, como también se ha observado en otras especies de peces (Lazo et al., 2000; Zambonino Infante et al., 2008). Además, aunque las diferencias no fueron estadísticamente significativas debido a la alta variabilidad individual, la actividad de la α-amilasa en *P. punctifer* tendió a disminuir durante el período larvario y a aumentar en respuesta a cambios en la composición de la dieta al destete (*cf.* 1.2). Tal respuesta puede estar relacionada con el tipo de carbohidrato más que con el contenido, ya que la *Artemia* y la dieta compuesta usados en el presente estudio contenían cantidades similares de carbohidratos (~10% y ~8%, respectivamente). Estos resultados contrastan con los estudios realizados en otras especies de peces tropicales como *O. bimaculatus* (Pradhan et al., 2013) o *A. tropicus* (Frías-Quintana et al., 2015), donde la α-amilasa tuvo una baja actividad específica en la eclosión y aumentó gradualmente después de la alimentación exógena.

La capacidad de sintetizar α-amilasa en estadios larvarios no sólo se considera un indicador de la maduración del páncreas exocrino (Cahu et al., 2004; Cahu y Zambonino Infante, 1994), sino que también se relaciona con diferencias en su fisiología digestiva y hábitos alimentarios (Kuz'mina, 1996; Solovyev et al., 2014). En particular, se ha reportado que la actividad de la α-amilasa es mayor en los peces omnívoros que en los carnívoros (Fernández et al., 2001; Hidalgo et al., 1999; Kim et al., 2014). También se ha observado un patrón creciente de expresión de *amy* durante el desarrollo en *Chelon labrosus* (Zouiten et al., 2008), y se ha sugerido que la expresión constitutiva de *amy* puede representar una verdadera especialización hacia hábitos alimentarios herbívoros y omnívoros en los peces de la familia Stichaeidae (Kim et al., 2014). En *P. punctifer*, se desconocen los hábitos alimenticios de las larvas y juveniles tempranos en el medio natural; sin embargo, considerando el perfil de expresión de *amy*, los carbohidratos probablemente sean importantes en su dieta larvaria. Durante este período de desarrollo, parece plausible que esta especie muestre un comportamiento de alimentación omnívoro con preferencia a la carnivoría y que el fitoplancton y los invertebrados sean un componente importante de la dieta. Aunque *P. punctifer* también presenta características digestivas histológicas y bioquímicas de una especie carnívora (*cf.* 1.2, A1), esta especie tiene la capacidad de producir altos niveles de amilasa durante el inicio de la digestión ácida, como se ha observado posteriormente durante la etapa juvenil temprana (*cf.* 2.1). Resultados similares se han observado en *Odax pullus*, un pez herbívoro que consume algas rojas ricas en almidón cuando es joven, pero no mucho almidón en la edad adulta y, sin embargo, presenta una actividad de amilasa elevada en la edad adulta (Skea et al., 2005). Entre 18 y 24 dpf (18 y 26 mm LT, respectivamente), *P. punctifer* mostró un aumento drástico en la expresión de *amy* coincidiendo con el período de destete con el fin de adaptar la actividad enzimática al contenido de carbohidratos (11% en *Artemia* vs. 20 % en dieta compuesta) de la nueva dieta suministrada (*cf.* 1.1).

Este aumento en la expresión de *amy* resultó en un aumento de la actividad de su enzima (*cf.* 1.2), mostrando que la modulación por la dieta de la actividad de la α-amilasa ocurre a nivel transcripcional durante la etapa juvenil en esta especie, tal y como se ha observado en otras especies de peces como *Dicentrarchus labrax* (Péres et al., 1998). La capacidad de *P. punctifer* para digerir carbohidratos durante la etapa larvaria podría representar una ventaja desde una perspectiva industrial ya que esto permitiría la formulación de alimentos larvarios con menor contenido proteico si se logra la sustitución total de presas vivas por dietas compuestas para esta especie (*cf.* A2). Sin embargo, un aumento en la expresión génica no significa necesariamente que el pez esté utilizando adecuadamente esta fuente de energía. De hecho, uno de los estudios realizados sobre las necesidades nutricionales de *P. punctifer* durante la etapa juvenil temprana (de 13 a 26 dpf) mostró que un contenido de carbohidratos en la dieta superior al 25% inducía la producción de α-amilasa a niveles más altos que las dietas con niveles más bajos de carbohidratos, pero redujo el crecimiento e indujo hígados grasos (*cf.* 2.1). Por lo tanto, los resultados de la expresión génica y de la actividad de las enzimas digestivas deben considerarse junto con respuestas fisiológicas adicionales, incluida la interacción de nutrientes.

La importancia de los fosfolípidos en la nutrición de las larvas de peces es ampliamente reconocida (revisado en Cahu et al., 2009). La PLA2 no discrimina tipos de ácidos grasos y necesita ácido biliar para realizar una actividad enzimática completa en la luz intestinal (Murakami et al., 2015). Al igual que con la α-amilasa, un aumento en la expresión del gen *plA2* durante el desarrollo larvario es un indicador de la maduración del páncreas exocrino (Cahu et al., 2003). De manera similar a nuestros resultados, también se ha observado un aumento en la expresión del gen y/o actividad de *plA2* durante el desarrollo en otras especies de peces como *Scophthalmus maximus* (Hoehne-Reitan et al., 2003), *Gadus morhua* (Sæle et al., 2011), *L. guttatus* (Moguel-Hernández et al., 2016), *Sparus aurata* (Mata-Sotres et al., 2016), *Larimichthys crocea* (Cai et al., 2017) y *Paralichthys californicus* (Fuentes-Quesada y Lazo, 2018). Además, el contenido de fosfolípidos también puede afectar la maduración tanto del páncreas como del intestino, y la regulación de la actividad de PLA2 ocurre principalmente a nivel transcripcional (Cahu et al., 2003). Por ejemplo, se ha observado la regulación de la expresión de *plA2* por la dieta exógena en *L. crocea* (Cai et al., 2017). El hecho de que la expresión del gen *plA2* en *P. punctifer* permaneciera alta y constante después del inicio de la digestión ácida indica que los niveles de expresión eran adecuados para digerir los fosfolípidos presentes en la dieta compuesta suministrada al destete (*cf.* 1.1). El nivel de expresión de *plA2* también podría considerarse un indicador del estado nutricional de los peces, ya que se ha observado una regulación a la baja de la expresión de *plA2* en individuos en ayunas (Benedito-Palos et al., 2014).

En *P. punctifer*, la expresión del gen *lpl* aumentó entre 3 y 10 dpf (6 y 11 mm LT, respectivamente), lo que coincide con la maduración completa del intestino (*cf.* 1.2, A1), y continuó aumentando a partir de entonces en respuesta al cambio de dieta en el destete. También se ha encontrado una regulación transcripcional de este gen por la dieta en otras especies de peces como *Epinephelus coioides* (Li et al., 2016), *L. guttatus* (Moguel-Hernández et al., 2016) y *L. crocea* (Cai et al., 2017). La

actividad de la lipasa está modulada por la composición lipídica (Cahu y Zambonino Infante, 2001; Morais et al., 2004; Zambonino Infante et al., 2008), y por lo tanto su análisis es una valiosa herramienta para determinar la composición lipídica adecuada a incluir en la dieta para un óptimo aprovechamiento (Uscanga-Martínez et al., 2011). Se ha demostrado que la actividad de la lipasa es mayor en especies de peces carnívoros que omnívoros o herbívoros (Chakrabarti et al., 1995; Solovyev et al., 2014; Tengjaroenkul et al., 2000) y generalmente aumenta durante la ontogenia (Zambonino Infante et al., 2008). En *P. punctifer*, la actividad de la lipasa fue alta al momento de la eclosión y luego disminuyó en la primera alimentación y aumentó nuevamente desde el destete hasta 27 dpf (*ca.* 36 mm LT) (*cf.* 1.2). La actividad temprana de la lipasa observada en *P. punctifer* está en línea con los hallazgos histológicos que mostraron digestión y absorción luminal en la primera semana de vida de las larvas, lo que refleja el desarrollo funcional del intestino y la vacuolización del citoplasma de los hepatocitos y la aparición de gotas lipídicas en las regiones intermedias del intestino y el hígado durante el período de alimentación mixta (*cf.* A1). La actividad temprana de la lipasa también ha sido reportada en *A. tropicus* (Frías-Quintana et al., 2015) y *L. guttatus* (Moguel-Hernández et al., 2014). El menor nivel de actividad de la lipasa observado en las larvas de *P. punctifer* durante el período de alimentación con *Artemia* (4-17 dpf) en comparación con el de la fase de alimentación con la dieta compuesta (17-27 dpf) podría estar relacionado con el diferente contenido de lípidos y/o composición de la *Artemia* (~15% materia seca–MS) y la dieta compuesta (~10% MS) utilizados en este estudio (*cf.* 1.2). La menor actividad de la lipasa puede indicar que la *Artemia* tiene una composición lipídica inadecuada para esta especie, ya que se observó un mejor crecimiento y supervivencia y un metabolismo lipídico más equilibrado de las larvas y los juveniles tempranos de *P. punctifer* cuando se alimentaron con *Artemia* enriquecida y dietas compuestas con un enriquecedor comercial que contenía altos niveles de DHA (*cf.* 2.2). Esto estaría de acuerdo con las observaciones de que los nauplios de *Artemia* no cubren por completo las necesidades nutricionales de las larvas (*cf.* 1.2, A1, A2) y, por lo tanto, esto también podría estar explicando la menor tasa de crecimiento encontrada en esta etapa de desarrollo. Los cambios en la actividad de la lipasa después del destete pueden ser indicativos de cambios en los requerimientos nutricionales, que se reflejan en la tasa de crecimiento. En *P. punctifer,* el aumento de la actividad específica de la lipasa a 27 dpf (*ca.* 36 mm LT) puede estar relacionado con la adaptación de los juveniles tempranos a la dieta formulada y con la adquisición de la plena capacidad digestiva en esta especie. El aumento en la capacidad de digerir lípidos durante el desarrollo también se ha descrito en algunas otras especies de bagre como *Silurus soldatovi* (Liu et al., 2010), *P. hypophthalmus* (Rangsin et al., 2012) y *O. bimaculatus* (Pradhan et al., 2013).

La expresión de *try* en *P. punctifer* siguió un perfil de expresión observado en varias especies de peces, el cual se caracteriza por un aumento de su expresión hasta la completa formación y funcionalidad del estómago (Darias et al., 2007b; García-Gasca et al., 2006; Kortner et al., 2011; Kurokawa et al., 2002; Mir et al., 2018; Murray et al., 2004; Parma et al., 2013; Péres et al., 1998; Srichanun et al., 2013; Srivastava et al., 2002). De forma similar a lo que se ha reportado en varias

especies de peces (Darias et al., 2007b, 2005; Galaviz et al., 2011; Mir et al., 2018; Suzer et al., 2006), la expresión de *try* en *P. punctifer* disminuyó a partir de 10 dpf (*ca.* 11 mm LT), concomitante con el aumento de la expresión de *pga*, lo que indica el cambio de la digestión básica y menos eficiente de proteínas en el intestino anterior a la digestión ácida en el estómago que permite una digestión más eficiente de proteínas complejas (*cf.* 1.1). Sin embargo, este patrón puede ser modulado por la composición nutricional de la dieta, como mostró el presente estudio en el destete, donde aumentaron las expresiones *try* y *pga* (*cf.* 1.1). Esto no es sorprendente considerando que ambas enzimas trabajan juntas: la pepsina hace una primera hidrólisis de moléculas de proteína por escisión selectiva en Phe, Tyr y otros aminoácidos aromáticos, y la tripsina completa el proceso actuando sobre los residuos de lisina y arginina. El perfil de expresión de *try* coincidió con el de la actividad de la tripsina (*cf.* 1.2), mostrando que el cambio de *Artemia* a la dieta compuesta indujo una modulación de la actividad de esta enzima a nivel transcripcional, como también se ha observado en otras especies (Péres et al., 1998). En el caso de *ctr*, este siguió un perfil de expresión similar al de *try* durante la etapa larvaria de *P. punctifer* (*cf.* 1.1), lo que denota la acción complementaria de sus enzimas correspondientes tripsina y quimotripsina hasta el inicio de la digestión ácida (Rønnestad et al., 2013). Sin embargo, el patrón opuesto observado después de 13 dpf (disminución de la expresión de *ctr*) no coincidió con el aumento de la actividad de la quimotripsina (*cf.* 1.2), lo que indicaría que la actividad de esta enzima estaba regulada a nivel postranscripcional durante la etapa juvenil en respuesta al cambio de dieta. Alternativamente, esta diferencia en el perfil de expresión entre *try* y *ctr* podría estar relacionada con el papel de la tripsina en la activación de la quimotripsina. De hecho, el conocimiento sobre la dinámica de la quimotripsina y la importancia de esta enzima en los procesos digestivos durante las primeras etapas de la vida de los peces aún es limitado (Rønnestad et al., 2013). Cabe destacar que la actividad de la tripsina y de la quimotripsina se detectó en el momento de la eclosión (*cf.* 1.2). La importancia de estas enzimas durante las primeras etapas de desarrollo podría explicarse por su participación en la degradación de las proteínas contenidas en el vitelo (Zambonino Infante et al., 2008) y/o la digestión y rotura del corion del huevo durante el proceso de eclosión (Gisbert et al., 2009). Luego, la actividad de tripsina y quimotripsina se mantuvo constante hasta el final del estudio (27 dpf, *ca.* 36 mm LT; *cf.* 1.2). Se observaron perfiles de actividad de tripsina y quimotripsina similares a los de *P. punctifer* en *A. tropicus* (Frías-Quintana et al., 2015), siendo ambas especies alimentadas con una dieta con un contenido de proteína del 45%. En otras especies, las actividades de tripsina y quimotripsina aumentaron durante el desarrollo larvario y alcanzaron su punto máximo en la transición de la etapa larvaria a la juvenil, como en *Cichlasoma urophthalmus* (López-Ramírez et al., 2011) y *L. guttatus* (Moguel-Hernández et al., 2014), que ocurrió alrededor de los 24-25 dpe. Parece que un patrón de actividad creciente de tripsina y quimotripsina es más fácilmente detectable en especies que exhiben un período larvario más largo y que este aumento podría estar enmascarado por cambios en la composición de la dieta durante el destete en especies de desarrollo más rápido.

La funcionalidad del estómago se caracteriza por la expresión de genes pepsinógeno y bomba de protones (H^+/K^+-ATPasa) en las glándulas gástricas, que son responsables de la producción de pepsina y ácido clorhídrico, respectivamente (Darias et al., 2007a, 2007c, 2005; Douglas et al., 1999; Gawlicka et al., 2001). El inicio de la digestión ácida puede estar sincronizado o no con el desarrollo morfológico de las glándulas gástricas (p.e., Darias et al., 2005; Huang et al., 1998; Murray et al., 2006; Perez-Casanova et al., 2006). En *P. punctifer*, el aumento de expresión de *pga* observado entre 10 y 13 dpf estuvo sincronizado con la aparición de las glándulas gástricas (*cf.* A1), como también se ha observado en otras especies de peces, como *Pleuronectes americanus* (Douglas et al., 1999), *E. coioides* (Feng et al., 2008), *L. guttatus* (Galaviz et al., 2012) o *Sparidentex hasta* (Nazemroaya et al., 2020). Estos resultados también coincidieron con los observados a nivel de actividad enzimática (*cf.* 1.2), indicando la madurez funcional de las glándulas gástricas, y por lo tanto la transición de la etapa larvaria a la juvenil. El nivel de expresión de *pga* generalmente aumenta con el desarrollo y el crecimiento de las larvas, lo que se asocia con el número creciente de glándulas gástricas en el estómago (Darias et al., 2007c, 2005; Galaviz et al., 2012; Gao et al., 2013; Mir et al., 2018; Moguel-Hernández et al., 2016; Murray et al., 2006), lo que conduce a una capacidad de acidificación total y, por lo tanto, a una digestión de proteínas más eficiente que se puede lograr varias semanas después del inicio de la digestión ácida (Darias et al., 2005; Hoehne-Reitan et al., 2001; Yúfera et al., 2004). En *P. punctifer*, el aumento exponencial de la expresión de *pga* después de 13 dpf (*ca.* 14 mm LT) está de acuerdo con el mayor desarrollo, tanto en número como en tamaño, de las glándulas gástricas del estómago observado histológicamente (*cf.* A1), así como con el nivel de su actividad enzimática proteolítica (*cf.* 1.2).

Aunque, como mencionado anteriormente, la aparición de las glándulas gástricas en *P. punctifer* se observó a 8 dpf (*cf.* A1), la expresión de *pga* se detectó sorprendentemente a 3 dpf (*ca.* 5 mm LT) y la actividad de la pepsina a 4 dpf (*ca.* 6 mm LT; *cf.* 1.1, 1.2). Sin embargo, estos datos no se asociaron a la función gástrica, sino a la presencia de otras proteasas ácidas como las catepsinas lisosomales, que podrían estar implicadas en la digestión de las proteínas del vitelo (Carnevali et al., 2001). El aumento en la actividad de la proteasa ácida entre 4 dpf y 12 dpf (*ca.* 12 mm LT) sí se relacionó con la aparición de glándulas gástricas, tal y como indicaron los datos histológicos (*cf.* A1).

A nivel intestinal, la aparición de la funcionalidad del borde en cepillo de los enterocitos constituye un paso crucial durante el desarrollo larvario de los peces, ya que caracteriza la maduración de la mucosa intestinal y la adquisición de un modo de digestión adulto (Gisbert et al., 2018; Zambonino Infante et al., 2008). La proporción entre las enzimas intestinales del borde en cepillo y citosólicas se considera un indicador del desarrollo de la digestión intestinal y describe el cambio del modo de digestión larval (intracelular) al adulto (lumen intestinal). Los valores de esta relación se consideran importantes para la preparación a la introducción de dietas formulada en los protocolos de cultivo larvario (Cahu y Zambonino Infante, 2001). Las diferentes ratios de maduración intestinal en *P. punctifer* fueron bajas a 4 dpf (*ca.* 6 mm LT) (*cf.* 1.2), lo que podría deberse a la presencia de un intestino recto indiferenciado y sin pliegues (*cf.* A1). Más adelante, las enzimas del borde en cepillo,

particularmente las actividades de la fosfatasa alcalina (AP) y de la aminopeptidasa N (AN), aumentaron a 12 dpf (*ca.* 12 mm LT), lo que indicó el inicio de la maduración de los enterocitos, así como el aumento de su proliferación, lo que resultó en un incremento de la superficie de digestión y absorción luminal (*cf.* 1.2, A1). El aumento de la actividad de las enzimas del borde en cepillo hasta 20 dpf (*ca.* 21 mm LT) también coincidió con la formación del asa intestinal para acomodar la cada vez mayor longitud del intestino dentro de la cavidad abdominal, así como el desarrollo de un sistema digestivo morfológicamente completo durante esta etapa (*cf.* 1.2, A1).

Según los estudios de ontogenia morfológica y funcional del sistema digestivo de *P. punctifer* (*cf.* Capítulo 1, A1), el estómago se completó morfológica y funcionalmente a los 12 dpf (*ca.* 12 mm LT), adquiriendo así el modo de digestión similar al adulto. Por lo tanto, se puede considerar que 12 mm LT es un tamaño óptimo para el destete. Las fluctuaciones en la actividad de la pepsina y de las enzimas del borde en cepillo después del período de destete podrían indicar la adaptación de los individuos a la composición del nuevo alimento. Resultados similares (perfil de dientes de sierra) han sido encontrados en otras especies de silúridos con varias diferencias en el momento de la diferenciación y el desarrollo de los órganos digestivos, como en *O. bimaculatus* (Pradhan et al., 2013), *Silurus glanis* (Kozarić et al., 2008) y *P. fulvidraco* (Yang et al., 2010).

Estudio de las necesidades nutricionales de *Pseudoplatystoma punctifer* durante las primeras etapas de vida

Tras analizar el desarrollo morfológico y funcional del sistema digestivo de *P. punctifer*, el siguiente paso fue realizar dos estudios nutricionales con el fin de estudiar el efecto de 1) diferentes proporciones de proteínas y lípidos (y sus ratios) y 2) del enriquecimiento de la *Artemia* y el alimento balanceado con DHA en el crecimiento, la supervivencia, la incidencia de canibalismo, la incorporación de los nutrientes en los tejidos y la función digestiva de los animales, con el fin de contribuir al desarrollo de protocolos de alimentación adaptados a las capacidades digestivas y necesidades nutricionales de esta especie que maximicen el crecimiento, la supervivencia y la calidad de los individuos durante las primeras etapas de vida.

En el capítulo 2.1 y anexo 2 se formularon cuatro dietas experimentales con diferentes niveles proteínas (45 *vs* 30% MS) y lípidos (15 *vs* 10% MS) (45:15, 45:10, 30:15 y 30:10) y se utilizó un protocolo de alimentación basado en los conocimientos adquiridos sobre el desarrollo de las capacidades digestivas de *P. punctifer*. Así, los individuos fueron destetados con las diferentes dietas compuestas experimentales a 13 dpf, cuando se completó el desarrollo morfológico y funcional del sistema digestivo (*cf.* Capítulo 1, A1). Además, se incluyó, a modo comparativo, un grupo control (C) usando el mismo protocolo de alimentación utilizado en los estudios de ontogenia (destete a 17 dpf y dieta compuesta 45:10; *cf.* Capítulo 1).

En el capítulo 2.2 se estudió el efecto del enriquecimiento de la dieta con DHA y evaluar a la vez si los requisitos de DHA variaban a lo largo del desarrollo. Para ello se crearon cuatro grupos

experimentales: un grupo C (control) alimentado con *Artemia* no enriquecida (4-18 dpf) y destetados con un alimento compuesto no enriquecido (16-26 dpf), un grupo T1 alimentado con *Artemia* enriquecida y alimento compuesto no enriquecido, un grupo T2 alimentado con *Artemia* no enriquecida y alimento compuesto enriquecido y un grupo T3 alimentado con *Artemia* enriquecida y alimento compuesto enriquecido.

***Efecto de la composición de la dieta en el desempeño de* P. punctifer**

En el estudio presentado en el anexo 2, se observó que la extensión de la fase de alimentación con *Artemia* (hasta 17 dpf en grupo C *vs* 12 dpf en los demás grupos) no proporcionó ninguna ventaja en términos de crecimiento y supervivencia, a la vista de los resultados observados en los grupos C y 45:10, a pesar de ser alimentados con la misma dieta compuesta. A medida que las larvas crecieron, la diferencia de crecimiento entre ambos grupos aumentó (250 *vs* 350 mg peso húmedo–PH, a 27 dpf) y el grupo C no mostró signos de crecimiento compensatorio. Este hallazgo es de especial relevancia considerando el alto costo de los quistes de *Artemia*, así como los costos asociados a la producción de nauplios. Estos resultados son consistentes con nuestra hipótesis previa de que la *Artemia* no cubre las necesidades nutricionales de las larvas de *P. punctifer* (*cf.* Capítulo 1, A1), ya que el crecimiento y la supervivencia de los especímenes del grupo C fueron similares a los de las larvas del grupo 30:15 y sólo más altos que el grupo alimentado con la dieta 30:10. Estos resultados indican que el protocolo de alimentación utilizado anteriormente (*cf.* A1) permitió que las larvas crecieran, pero no sirvió para aprovechar al máximo su potencial de crecimiento. Además, la gran acumulación de depósitos de lípidos que se encontró en el intestino y el hígado de los individuos del estudio previo (*cf.* A1) sugirió un desequilibrio nutricional con respecto al contenido de proteínas y lípidos de la dieta compuesta suministrada. Por otro lado, los resultados del capítulo 2.2 mostraron que el enriquecimiento de DHA durante el período de alimentación con *Artemia* (período larvario) tampoco tuvo un efecto positivo sobre el crecimiento. Esto podría estar relacionado con el hecho de que las especies de agua dulce son capaces de sintetizar DHA *de novo* a partir de precursores como el ácido alfa linoleico (18:3, ALA) y, por lo tanto, a menudo tienen requisitos más bajos en HUFA n-3 que las larvas de peces marinos (Bell y Sargent, 2003). De manera similar, un estudio nutricional realizado en *C. gariepinus* mostró que los cambios en la composición de ácidos grasos de la dieta no afectaron el crecimiento de las larvas (Verreth et al., 1994). En cambio, durante la fase juvenil temprana, la dieta enriquecida con DHA si mejoró el crecimiento de los individuos con respecto a los que fueron alimentados con la dieta no enriquecida (50 *vs* 80 mg PH a 26 dpf).

La presente tesis constituye el primer estudio sobre los requerimientos en macronutrientes durante la etapa juvenil temprana (a partir de 12 dpf, 12 mm LT, 7 mg PH) de una especie del género *Pseudoplatystoma*. La literatura muestra una diferencia notable en los requerimientos de proteínas, lípidos y carbohidratos para juveniles de mayor tamaño (PH inicial de 1 a 120 g) de varias especies de este género. Así, los niveles óptimos varían del 36 al 49% para las proteínas (Campos et al., 2006;

Cornélio et al., 2014; Gonçalves, 2014; Silva, 2013; Zanardi et al., 2008), del 8 al 19% para los lípidos (Arslan et al., 2013; Campos et al., 2006; Martino et al., 2005; Silva, 2013) y del 13% al 25% para los carbohidratos (Gonçalves, 2014; Lundstedt et al., 2004; Okamura, 2009) dependiendo de la calidad de los ingredientes y sus proporciones relativas. En particular, Gonçalves (2014) encontró trabajando con *P. reticulatum* que el contenido de proteína se podía reducir hasta un 36% cuando se equilibraban otros nutrientes energéticos (15% de carbohidratos y 8% de lípidos). Asimismo, nuestro estudio mostró que se puede obtener un muy buen crecimiento con una dieta que contenga un 45% de proteínas, provenientes de la harina de pescado (entera e hidrolizada), y un 15% de lípidos, en forma de fosfolípidos (lecitina marina y de soja) y lípidos neutros (incluidos en el aceite de pescado). Esta dieta (45:15) mostró pronto un efecto positivo evidente en el crecimiento (1 semana después del destete) y al final del experimento permitió mejorar el crecimiento (seis veces en términos de PH y dos veces en términos de LT) y la supervivencia (dos veces) en comparación con los protocolos anteriores utilizados en condiciones de cultivo similares (*cf.* A1; MJ Darias, datos no publicados).

Uno de los principales objetivos en investigación en nutrición de peces es reducir el contenido de proteínas de la dieta mediante la incorporación de otras fuentes de energía, como lípidos y carbohidratos, lo que permite mejorar la utilización de proteínas para el crecimiento y un ahorro en el coste económico en la fabricación de los alimentos. El efecto ahorrador de proteínas a través del uso de lípidos ha sido reportado en muchas especies de peces, incluso en la etapa juvenil (Li et al., 2012; Vergara et al., 1996). Sin embargo, es necesario determinar el nivel óptimo de estos nutrientes en la dieta para cada especie, ya que pueden variar de una especie a otra. Por ejemplo, un nivel de lípidos superior al 19% no sólo no mejoró el crecimiento ni tuvo efecto ahorrador de proteínas en los juveniles de *P. corruscans*, sino que resultó en un aumento del contenido de lípidos viscerales (Martino et al., 2005). Las diferencias en el crecimiento y la supervivencia entre los grupos del capítulo 2.1 y anexo 2 se asociaron principalmente al contenido de proteínas de la dieta y, en segundo lugar, al nivel de lípidos. El menor rendimiento de crecimiento en los grupos 30:10 y 30:15 indicó una disponibilidad insuficiente de proteínas para mantener un crecimiento y metabolismo adecuados en los juveniles tempranos de *P. punctifer* y fuentes de energía insuficientes y/o inadecuadas (lípidos y carbohidratos) para compensar esta deficiencia de proteínas. Los carbohidratos de la dieta, que se utilizaron para completar la fórmula de manera que las dietas bajas en proteínas contuvieron cantidades de carbohidratos más elevadas (> 25%) que las dietas con mayor contenido proteico (<8%), influyeron también en la fisiología general de los peces alimentados con estas dietas. Tanto los depósitos de glucógeno como de lípidos en el hígado se correlacionaron con el contenido de carbohidratos de las dietas. Se sabe que niveles altos de carbohidratos (> 30%) aumentan la deposición de lípidos en el hígado de los peces al mejorar la lipogénesis y el potencial de captación de lípidos (Brauge et al., 1994; Li et al., 2019; Tan et al., 2009). Además, los alimentos para peces con un contenido excesivo de carbohidratos pueden provocar depósitos de grasa desequilibrados, una función inmunológica suprimida y comprometer la salud de los animales (Gao et al., 2010). Así, un exceso en carbohidratos es convertido en azúcares simples por

digestión y el exceso de glucosa puede almacenarse como glucógeno (glucogénesis) o convertirse en lípidos (lipogénesis) (Moreira et al., 2008; Panserat et al., 2009; Polakof et al., 2012, 2010), desempeñando un papel clave en la retención de nutrientes. El aumento de los depósitos de glucógeno y lípidos observados en los hígados de los individuos alimentados con las dietas 30:10 y 30:15 sugiere que tanto la glucogénesis como la lipogénesis podrían haber ocurrido en el hígado para regular la homeostasis de la glucosa en *P. punctifer*. Sin embargo, estos resultados indican que más investigaciones son necesarias sobre la regulación de los genes relacionados con el metabolismo de la glucosa para dilucidar el metabolismo de los carbohidratos en *P. punctifer* y para determinar mejor la proporción óptima de carbohidratos:lípidos (C:L) en la dieta que favorezca el ahorro de proteínas y un rendimiento fisiológico general adecuado. De hecho, aunque todas las dietas fueron isoenergéticas, una alta proporción C:L redujo el crecimiento, lo que indica que diferentes fuentes de energía influyen en la utilización de lípidos y carbohidratos. Esto se debe a la interconexión entre estas fuentes de energía a través de la posible conversión de lípidos en glucosa mediante la gluconeogénesis y la posible conversión de glucosa en lípidos a través de la lipogénesis. También se han observado resultados similares en otras especies de peces (Erfanullah y Jafri, 1998; Gao et al., 2010; Jantrarotai et al., 1994). La digestión y el metabolismo de los carbohidratos de la dieta están estrechamente relacionados con los hábitos alimenticios de los peces, siendo los peces omnívoros y herbívoros capaces de utilizar mejor los carbohidratos de la dieta (Figueiredo-Silva et al., 2013; Li et al., 2013; Panserat et al., 2000) que los peces carnívoros. Este estudio mostró que *P. punctifer* tiene una clara preferencia por los lípidos como fuente de energía en lugar de los carbohidratos. Como se observó en *Oncorhynchus mykiss* (Brauge et al., 1994), los lípidos de la dieta tuvieron un mejor efecto ahorrador de proteínas que los carbohidratos a un nivel similar de ingesta de energía digestible en *P. punctifer*, como lo demuestra el mejor crecimiento y supervivencia de los individuos alimentados con la dieta 45:15 (que contenía un 2% de carbohidratos) en comparación con los individuos alimentados con la dieta 45:10 (que contenía un 8% de carbohidratos). Igualmente, Gonçalves (2014) no encontró un efecto ahorrador de proteínas a través del uso de carbohidratos en juveniles de *P. reticulatum* alimentados con dos niveles de carbohidratos (15 y 25%) y tres de proteínas (44, 40 y 36%) en la dieta. Okamura (2009) encontró que los juveniles de *Pseudoplatystoma* spp. híbrido (*P. corruscans* × *P. fasciatum*) presentaron hiperglucemia persistente cuando se les administró un 20% de almidón de maíz y concluyó que el nivel óptimo estaría alrededor del 15%. Por el contrario, se ha observado una preferencia de los carbohidratos sobre los lípidos como fuente de energía en algunos peces omnívoros de agua dulce (Erfanullah y Jafri, 1998; Honorato et al., 2010). Según los resultados generales, la proporción óptima de C:L en la dieta para *P. punctifer* se encontró entre 0.2 (dieta 45:15) y 0.8 (dieta 45:10).

La relación energía:proteína (E:P) puede afectar el rendimiento del crecimiento, la eficiencia alimentaria y la composición corporal de los peces. En el capítulo 2.1 y anexo 2, el rendimiento del crecimiento y el metabolismo de *P. punctifer* se vieron afectados significativamente por la proporción E:P en las dietas experimentales. En particular, una relación E:P de 11 kcal g^{-1} de proteína cruda en las

dietas 30:15 y 30:10 indujo una deposición de lípidos en el hígado y disminuyó el crecimiento somático de los peces. También se ha observado un mayor contenido de lípidos en el hígado (o corporal) relacionado con un aumento de la relación E:P en otras especies de peces, como en *Ctenopharyngodon idella* (Du et al., 2009), *Siniperca chuatsi* (Alam et al., 2019), *Oreochromis aureus* (Winfree y Stickney, 1981) e *Ictalurus punctatus* (Garling y Wilson, 1976). Se ha observado una relación E:P óptima para el crecimiento y el rendimiento general similar a la encontrada para *P. punctifer* (aproximadamente 7 kcal g^{-1} de proteína cruda) en *Lates calcarifer* (Catacutan y Coloso, 1995), *Clarias gariepinus* (Henken et al., 1986) y *C. idella* (Du et al., 2009), mientras que la relación E:P más alta que fue subóptima para *P. punctifer* (11 kcal g^{-1} de proteína cruda) resultó ser óptima para *I. punctatus* (Garling y Wilson, 1976). En *P. punctifer*, los niveles de proteína en las dietas con E:P elevada (30:10 y 30:15) estuvieron por debajo del nivel óptimo para esta especie y etapa de desarrollo, y se utilizaron principalmente para el mantenimiento de proteínas corporales; y los lípidos y carbohidratos de la dieta no se utilizaron de manera eficiente o resultaron en cantidades insuficientes para fines energéticos. Además, una posible interacción del almidón de la dieta con la digestibilidad de las proteínas no puede descartarse en estos grupos dietéticos ricos en carbohidratos, ya que en otros peces se ha demostrado una disminución de la digestibilidad de las proteínas relacionada con un aumento del contenido de almidón en la dieta (Couto et al., 2012). El alto contenido de carbohidratos de estas dietas contribuyó al aumento de los niveles de actividad de la α-amilasa en estos grupos. Sin embargo, no contribuyeron con la eficiencia suficiente para promover el crecimiento. Esto pudo haberse debido, en parte, a una disponibilidad de glucosa limitada. Desafortunadamente, la actividad maltasa, encargada de hidrolizar las moléculas de maltasa liberadas por la amilasa en glucosa, no fue evaluada en este estudio y, por consiguiente, no se pudo comprobar esta hipótesis. En otras especies de peces se ha observado un crecimiento reducido como resultado de la sobrecarga de carbohidratos en la dieta (Brauge et al., 1994; Tan et al., 2009; Zhou et al., 2015). Por lo tanto, una baja síntesis de proteínas da como resultado un rendimiento de crecimiento bajo y un metabolismo deficiente, mientras que la tendencia opuesta ocurre cuando los peces se alimentan con dietas con una relación E:P baja (Alam et al., 2019).

Si una dieta es deficiente en un nutriente esencial, los peces consumen más alimento para satisfacer las demandas de ese nutriente específico (Alam et al., 2019). De acuerdo con esto, se ha demostrado que los peces alimentados con dietas altas en carbohidratos necesitan aumentar su ingesta de alimento para obtener niveles adecuados de aminoácidos para promover el crecimiento (Boonanuntanasarn et al., 2018). Suponiendo que este también fuera el caso de *P. punctifer* alimentado con las dietas 30:10 y 30:15, se puede plantear la hipótesis de que la energía suministrada en estas dietas bajas en proteínas fue insuficiente en cantidad y/o calidad para compensar los costos energéticos adicionales asociados a la desaminación, así como a los comportamientos de natación y búsqueda de alimento, reduciendo, en consecuencia, la energía disponible para el crecimiento. Entre las dietas probadas, cuanto menor fue la relación E:P, mejor crecimiento y supervivencia se observó en *P. punctifer*. Lo contrario se ha encontrado en la omnívora *Oreochromis niloticus* (Boonanuntanasarn et

al., 2018) y también en peces carnívoros como *Sparus macrocephalus* (Zhang et al., 2010). Esto se ha atribuido a un exceso de proteína en la dieta que daría lugar a la pérdida de energía para la desaminación de los aminoácidos hacia el catabolismo proteico (Boonanuntanasarn et al., 2018).

***Influencia de la composición proximal y de ácidos grasos de la dieta en la composición proximal y de ácidos grasos corporales de* P. punctifer**

La composición proximal de *P. punctifer* fue modulada por los diferentes niveles de proteínas:lípidos:carbohidratos de la dieta (*cf.* 2.1, A2). La proteína corporal fue la misma en todos los grupos dietéticos, lo que significa que los individuos alimentados con dietas con bajo contenido proteico tuvieron que sintetizar más proteínas de las que se les proporcionó a través de la dieta para alcanzar las cantidades requeridas en sus tejidos (*ca.* 45%). Por el contrario, se ha encontrado que el contenido de proteínas corporales se correlaciona positivamente con el contenido de proteínas de la dieta en otras especies de peces (Islam y Tanaka, 2004; Kim et al., 2002; Luo et al., 2004; Shyong et al., 1998; Yang et al., 2002; Zhang et al., 2010). Los grupos alimentados con dietas bajas en proteínas presentaron un mayor contenido de carbohidratos en los tejidos que los otros grupos dietéticos, y el hecho de que los individuos alimentados con la dieta 45:15 presentaran el menor contenido de carbohidratos y lípidos sugiere que la relación E:P y/o C:L promovió una mayor acumulación de lípidos en los otros grupos experimentales. También se ha observado una modulación dietética de la composición corporal en juveniles de tilapia *Oreochromis niloticus* × *O. aureus* (Wang et al., 2005), mientras que no se observó ningún efecto en la tilapia del Nilo *O. niloticus* durante la fase de crecimiento (Boonanuntanasarn et al., 2018). Esto podría indicar diferencias en la regulación del metabolismo de los nutrientes según la especie y las etapas de desarrollo. De hecho, se ha sugerido que los procesos de anabolismo y catabolismo podrían tener lugar en etapas posteriores del desarrollo para estabilizar la composición proximal corporal (Boonanuntanasarn et al., 2018).

Con respecto a la composición de ácidos grasos del capítulo 2.1, el contenido de DHA de los individuos de los grupos 30:10 y 45:10 (aproximadamente 23%) reflejó el de la dieta ingerida (aproximadamente 21%) y representó la mayoría del contenido total de PUFA n-3 de los tejidos. Sin embargo, los peces alimentados con las dietas 30:15 y 45:15, que contenían niveles más bajos de DHA (*ca*. 9%), mostraron un contenido de DHA considerablemente más alto en sus tejidos (*ca.* 18%). Esto sugiere que en esos grupos experimentales tuvo lugar la síntesis *de novo* de DHA, lo que está de acuerdo con el agotamiento del ácido α-linolénico (ALA), el precursor de la biosíntesis de DHA, observado en estos grupos (Brodtkorb et al., 1997). El contenido de DHA en los individuos de los grupos 30:15 y 45:15 fue significativamente menor (ca. 18%) que el de los grupos 30:10 y 45:10 (ca. 23%). Estos resultados sugieren que niveles más altos de DHA en la dieta resultaron en niveles aparentemente más altos de DHA de lo podría ser necesario en los tejidos.

Al igual que en las dietas, el ácido linoleico (LA) fue el principal responsable de las diferencias observadas en el contenido total de PUFA n-6 en el tejido del pez. El contenido total de PUFA n-6 fue

entre 5 y 7 veces mayor en las dietas 30:15 y 45:15 en comparación con las dietas 30:10 y 45:10. Este perfil en el contenido total de PUFA n-6 también se encontró en los grupos experimentales, aunque las diferencias fueron menores (*ca.* 2 veces). Al comparar el contenido total de PUFA n-6 de la dieta con el de los tejidos de cada grupo dietético, el contenido total de PUFA n-6 (y por lo tanto de LA) permaneció igual en los grupos 30:10 y 45:10, mientras que fue 2 y 3 veces menor en los grupos 45:15 y 30:15, respectivamente. Esto indica que un mayor contenido de PUFA n-6 (en particular de LA) en la dieta permitió a los grupos 30:15 y 45:15 usar una parte para el funcionamiento fisiológico y almacenar una cantidad mayor en sus tejidos que la permitida por las dietas 30:10 y 45: 10. Además, parecía esencial para los grupos 30:10 y 45:10 retener la totalidad del bajo contenido de PUFA n-6 (*ca.* 5%) de las dietas 30:10 y 45:10 en sus tejidos. Dado que el grupo 45:10 mostró el segundo mayor crecimiento de los grupos, parece que un contenido de PUFA n-6 más bajo en los tejidos no compromete el crecimiento de los individuos. Los grupos 30:15 y 45:15 mostraron valores de LA más bajos que los de las dietas, aunque no resultó en un aumento del contenido de ARA. El uso diferente de LA entre ambos grupos alimentados con las distintas dietas dio como resultado que el grupo de 45:15 presentara el contenido más alto de PUFA n-6 total. La suma de los contenidos de PUFA n-3 y n-6 totales de cada grupo dietético condujo a un contenido de PUFA total más alto en el grupo 45:15 y más bajo en los grupos 30:10 y 45:10, aunque las diferencias de contenido no fueron muy elevadas (43% *vs* 40%). Las únicas diferencias claras entre el grupo con mayor crecimiento (45:15) y el resto de los grupos dietéticos fueron las relaciones PUFA n-3/n-6, LA/PUFA y ALA/PUFA, para los cuales el 45:15 mostró valores intermedios. Sin embargo, estos perfiles por sí solos no explican las diferencias en el crecimiento, ya que el grupo 45:10, que presentó el segundo mayor crecimiento, mostró la relación n-3/n-6 más alta junto con las relaciones LA y ALA/PUFA más bajas. El sutil equilibrio entre todos los componentes de la dieta 45:15 ciertamente explicó los mejores resultados observados en los juveniles tempranos de *P. punctifer* en el presente estudio, ya que las otras dietas que indujeron un desarrollo menos eficiente tenían uno o varios compuestos en una proporción diferente en comparación con la composición de la fórmula 45:15.

A diferencia de los peces marinos (Sargent et al., 1997), una proporción DHA/EPA de la dieta más cercana a 1 probablemente contribuyó a un mejor desempeño en *P. punctifer*, como fue el caso de la dieta 45:15. Independientemente de la proporción DHA/EPA proporcionada en las dietas, los juveniles tempranos de *P. punctifer* mostraron una proporción DHA/EPA similar en sus tejidos (*ca*. 2.5), lo que sugiere que esta es la proporción necesaria para mantener la homeostasis fisiológica correcta. De manera similar, la relación DHA/EPA fue consistentemente más alta en los triacilgliceroles depositados en *Salmo salar* de cultivo que en los aceites de pescado utilizados en los alimentos (Bell et al., 1998); y, en *S. salar*, la relación DHA/EPA también aumentó en el músculo en comparación con la de la dieta, de acuerdo con la oxidación selectiva de EPA, lo que resultó en una retención selectiva de DHA por parte del pez (Brodtkorb et al., 1997). En el caso de *P. punctifer*, el crecimiento no pareció depender del EPA o DHA, sino más bien del ALA, como se observó en los grupos 30:15 y 45:15, donde el ALA

proporcionado por la dieta se utilizó para sintetizar DHA para satisfacer los requisitos a nivel de tejido, los cuales son vitales para la correcta estructura y función de las membranas celulares (*ca*. 18% TFA). Sin embargo, cuando los niveles de DHA en la dieta fueron lo suficientemente altos para sus necesidades tisulares, como fue el caso de las dietas 30:10 y 45:10, la vía de biosíntesis de DHA *de novo* de su precursor ALA pareció inhibirse. Otros peces de agua dulce como *C. gariepinus* y *Coptodon zillii* también convierten PUFA C18 en HUFA y, en el caso de *C. gariepinus*, se ha demostrado que los niveles altos o bajos de HUFA n-3 en la dieta no influyen en el crecimiento (Işik et al., 1999; Verreth et al., 1994). Esto último no se pudo confirmar en *P. punctifer*, ya que todas las dietas experimentales contenían niveles altos de DHA (9-24% de TFA). Debido al bajo contenido de proteínas y lípidos de la dieta 30:10, los lípidos se utilizaron principalmente para mantener los niveles adecuados de lípidos y ácidos grasos en los tejidos, que era similar a los de los otros grupos dietéticos, en lugar de promover el crecimiento somático.

Igualmente, en el capítulo 2.2, la biosíntesis y el metabolismo de los ácidos grasos difirieron en los grupos alimentados con *Artemia* enriquecida y no enriquecida. A pesar de haberles ofrecido la misma cantidad de LA en la dieta, los grupos alimentados con *Artemia* enriquecida mostraron un mayor contenido de ARA que los grupos alimentados con *Artemia* no enriquecida. Los grupos alimentados con *Artemia* enriquecida biosintetizaron DPA y ARA a partir de LA, lo que resultó en una menor acumulación de LA en los tejidos que en los grupos alimentados con *Artemia* no enriquecida. Por el contrario, los grupos alimentados con *Artemia* no enriquecida mostraron cierta síntesis de ARA y acumulación de lípidos en los tejidos, pero no DPA. Como consecuencia, los contenidos de ARA y LA fueron menores y mayores, respectivamente, en los grupos C y T2 que en aquellos alimentados con *Artemia* enriquecida (T1, T3). Las diferencias en la biosíntesis de DPA n-6 entre ambos grupos dietéticos podrían deberse a un proceso de desaturación (delta 4 desaturasa) de 22:4n-6 contenido en la *Artemia* enriquecida, que estaba ausente en la *Artemia* no enriquecida. Esto indicaría que la delta 4 desaturasa también existe en esta especie (Monroig et al., 2018; Monroig y Kabeya, 2018). Los niveles de ácido oleico (18:1, OA) fueron más altos en la *Artemia* no enriquecida que en la enriquecida y este mismo patrón se reflejó en el tejido de los peces. El contenido de ácido eicosatetranoico (20:4n-3) y ácido docosapentanoico (22:5n-3), ambos sustratos para la elongasa Elovl2 (Monroig et al., 2018), fue similar en la *Artemia* enriquecida y no enriquecida. Sin embargo, los grupos alimentados con *Artemia* enriquecida presentaron contenidos más bajos de 20:4n-3 y 22:5n-3 que los alimentados con *Artemia* no enriquecida, lo que sugiere que se promovió la elongación de estos PUFAs en estos grupos dietéticos para producir DHA. El mayor contenido de EPA de la *Artemia* enriquecida (3% TFA) *versus* no enriquecida (1% TFA) explicó esta vía biosintética, ya que todos los grupos dietéticos presentaron un contenido similar de EPA en sus tejidos (3% TFA). Los grupos alimentados con *Artemia* no enriquecida tuvieron que sintetizar EPA *de novo* para acumular un 3% de TFA en sus tejidos, mientras que los grupos alimentados con *Artemia* enriquecida cubrieron sus requisitos de EPA a través de la dieta.

La cantidad de ALA y de DPA de las dietas enriquecida y no enriquecida utilizadas a partir del destete (fase juvenil) fue similar, mientras que el contenido en EPA y DHA fue mayor en la dieta no enriquecida y enriquecida, respectivamente. A nivel de tejidos, el contenido en ALA fue similar en ambos grupos (C, T2), al igual que el contenido en EPA y DHA (*ca.* 21 % TFA). Esto mostró que el EPA de la dieta no enriquecida se utilizó para sintetizar DHA a los niveles requeridos en los tejidos, que eran visiblemente superiores a los contenidos en la dieta. Por otro lado, el mayor contenido de LA de la dieta no enriquecida promovió una mayor acumulación de ARA en los tejidos de los peces alimentados con esta dieta, lo que se vio reflejado en el menor contenido de LA en los tejidos de este grupo que en los individuos alimentados con la dieta enriquecida. El menor contenido de DPA en los tejidos de los individuos alimentados con la dieta enriquecida sugiere, una vez más, un proceso de desaturación a partir del ARA de la dieta, lo que se vio reflejado en el menor contenido de ARA en los tejidos de los peces alimentados con esta dieta enriquecida. Estos resultados sugieren que la mayor ratio DHA/EPA y PUFA/saturados de la dieta enriquecida pudo haber contribuido a la mejora en crecimiento observada en los individuos de este grupo experimental.

***Influencia de la composición de la dieta en la función digestiva de* P. punctifer**

La actividad de las enzimas pancreáticas e intestinales son marcadores útiles y fiables para evaluar el desarrollo y el estado de la función digestiva en los peces (Zambonino Infante et al., 2008). Como en muchos otros peces, la actividad específica de la AP aumentó concomitantemente con la disminución de la actividad de la LAP durante el desarrollo larvario de *P. punctifer* (*cf.* 1.2). En la presente tesis, la relación AP/LAP a 26 dpf (33-47 mm LT) medida en los cuatro grupos dietéticos del capítulo 2.1 indicó un menor grado de maduración del intestino en los grupos 30:10 y 30:15, mientras que el intestino más maduro correspondió a individuos del grupo 45:15. Estos resultados indican que las distintas dietas administradas estaban afectando directamente el proceso de desarrollo de *P. punctifer*, lo que también se demostró a nivel histológico. Las diferencias fueron particularmente evidentes con respecto al número de enterocitos a lo largo de las diferentes partes del intestino, el número y la longitud de los pliegues intestinales, el grado de acumulación de lípidos en el intestino posterior y el número de células caliciformes en el intestino anterior. En particular, cuanto menor es la diferencia en el número de enterocitos entre las partes intestinales, mejor es el rendimiento general, como se observó en el grupo 45:15. En este sentido, y considerando los resultados presentes, una relación entre el número de enterocitos en el intestino anterior y el posterior superior a 1.2 podría considerarse un indicador de retraso en el desarrollo de esta especie. Estas observaciones histológicas relativas al desarrollo intestinal se correlacionaron con varios indicadores de la función digestiva, como la AP/LAP, la actividad de la lipasa activada por sales biliares y de la pepsina y el crecimiento. En cuanto al metabolismo de los lípidos, los grupos 30:15 y 30:10 presentaron el menor número de depósitos de lípidos en el intestino posterior, lo que se interpreta como un indicador de un retraso en la maduración intestinal en estos grupos (Gisbert et al., 2008). Además, tanto el grupo 30:15 como el 30:10 presentaron

los hígados más grasos de todos los grupos, lo que, como se discute a continuación, pareció estar asociado al alto nivel de carbohidratos contenido en estas dietas. Además, el mayor número de células caliciformes encontradas en el intestino anterior del grupo 45:15 se correlacionó con el mejor desarrollo y crecimiento de estos individuos, ya que una de las diversas funciones de las mucinas neutras secretadas por las células caliciformes es contribuir a la digestión y transformación del quimo, así como a la absorción de moléculas fácilmente digeribles como los disacáridos y los ácidos grasos de cadena corta (Sarasquete et al., 2001).

El número de hepatocitos en el parénquima hepático estuvo en consonancia con el grado de desarrollo fisiológico de los individuos alimentados con los diferentes tratamientos dietéticos del capítulo 2.1. Así, los individuos alimentados con la dieta 45:15 presentaron el mayor número de hepatocitos y los del grupo 30:10 el menor. En términos de acumulación de lípidos, los individuos alimentados con dietas bajas en proteínas (30:15 y 30:10) mostraron los hígados más grasos, probablemente debido a un exceso de carbohidratos en la dieta que pudo promover la lipogénesis en el hígado (He et al., 2015; Li et al., 2019; Tan et al., 2009; Zamora-Sillero et al., 2013). Como se mencionó anteriormente, este patrón también se reflejó en el contenido total de lípidos y ácidos grasos de los individuos. En particular, los individuos del grupo 30:10, aunque recibieron el contenido más bajo de lípidos (y proteínas), acumularon lípidos y ácidos grasos similares a los niveles observados en los grupos 30:15 y 45:10. En *O. niloticus*, se encontró una respuesta metabólica hepática similar, donde la lipogénesis aumentaba en el hígado cuando la ingesta de lípidos era limitada y la síntesis *de novo* de ácidos grasos aumentaba el contenido de ácidos grasos hepáticos (He et al., 2015). En esa especie, se sugirió que la glucólisis estimulada por la deficiencia de lípidos en la dieta o por un alto contenido de carbohidratos en la dieta proporciona los sustratos para la lipogénesis (He et al., 2015). Una situación similar podría estar sucediendo en *P. punctifer* alimentados con las dietas 30:15 y 30:10. La reducción de las capacidades de hidrólisis de triglicéridos y fosfolípidos en estos individuos, como mostró la expresión reducida de *lpl* y *plA2* podrían ser indicadores del metabolismo lipídico alterado observado en estos individuos, hipótesis apoyada por la gran acumulación de lípidos en los hepatocitos observada histológicamente. Además, se observó una disminución en la actividad de la lipasa en estos grupos dietéticos, que se correlacionó con los niveles de inclusión de lípidos en el intestino posterior. La actividad reducida de la lipasa observada en el grupo 30:10 junto con el número y área reducidos de depósitos lipídicos en el intestino posterior sugiere una alteración de la digestión y absorción de lípidos a nivel intestinal en estos individuos. La dieta 30:10 parecía tener un contenido de lípidos insuficiente y un contenido excesivo de carbohidratos para los juveniles tempranos de *P. punctifer*. El aumento de la actividad de la α-amilasa junto con una disminución de la actividad de la lipasa y la expresión de *lpl* sugiere que ambos macronutrientes inducen cierta disfunción en el metabolismo lipídico. En ratas se han observado resultados similares, en los que niveles superiores de insulina producidos por niveles elevados de glucosa incrementaron la actividad de α-amilasa pancreática junto con una disminución de la actividad del quimotripsinógeno y de la lipasa (Henderson et al., 1981). En humanos se ha demostrado

que la expresión de *lpl* es estimulada en mayor medida por los carbohidratos que por los lípidos (Yost et al., 1998). En *P. punctifer*, la expresión de *lpl* fue particularmente baja en los grupos alimentados con las dietas 30:15 y 30:10, lo que podría estar relacionado con los niveles más altos de carbohidratos de las dietas, pero también con su bajo contenido de proteínas. De hecho, se ha demostrado en ratas que un bajo contenido de proteínas en la dieta disminuye la actividad de LPL y perjudica la exportación de VLDL-TAG desde el hígado hacia otros tejidos del organismo (Boualga et al., 2000). De acuerdo con esto, el nivel más bajo de expresión del gen *lpl* observado en individuos de *P. punctifer* alimentados con las dietas 30:10 y 30:15 podría estar indicando una exportación limitada de VLDL (del inglés *very low-density lipoproteins*) desde el hígado, lo que resultó en el desarrollo de hígados grasos en estos individuos. Además, la deficiencia de proteínas puede inducir resistencia a la insulina asociada con una actividad reducida de LPL, una sobreproducción de TAG y un catabolismo deficiente de las VLDL (Ginsberg, 1991). En humanos, la insulina regula a la baja la expresión de *lpl* en individuos resistentes a la insulina, que presentan un metabolismo lipídico intracelular alterado (Boucher et al., 2002). Los diabéticos muestran una disminución de la insulina después de una dieta alta en carbohidratos (Gutniak et al., 1986). En algunos peces, como en *O. mykiss*, se produjo una secreción insuficiente de insulina cuando se alimentaron con una dieta alta en carbohidratos (Polakof et al., 2010). Del presente estudio se desprende que los contenidos de carbohidratos y proteínas de las dietas 30:10 y 30:15 fueron inadecuados para *P. punctifer*. Sin embargo, se necesita más investigación para descifrar la participación y la interconectividad de los mecanismos de acción de estos macronutrientes en lo que respecta al metabolismo lipídico hepático en estos peces y si estas dietas bajas en proteínas y ricas en carbohidratos inducen resistencia a la insulina o no en *P. punctifer*. Con respecto a los grupos 45:10 y 45:15, las diferencias en la relación de contenido de lípidos:carbohidratos de las dietas proporcionadas (1 frente a 5, respectivamente) podrían ser responsables de la mayor expresión de *lpl* en el grupo 45:10 en comparación con el grupo 45:15. Una mayor ingesta de carbohidratos en la dieta puede tener un efecto más fuerte en el almacenamiento de grasa en el hígado, como se observó en individuos del grupo 45:10 en comparación con el grupo 45:15. Los niveles más altos de expresión de *lpl* observados en los grupos 45:10 y 45:15 reflejan un mejor transporte de lípidos desde el sistema vascular a los tejidos, y la menor acumulación de lípidos en el hígado de estos grupos dietéticos indica un metabolismo de lípidos más equilibrado que el resto de los grupos dietéticos. Esto está en consonancia con el nivel de maduración intestinal de estos grupos en comparación con los demás.

La composición proximal de las dietas moduló de manera diferente la expresión génica de las enzimas pancreáticas y gástricas, así como la regulación postranscripcional de la producción de la enzima. En este sentido, la expresión de *amy* fue mayor en el grupo 45:15 en comparación con el resto de los grupos, mientras que la actividad de la α-amilasa mostró la tendencia opuesta. Estos resultados sugieren que la actividad de la α-amilasa fue modulada a nivel postranscripcional en todos los grupos dietéticos de acuerdo con el contenido de carbohidratos de las dietas. Así, los grupos alimentados con las dietas 30:10 y 30:15, que contenían los niveles más altos de carbohidratos (31% y 25%,

respectivamente), mostraron la mayor actividad de α-amilasa. Se ha observado una respuesta similar en *D. labrax* (Péres et al., 1998). A partir de los datos obtenidos sobre la expresión génica y la regulación de la actividad enzimática de los diferentes grupos dietéticos, parece que los juveniles de *P. punctifer* tienen la capacidad de responder a niveles tan altos de carbohidratos aumentando en cierta medida la actividad de la α-amilasa. De hecho, acumularon un mayor contenido de carbohidratos en sus tejidos que los otros grupos dietéticos. Sin embargo, la acumulación excesiva de lípidos en el hígado de estos individuos sugiere que el contenido de carbohidratos era excesivo en estas dietas. Además, el bajo nivel de expresión de *amy* observado en los grupos 30:10 y 30:15, e incluso en el grupo 45:15, podría estar indicando una regulación negativa de la expresión génica en respuesta a niveles excesivos de carbohidratos en la dieta.

Las dietas con mayor contenido de lípidos promovieron el desarrollo del sistema digestivo, como lo refleja el mayor número de enterocitos en el intestino anterior y el intestino medio encontrados en los grupos 30:15 y 45:15. Esto podría explicar el mejor crecimiento del grupo 30:15 en comparación con el grupo 30:10, a pesar de tener el mismo contenido de proteínas. Este resultado estaría también respaldado por el mayor nivel de actividad de la lipasa en el grupo 30:15, actividad que fue similar al del grupo 45:10, en comparación con el grupo 30:10. El nivel de esta enzima lipolítica está generalmente modulado por el contenido de lípidos de la dieta (Martínez et al., 1999), si bien hay trabajos que describen que dicha regulación no vendría tanto relacionada con el nivel de grasa de la dieta como por el perfil de ácidos grasos de esta (Morais et al., 2004). Sin embargo, en el presente estudio, el grupo 45:15 mostró un mayor nivel de actividad de lipasa que el grupo 30:15, lo que podría explicarse por el hecho de que, aunque el contenido de lípidos totales fue similar en ambas dietas, la dieta 45:15 contenía una mayor cantidad de TAG que la dieta 30:15 (*cf.* A2), así como un sistema digestivo más maduro desde un punto de vista funcional. Al igual que en los grupos alimentados con bajo contenido en proteínas, la actividad de la lipasa parece estar modulada también por el contenido de proteínas en los grupos alimentados con niveles más altos de proteínas, ya que el grupo 45:10 presentó una mayor actividad de la lipasa que el grupo 30:10 (que también presentaba una composición similar de ácidos grasos) y una actividad similar a los grupos 45:15 y 30:15.

La eficiencia de la utilización de PUFAs en la dieta depende de la clase de lípidos de la dieta (lípidos neutros o fosfolípidos). Los ingredientes lipídicos utilizados para formular las cuatro dietas del capítulo 2.1. fueron soja y lecitina marina como fuentes de lípidos neutros y fosfolípidos, respectivamente. Las primeras etapas de los peces tienen altas necesidades de fosfolípidos (Cahu et al., 2003) que son nutrientes esenciales para el crecimiento, la supervivencia y la maduración de las funciones intestinales (Bell y Sargent, 2003; Cahu et al., 2003). El nivel de expresión de *plA2* se correlacionó con el contenido de fosfolípidos de las dietas. El mayor contenido de fosfolípidos de la dieta 45:15 en comparación con la dieta 45:10 también podría explicar el mejor crecimiento y desempeño de los individuos alimentados con la dieta 45:15, así como los cambios en la acumulación de lípidos hepáticos. Además, el mayor contenido de fosfolípidos de la dieta 30:15 podría contribuir a

las medidas similares de LT y PH (a 20 dpf) con el grupo 45:10, a pesar de la cantidad considerablemente menor de proteína dietética disponible. Los fosfolípidos son especialmente importantes en los peces marinos, ya que utilizan los PUFAs n-3 de la dieta de la fracción de fosfolípidos de manera más eficaz que los de la fracción de lípidos neutros (Gisbert et al., 2005). De manera similar, se ha demostrado en algunos peces de agua dulce, como *C. gariepinus* y *Carassius auratus*, que los fosfolípidos son los principales lípidos catabolizados (Verreth et al., 1994; Wiegand, 1996). Aunque no se ha determinado la preferencia por las clases de lípidos en *P. punctifer*, el mayor contenido de fosfolípidos de la dieta 30:15 en comparación con el resto de las dietas podría haber contribuido a promover el crecimiento en los individuos de este grupo hasta los niveles del grupo 45:10, a pesar de la cantidad reducida de proteína en la dieta.

El nivel de expresión de *try* fue mayor en el grupo 45:15 a 26 dpf, mientras que la actividad de la tripsina fue similar en los cuatro grupos dietéticos a esa edad. Sin embargo, se observaron diferencias en la actividad de la tripsina a 20 dpf; los grupos alimentados con el mayor contenido de proteína en la dieta mostraron el nivel más alto de actividad. Estos resultados indican que, si bien *try* y *pga* contribuyeron a la digestión de proteínas a 20 dpf y, en consecuencia, se observa una modulación de la actividad enzimática en función de la dieta, la actividad de la pepsina aumentó a 26 dpf y los niveles de actividad de la tripsina se mantuvieron estables en el tiempo e invariables entre los regímenes dietéticos. A 26 dpf, la expresión *try* fue más alta en el grupo 45:15 y más baja en el grupo 30:10. Inesperadamente, el nivel de expresión *try* en el grupo 45:10 fue más bajo que el del grupo 45:15, pero similar al del grupo 30:15. Estos hallazgos sugieren que, en *P. punctifer*, la modulación de la expresión de *try* no sólo depende del nivel de proteína en la dieta, sino más bien de la combinación de nutrientes e incluso de la regulación de hormonas digestivas (i.e. colecistoquinina, CCK) que también participan en su síntesis y secreción (Rønnestad et al., 2013). De hecho, en ratas se ha demostrado que la expresión de *try* aumenta cuando se ofrece una dieta rica en carbohidratos sin proteínas (Dakka et al., 1990). Además, en *G. morhua* alimentados hasta la saciedad, la tripsina fue la única enzima examinada por Lemieux et al. (1999) que podría limitar potencialmente la eficiencia de conversión de alimentos y, por lo tanto, limitar la tasa de crecimiento. En general, en esta especie, la tripsina mostró una correlación más fuerte con la tasa de crecimiento que con la ingesta de alimentos, y en *P. punctifer*, la dieta alta en proteínas dio los mejores resultados de crecimiento y la mayor expresión de *try* a 26 dpf (Lemieux et al., 1999). Los niveles de actividad de la tripsina fueron similares en todos los grupos dietéticos, lo que indica una regulación postranscripcional (Péres et al., 1998). El nivel similar de actividad de la tripsina entre los cuatro grupos dietéticos indica que se utilizaron niveles similares para digerir el contenido de proteínas que iban del 30% al 43% y/o que hay otros nutrientes que inducen la actividad de esta enzima en los grupos bajos en proteínas. Investigaciones adicionales sobre la influencia de los carbohidratos en la regulación de la actividad de la tripsina, así como sobre la participación de la hormona digestiva colecistoquinina en la regulación de la secreción de *try* (Koven et al., 2002), contribuirán a comprender mejor la regulación de la actividad de la tripsina a nivel holístico en esta especie.

En *P. punctifer*, la expresión de *pga* resultó estar modulada por el contenido de proteína de la dieta. Por lo tanto, los grupos experimentales alimentados con niveles altos de proteínas mostraron niveles más altos de expresión de *pga* que los grupos alimentados con niveles bajos de proteínas. Sin embargo, a nivel de enzima, el grupo 45:15 mostró la mayor actividad de pepsina y el grupo 30:10 la más baja, mientras que los grupos 45:10 y 30:15 mostraron valores intermedios. Estos resultados podrían estar relacionados con la alta variabilidad interindividual obtenida en los ensayos enzimáticos que dieron como resultado diferencias no significativas de la actividad de la pepsina entre esos grupos dietéticos, aunque se observó que la actividad de la pepsina tendía a ser mayor en el grupo 45:10 que en el 30:15. Alternativamente, estos resultados podrían mostrar una regulación postranscripcional de este gen para adaptarse a una combinación de nutrientes específica, aunque algunos autores consideran que la actividad de la pepsina está pobremente modulada por la proteína de la dieta (Zambonino Infante y Cahu, 2007). En este caso hipotético, además del contenido de proteínas, el diferente contenido de energía de las dietas 45:10 y 30:15 podría ser responsable de un nivel similar de actividad de la pepsina pues, en función de la energía de la dieta, ésta es consumida en mayor o menor grado, y el grado de ingesta del alimento regula el nivel de distensión de las paredes del estómago y, esta, a su vez, la producción de pepsina (Rønnestad et al., 2013). Sin embargo, se necesitan más conocimientos sobre la interacción y la comunicación entre los nutrientes y los diferentes mecanismos enzimáticos para confirmar estas hipótesis y comprender mejor los complejos mecanismos que subyacen a la nutrición en peces.

En el capítulo 2.2, los grupos alimentados con *Artemia* no enriquecida mostraron una mayor deposición de lípidos en el hígado que los individuos alimentados con *Artemia* enriquecida, lo que podría estar asociado con las proporciones más bajas de PUFA/saturados y OA/PUFA observadas en estos grupos en comparación con los alimentados *Artemia* enriquecida (Boglino et al., 2012a). La mayor expresión de *lpl* observada en los grupos de *Artemia* no enriquecidos podría haber ayudado a prevenir la esteatosis hepática en estos grupos, ya que la LPL es esencial para amortiguar la carga circulatoria de TAG, que protege contra la acumulación ectópica de TAG (Frayn, 2002). La acumulación de lípidos en el intestino similar en todos los grupos dietéticos indicó una regulación diferencial en la captación de lípidos hepáticos entre los grupos dietéticos enriquecidos y no enriquecidos. De hecho, se observó una regulación diferencial en la expresión de genes relacionados con los lípidos entre estos grupos, donde la expresión *de plA2* y *lpl* fue mayor en los grupos no enriquecidos que en los enriquecidos. Esto podría estar asociado a un mayor contenido de OA, LA y ALA de los grupos alimentados con *Artemia* no enriquecida (grupos C y T2). Por lo tanto, a pesar de que el DHA en la dieta no tuvo un efecto significativo sobre el crecimiento durante la fase larvaria de *P. punctifer*, sí parece ser importante para preservar la calidad de las larvas y la salud de los tejidos. Los ácidos grasos juegan un papel en el control de la actividad de LPL para asegurar que los ácidos grasos no se formen más rápidamente de lo que pueden ser absorbidos por el tejido periférico (Saxena et al., 1989). En el capítulo 2.2 hubo una correlación inversa entre la acumulación de lípidos en el hígado y la expresión del gen *lpl* en todos los

grupos dietéticos. Por ejemplo, los grupos alimentados con *Artemia* no enriquecida mostraron niveles más altos de expresión de *lpl* junto con una menor acumulación de lípidos en el hígado que sus congéneres alimentados con *Artemia* enriquecida. En ausencia de diferencias en las clases de lípidos (incluidos PL y TAG) entre la *Artemia* enriquecida y no enriquecida, la diferencia más importante en la composición de ácidos grasos fue en su contenido de DHA. Estos resultados sugieren que el DHA moduló la expresión de *lpl*. Específicamente, el DHA disminuyó la expresión de *lpl*, lo que probablemente controló la captación de lípidos en el hígado, y que podría explicar los niveles más bajos de acumulación de lípidos en el hígado observados en los grupos enriquecidos (T1, T3). También se observó una tendencia similar para la expresión de *plA2* entre los grupos dietéticos. Sin embargo, en la etapa juvenil temprana, el grupo C mostró una importante acumulación de lípidos en el intestino posterior (esteatosis) en comparación con el hígado, al contrario de los otros grupos que presentaron cantidades similares de lípidos en ambos tejidos. La acumulación importante de depósitos lipídicos (que consisten principalmente en TAG) en el intestino de los peces generalmente se debe a una reducción de la exportación de lípidos desde la mucosa intestinal al sistema circulatorio (Fontagné et al., 1998; Gisbert et al., 2008). En este sentido, aunque las diferencias no fueron estadísticamente significativas entre los grupos experimentales, la expresión de *lpl* en el grupo C tendió a ser menor, lo que podría estar indicando su acción en la modulación de la deposición de TGA en el hígado en estos individuos.

El cambio de *Artemia* enriquecida a la dieta compuesta no enriquecida (grupo T1) tuvo un marcado impacto en la expresión de las enzimas digestivas analizadas. A pesar de que el contenido de carbohidratos fue similar en las dietas compuestas enriquecidas y no enriquecidas, solo se observó un aumento en la expresión de *amy* en el grupo T1, pero no en el grupo T3. Además, aunque todas las dietas enriquecidas y no enriquecidas tenían contenidos de proteínas similares, la expresión de *pga*, *try* y *ctr* fue notablemente más elevada en el grupo T1. Estos resultados evidencian una clara interacción entre los ácidos grasos de la dieta y los genes involucrados en la digestión de carbohidratos y proteínas. Estos hallazgos están en línea con los resultados obtenidos en los estudios nutricionales previos (*cf.* 2.1, A2). Al comparar la composición de ácidos grasos de la *Artemia* enriquecida y la dieta compuesta no enriquecida, se encontraron LA y EPA en cantidades más altas en la dieta compuesta no enriquecida que en la *Artemia* enriquecida. Por lo tanto, estos dos ácidos grasos probablemente explicarían el aumento de la expresión de *amy*, *pga*, *try*, *plA2* y *ctr* observado en el grupo T1. De hecho, se ha demostrado que el LA de la dieta induce la expresión de los genes *amy*, *try* y *ctr* en el hepatopáncreas de *Ctenopharyngodon idellus* (Zeng et al., 2016). Estos autores también demostraron que una proporción óptima de LA/ALA en la dieta de 1.03 era necesaria para promover el crecimiento en esta especie, lo que consideraron que se atribuía en parte a la mejora de las actividades de tripsina, quimotripsina, lipasa y α-amilasa. En el presente estudio, el grupo T1 presentó, junto con el grupo C, el menor crecimiento a 26 dpf. Esto sugiere una regulación negativa postranscripcional de estas enzimas y/o la influencia de otros ácidos grasos en el metabolismo de nutrientes en el grupo T1, lo que conduce a un menor aumento de peso. Por ejemplo, al igual que en el grupo C, la proporción de PUFAs n-3/n-6 fue mayor en los peces

del grupo T1 que en los grupos T2 y T3. Estos resultados sugieren que una dieta rica en PUFA n-6 promovió el aumento de peso en *P. punctifer*. Aunque el crecimiento al final del experimento fue similar en T2 y T3, al considerar todas las variables analizadas, el protocolo de alimentación utilizado en el grupo T3 favoreció una fisiología digestiva más equilibrada en comparación con los otros tratamientos dietéticos, incluyendo un metabolismo lipídico y un almacenamiento de grasa en el parénquima hepático y el epitelio intestinal más equilibrados y, por consiguiente, una mejor condición nutricional de los animales. Además, la *Artemia* enriquecida en el grupo T3 contribuyó a reducir la incidencia de canibalismo, lo que condujo a una mejor tasa de supervivencia al final del experimento (*cf.* 2.2).

***Influencia de la composición de la dieta en la incidencia del canibalismo en* P. punctifer**

El estudio de la gestión del comportamiento caníbal en *P. punctifer* mediante estrategias nutricionales también fue objeto de interés en la presente tesis doctoral. Así, en el estudio presentado en el anexo 2, se observó una incidencia variable de canibalismo entre los diferentes tratamientos (3.6-6.4%), aunque se redujo considerablemente en comparación con estudios anteriores (16-30%, Arslan et al., 2013; Baras et al., 2011; *cf.* A1). En el presente estudio, el canibalismo tipo I (parcial) fue predominante, pero no exclusivo, en las primeras etapas; el canibalismo tipo II (completo) fue más importante a partir de los 15 dpf (*ca.* 16 mm LT), coincidiendo con la completa formación de las glándulas gástricas y el final del destete. Se observaron dos picos de canibalismo: el primer día de destete (13 dpf, 13 mm LT) y 2 días después del final del destete (17 dpf, *ca.* 17 mm LT). El canibalismo luego disminuyó gradualmente hasta desaparecer a los 21 dpf (*ca.* 22 mm LT). Estudios previos (Fernández-Méndez et al., 2015; *cf.* A1) mostraron que el destete constituía una etapa crítica que promovía el comportamiento caníbal. Sin embargo, la menor incidencia de canibalismo observada en el presente estudio y, en particular, su desaparición gradual después del destete, mostró que una dieta mejor adaptada podría en gran medida reducir este comportamiento. El canibalismo no se relacionó consistentemente con el crecimiento o la supervivencia. Por ejemplo, los especímenes más pequeños (grupo 30:10) presentaron la mayor incidencia de canibalismo y los grupos que mostraron una baja incidencia de canibalismo mostraron bajas tasas de supervivencia (grupos 30:15 y C). Aunque generalmente se encontró una distribución de tamaño más alta en grupos que mostraban un mayor canibalismo en experimentos anteriores, en el presente estudio no se observó ningún efecto del canibalismo en la distribución de talla de la cohorte entre los grupos experimentales. En cualquier caso, su influencia en esos parámetros pudo haber sido muy limitada debido a la baja incidencia de canibalismo registrada. De hecho, se observó un efecto beneficioso de todas las dietas probadas en el comportamiento de *P. punctifer*, siendo los peces más tranquilos y menos agresivos que en los experimentos anteriores (*cf.* Capítulo 1, A1). Esto sugiere que, además del valor nutricional general de las dietas, la inclusión de nutrientes específicos inductores de efectos relajantes, como los fosfolípidos, podría contribuir a atenuar el canibalismo. De hecho, se ha demostrado que los fosfolípidos de la dieta reducen la actividad locomotora, el estrés y la agresividad en humanos y ratas cuando se administran en

cantidades suficientes (Chalon et al., 1998; DeMar et al., 2006; Hamazaki y Hamazaki, 2008), mientras que la deficiencia promueve un perfil de comportamiento ansioso en peces (Lund et al., 2014). En este sentido, los resultados obtenidos en el capítulo 2.2, discutidos a continuación, contribuyeron a apoyar esta hipótesis. Además de ser esencial para el crecimiento y el rendimiento de muchas especies de peces, incluidos los juveniles de *P. fasciatum* (Arslan et al., 2009; Cahu et al., 2009), los fosfolípidos también podrían contribuir a mejorar la palatabilidad de las dietas (Gong et al., 2014; Hadas et al., 2003; Tocher et al., 2008). Esto es particularmente importante para *P. punctifer* ya que el gusto y el olfato parecen tener un papel clave en la alimentación, como lo sugiere la presencia de válvulas orales de gran tamaño equipadas con papilas gustativas, que se cree sirven para evaluar la calidad de los alimentos (Gamal et al., 2012; Yashpal et al., 2006), y un gran desarrollo del órgano olfativo (*cf.* A1). De hecho, las larvas y los juveniles tempranos de *P. punctifer* demostraron una sensibilidad a la textura de los alimentos, siendo más atraídos por las dietas compuestas húmedas que por las secas (Fernández-Méndez et al., 2015), lo que probablemente esté relacionado con la palatabilidad y/o el olor asociado a los atrayentes liberados.

En el capítulo 2.2, los metanauplios de *Artemia* enriquecidos con DHA redujeron la incidencia de canibalismo y probablemente contribuyeron a favorecer la supervivencia observada al final del período experimental. Las deficiencias nutricionales pueden favorecer la dispersión de tallas y promover el canibalismo (Baras y Jobling, 2002). En este estudio, sin embargo, la dispersión de talla fue similar entre todos los tratamientos dietéticos a lo largo del ensayo. La incidencia de canibalismo en *P. punctifer* aumentó en el destete, alcanzó su punto máximo dos días después del final del período de destete y desapareció cinco días después. Por lo tanto, el comportamiento caníbal en el destete parece ser más una respuesta al cambio de dieta que a la dispersión de tallas.

El DHA es esencial para el desarrollo del cerebro y la ontogenia del comportamiento en peces (Benítez-Santana et al., 2012, 2007; Ishizaki et al., 2001; Masuda et al., 1999). Se ha demostrado que la reducción de los PUFAs n-3 en la dieta y, por tanto, en los tejidos de los peces, reduce el comportamiento de huida de las larvas (Benítez-Santana et al., 2012). En los mamíferos, la ingesta inadecuada de DHA se asocia con índices conductuales de ansiedad, agresión y depresión elevados (Fedorova y Salem, 2006) y, tanto en mamíferos como en peces, se sabe que el DHA reduce el estrés (Lund et al., 2012; Lund y Steenfeldt, 2011; Takeuchi et al., 2003; Xu et al., 2016). Teniendo en cuenta que los grupos alimentados con *Artemia* enriquecida contenían niveles más altos de DHA, así como proporciones más altas de PUFA/saturados y menores de LA/PUFA y OA/PUFA en sus tejidos que los grupos alimentados con *Artemia* no enriquecida, se podría especular que la incidencia reducida de canibalismo en los primeros grupos podría estar asociada con un sistema nervioso y un comportamiento de huida mejor desarrollados, así como con niveles de estrés reducidos en esos especímenes. Esta hipótesis se basa en los resultados de canibalismo observados en los juveniles del grupo T1 (alimentados con *Artemia* enriquecida y dieta compuesta no enriquecida), donde la incidencia de canibalismo se redujo a los niveles de los grupos T2 y T3 después del destete, a pesar de ser alimentados con una dieta compuesta no enriquecida.

Durante el destete se pudo observar un período de transición en el comportamiento caníbal, en el que la introducción de la dieta compuesta enriquecida redujo la incidencia de canibalismo (grupos T2 y T3), mientras que la transición de *Artemia* enriquecida a una dieta no enriquecida aumentó temporalmente el comportamiento caníbal (grupo T1). El hecho de que el comportamiento caníbal del grupo T1 en el destete (14 dpf) no duró y disminuyó a 26 dpf a niveles de canibalismo similares a los de los grupos T2 y T3 sugirió que el cambio de dieta indujo un estrés transitorio y que el DHA proporcionado durante la fase larvaria tuvo un efecto condicionante hacia el desarrollo de juveniles menos caníbales. En consonancia con esto, se ha observado el efecto a largo plazo del historial nutricional temprano de deficiencias de DHA y EPA sobre el comportamiento ansiolítico en peces (Lund et al., 2012). En particular, las larvas de *Sander lucioperca* alimentadas por primera vez con una dieta deficiente en DHA aumentaron la sensibilidad al estrés en los futuros juveniles, mientras que una dieta rica en DHA aumentó su tolerancia frente al estrés (Lund et al., 2012). En *P. punctifer*, la hipótesis de que un bajo contenido de DHA en la dieta temprana promovió el desarrollo de juveniles más estresados o menos resilientes al estrés fue respaldada por el hecho de que el canibalismo total fue mayor en el grupo C al final del ensayo y por la mayor incidencia de canibalismo tipo II en este grupo en comparación con el grupo T1 durante la fase de alimentación con dieta compuesta. Otro indicio sobre el condicionamiento nutricional durante la etapa larvaria fueron los niveles más altos de expresión de la mayoría de los genes digestivos analizados en el grupo T1 en comparación con el grupo C, ya que se habría esperado un perfil de expresión génica similar a 26 dpf en estos grupos alimentados con una dieta compuesta no enriquecida. Estos niveles más altos de expresión génica en el grupo T1 a 26 dpf no explicaron un mejor desempeño en términos de crecimiento, ya que este grupo presentó un peso húmedo similar al del grupo C, que fue menor que el de los grupos alimentados con la dieta enriquecida.

CONCLUSIONES

1. *Pseudoplatystoma punctifer* mostró un patrón de crecimiento exponencial con dos periodos de crecimiento diferentes: una etapa más lenta desde la eclosión hasta los 12 dpf (*ca.* 12 mm LT), seguida de una más rápida hasta 27 dpf (*ca.* 36 mm LT). El crecimiento lento en la fase inicial se atribuye a que la energía se utilizó para desarrollar los órganos y sistemas, mientras que, una vez completada la ontogenia de los sistemas, la energía se asignó para el crecimiento.

2. Las larvas de *P. punctifer* mostraron una ontogenia funcional del sistema digestivo rápida y sincronizada con el desarrollo histológico.

3. La expresión génica y actividad de las enzimas digestivas analizadas durante el desarrollo de *P. punctifer* siguió el perfil típico de una especie tropical carnívora a excepción de *amy*, que aumentó durante el desarrollo. Se sugiere que *P. punctifer* muestra un comportamiento de alimentación omnívoro con preferencia carnívora durante la etapa temprana de la vida.

4. Los perfiles de actividad de las enzimas digestivas intestinales, pancreáticas y gástricas reflejaron los cambios funcionales asociados a su ontogenia y al régimen de alimentación.

5. Los resultados de expresión génica y de actividad enzimática mostraron que la maquinaria enzimática de *P. punctifer* está completamente preparada antes del inicio de la alimentación exógena a 4 dpf (*ca.* 6 mm LT) y alcanza su madurez entre 10 y 13 dpf (*ca.* 11-14 mm LT), cuando los individuos presentan un sistema digestivo con el modo de digestión adulto. Esto indica, desde la perspectiva de la fisiología digestiva, la transición del estadio larvario al juvenil, y el momento adecuado para el destete en condiciones de cultivo.

6. Entre las dietas compuestas experimentales probadas en esta tesis, la dieta 45:15 (proteína: lípido) promovió un desarrollo más rápido del sistema digestivo, como lo demuestran los indicadores histológicos y funcionales: mayor número de hepatocitos, mayor número de células caliciformes en el intestino anterior, mayor número de enterocitos en todas las secciones intestinales y pliegues más largos en el intestino posterior; la expresión más alta de *amy*, *lpl*, *plA2*, *try* y *pga*; y mayor actividad de lipasa y pepsina y mayor proporción AP:LAP. En conjunto, esto permitió a los individuos digerir, absorber y metabolizar mejor los nutrientes.

7. Las proteínas, seguidas de los lípidos, fueron los principales impulsores del crecimiento y la supervivencia en los juveniles tempranos de *P. punctifer*. Los fosfolípidos resultaron ser clave para mejorar el rendimiento del grupo de 45:15. Parece que un mejor uso de la fracción de fosfolípidos permitió a este grupo aprovechar todos los beneficios que estos lípidos aportan para el crecimiento, desarrollo y comportamiento.

8. El crecimiento y el metabolismo de *P. punctifer* se vieron afectados significativamente por la relación E:P de la dieta. A relaciones E:P altas (p. ej. 11 kcal g^{-1} de proteína), la energía se utilizó para asegurar los requerimientos de proteínas corporales y la energía restante no contribuyó con la suficiente eficiencia para promover el crecimiento.

9. *P. punctifer* tiene una clara preferencia por los lípidos como fuente de energía respecto a los carbohidratos. Además, los lípidos promueven un efecto ahorrador de proteínas en *P. punctifer* cuando existe una relación E:P adecuada.

10. Un contenido de carbohidratos en la dieta superior al 25% resultó ser excesivo para *P. punctifer*, ya que provocó un desequilibrio del metabolismo de los lípidos y la deposición de grasas en el hígado. Según los datos obtenidos en la presente tesis, una relación C:L de 0.2-0.8 es adecuada para *P. punctifer*.

11. Los niveles de DHA de la dieta influyeron en la fisiología de *P. punctifer* de manera dependiente del estado de desarrollo. En particular, la *Artemia* enriquecida con DHA durante la etapa larvaria contribuyó a reducir la incidencia de canibalismo y mejoró la supervivencia, mientras que la dieta compuesta enriquecida con DHA durante la etapa juvenil temprana sólo contribuyó a mejorar el crecimiento.

12. El grupo alimentado tanto con *Artemia* como con dieta compuesta enriquecidas con DHA (4.4% TFA en *Artemia*, 25% TFA en dieta compuesta; grupo T3) proporcionó los mejores resultados en términos de crecimiento, supervivencia, incidencia de canibalismo y fisiología digestiva.

13. La historia nutricional durante el período larvario afectó la nutrición y el comportamiento de los peces en la etapa juvenil temprana. Por ejemplo, el paso de *Artemia* enriquecida con DHA a una dieta compuesta no enriquecida (grupo T1) influyó notablemente en la expresión de las enzimas digestivas analizadas en la etapa juvenil temprana. Además, en este mismo grupo dietético, el DHA proporcionado durante la etapa larvaria contribuyó a reducir el comportamiento caníbal de los juveniles tempranos.

14. El cambio observado en la incidencia de canibalismo durante el período de transición de alimentación con *Artemia* a alimento compuesto demostró que el canibalismo se ve fuertemente afectado por la nutrición en *P. punctifer*. De hecho, la incidencia de ambos tipos de canibalismo (I y II) fue modulada por la composición nutricional de la dieta.

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ANEXOS

Artículos relacionados con la tesis publicados previamente

A1

Histological development of the digestive system of the Amazonian pimelodid catfish *Pseudoplatystoma punctifer*

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Resumen

Se describió la organogénesis del sistema digestivo del bagre pimelódido amazónico *Pseudoplatystoma punctifer* desde la eclosión (3.5 mm de longitud total, LT) hasta 41 días post- fertilización (dpf) (58.1 mm LT) cultivado a 28 °C. Las larvas recién eclosionadas mostraron un tracto digestivo simple, que apareció como un tubo recto indiferenciado y desplegado revestido por una sola capa de células epiteliales columnares (futuros enterocitos). Durante el período de alimentación endógena, comprendido entre 20 y 96 h post-fertilización (3,5 a 6,1 mm LT), el sistema digestivo de las larvas experimentó una rápida transformación con el desarrollo y diferenciación casi completos de la mayoría de los órganos digestivos (bucofaringe, esófago, intestino, hígado y páncreas exocrino). Las reservas vitelinas no estaban agotadas por completo al inicio de la alimentación exógena (4 dpf, 6.1 mm LT), y se observó un período de nutrición mixta hasta 6 a 7 dpf (6.8 a 7.3 mm LT) cuando el vitelo se agotó completamente. El estómago fue el último órgano en diferenciarse, caracterizado por el desarrollo de abundantes glándulas gástricas en el estómago fúndico entre 10 y 15 dpf (10,9 a 15,8 mm LT) y la formación del esfínter pilórico en la unión del estómago pilórico y el intestino anterior a 15 dpf (15,8 mm TL). Las características morfológicas e histológicas antes mencionadas sugirieron la obtención de un sistema digestivo característico de juveniles y adultos de *P. punctifer*. La ontogenia del sistema digestivo en *P. punctifer* siguió el mismo patrón general que en la mayoría de las especies siluriformes, aunque se observaron algunas diferencias específicas en el momento de la diferenciación de varias estructuras digestivas, que podrían estar relacionadas con diferentes desempeños reproductivos, tamaños de huevo y larva o incluso diferentes prácticas de cultivo. De acuerdo con los hallazgos sobre el desarrollo histológico del sistema digestivo en *P. punctifer*, también se brindan algunas recomendaciones sobre las prácticas de cultivo de esta especie para mejorar el cultivo larvario de esta especie de bagre neotropical de rápido crecimiento.

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Histological development of the digestive system of the Amazonian pimelodid catfish *Pseudoplatystoma punctifer*

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The organogenesis of the digestive system was described in the Amazonian pimelodid catfish species Pseudoplatystoma punctifer *from hatching (3.5 mm total length, TL) to 41 days post-fertilization (dpf) (58.1 mm TL) reared at 28°C. Newly hatched larvae showed a simple digestive tract, which appeared as a straight undifferentiated and unfolded tube lined by a single layer of columnar epithelial cells (future enterocytes). During the endogenous feeding period, comprised between 20 and 96 h post-fertilization (3.5 to 6.1 mm TL), the larval digestive system experienced a fast transformation with the almost complete development and differentiation of most of digestive organs (buccopahrynx, oesophagus, intestine, liver and exocrine pancreas). Yolk reserves were not completely depleted at the onset of exogenous feeding (4 dpf, 6.1 mm TL), and a period of mixed nutrition was observed up to 6 to 7 dpf (6.8 to 7.3 mm TL) when yolk was definitively exhausted. The stomach was the organ that latest achieved its complete differentiation, characterized by the development of abundant gastric glands in the fundic stomach between 10 and 15 dpf (10.9 to 15.8 mm TL) and the formation of the pyloric sphincter at the junction of the pyloric stomach and the anterior intestine at 15 dpf (15.8 mm TL). The above-mentioned morphological and histological features observed suggested the achievement of a digestive system characteristic of* P. punctifer *juveniles and adults. The ontogeny of the digestive system in* P. punctifer *followed the same general pattern as in most Siluriform species so far, although some species-specific differences in the timing of differentiation of several digestive structures were noted, which might be related to different reproductive guilds, egg and larval size or even different larval rearing practices. According to present findings on the histological development of the digestive system in* P. punctifer*, some recommendations regarding the rearing practices of this species are also provided in order to improve the actual larval rearing techniques of this fast-growing Neotropical catfish species.*

Keywords: histology, ontogeny, digestive system, catfish, larvae, Amazonia

Implications

The aquaculture of Amazonian native species requires the mastering of their biological cycle, especially the improvement of current larval rearing procedures. In this context, the present study provides a detailed and comprehensive description of the development of the digestive tract and accessory glands in the pimelodid catfish *Pseudoplatystoma punctifer*. This information will provide insight in the digestive physiology of this Amazonian fish species in order to synchronize the stage of larval development with rearing procedures and overcome actual larval rearing bottlenecks (e.g. diet formulation, weaning and cannibalistic behaviour).

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Introduction

Aquaculture in Latin America and the Caribbean is principally known for the salmon industry in Chile and shrimp farming in Ecuador. However, the benefits of this export-oriented and large-scale industrial production have eclipsed interest in rural aquaculture activities. Aquaculture in Latin America accounts for 3.1% of the world's production (FAO, Fisheries and Aquaculture Department, 2013) and is mostly based on the culture of exotic freshwater species, such as the common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*) and trout (*Oncorhynchus mykiss*) (Martínez-Espinosa and Pedini, 1998). However, different authors have highlighted an increasing concern over the impact of exotic species on native fish populations and aquatic habitats (Zambrano *et al.*, 2006; Gozlan *et al.*, 2010; Loebmann *et al.*, 2010

among others), whereas this area, especially the Neotropical region, has the most diverse freshwater fish fauna in the world (Goulding, 1980). Consequently, different authorities are paying more attention to the development of indigenous species aquaculture in rural habitats. In these areas, the aquaculture of indigenous species could contribute to the double goal of food security and income generation while conserving fisheries resources, as well as provide a feasible alternative to alien species introduction for aquacultural purposes (Nuñez *et al*., 2008; Garcia *et al*., 2009). In this sense, the Amazonian basin, with one of the worlds' richest hydrological and fish resources, is an area with major potential for the development of the aquaculture of indigenous freshwater species, that represent a source of good quality protein and an important income for local communities (Beeby, 2012). In addition, the development of the aquaculture of native freshwater fish species in Latin America is a challenge that may serve to reduce the impact of exotic species on native fish populations and aquatic habitats, as well as to contribute to food production of locally appreciated fish species, and land diversification in rural areas and coastal zones (NACA/FAO, 2001; Subasinghe *et al*., 2009; Loebmann *et al*., 2010). Freshwater aquaculture in South America has shown strong and continuous growth, particularly in Brazil and Peru (FAO, Fisheries and Aquaculture Department, 2013). In Peru, the culture of Amazonian fish species only represents 1.13% of the national aquaculture production. Among the vast number of fish species that are found in the Amazonian region, the culture of *Colossoma macropomum*, *Piaractus brachypomus*, *Brycon erythropterum* and *Prochilodus nigricans* are the best mastered, while research efforts are being focused in promising species like *Arapaima gigas*, *Pseudoplatystoma punctifer* or *Brachyplatystoma* spp. (Mendoza, 2011).

P. punctifer (Teleostei: Siluriformes, *Pimelodidae*) is an appreciated catfish species, because of the flavour and texture of its flesh, and the absence of intramuscular spines. Several studies have been performed to develop the culture of this species (Nuñez *et al*., 2008 and 2011; Baras *et al*., 2011 and 2012). One of the limiting factors of fish culture development in this area is the lack of sustained fingerling production to support this economical activity. Larval production of native South American fish is mainly conducted in semi-intensive systems, where newly hatched larvae are directly stocked in plankton-rich fertilized ponds. However, survival rates in these systems are generally low and quite unpredictable (Gomes *et al*., 2000; Mai and Zaniboni Filho, 2005, among others), as they are highly impacted by food availability and the presence of predators. In *P. punctifer*, the difficulty of larvae to accept inert diets at weaning and a strong cannibalistic behaviour hampers the aquaculture of this fast-growing Amazonian catfish species, even in recirculation systems (Baras *et al*., 2011; Nuñez *et al*., 2011). One of the first steps in order to enhance the success of *P. punctifer* larval rearing and facilitate the overcoming of the major bottleneck of its culture, is the description of the ontogeny of the digestive system in order to synchronize the larval stage of development and maturation of their digestive organs with the feeding protocol and rearing practices, as well as identifying limiting factors during larval rearing. Thus, the histological development of the digestive tract has been regularly used as a histological biomarker to assess the nutritional status of fish at early life stages of development (Gisbert *et al*., 2008 and 2013). Although the basic mechanisms of organ and system development are similar among teleosts, there are considerable interspecific differences regarding their relative timing of differentiation, development and functionality during early ontogeny (Treviño *et al*., 2011). The timing of development of organ and physiological function is affected by the general life history and reproductive strategy of each species, especially in tropical species with fast development (Zambonino-Infante *et al*., 2008; Lazo *et al*., 2011). Hence, there is a need to conduct specific studies on the ontogenesis of fish digestive system for each species to better understand their morphogenesis and nutritional physiology.

The present study aimed to describe the histological structure of digestive tract and accessory digestive organs during the ontogeny of *P. punctifer*, from hatching to 41 days post-fertilization (dpf). This new information is expected to provide fundamental knowledge for improving actual larval rearing practices for this catfish species.

Material and methods

Larvae were obtained by hormonally induced spawning of a sexually mature couple of *P. punctifer* (♀: 4.73 kg; ♂: 1.15 kg) from a broodstock maintained in captivity at the Instituto de Investigaciones de la Amazonía Peruana (IIAP, Iquitos, Peru). Female and male were injected intramuscularly with the synthetic hormone Conceptal® (Intervet, Huixquilucan, México) at 2.6 ml/kg and 1 ml/kg BW, respectively. Hormone injections were administered in two doses: a first one at 10% and 50% of the total dose, and a second one 12 h later at 90% and 50% of the total dose for female and male, respectively. Stripping of female, sperm collection and fertilization procedure was performed following the protocol described by Nuñez *et al.* (2008).

Spawned eggs (fertilization rate = 90%) were incubated at 28°C in 60 l tanks connected to a clear water recirculating system and hatched 18 ± 2 h later (hatching rate = 84%). Larvae were transferred at 3 dpf (5.6 mm total length, TL) into 30 l tanks connected to the same water recirculation system provided with mechanical, biological and UV filters. Water conditions throughout the larval experiment were as follows: temperature 28.3 ± 0.4°C, pH 6.9 ± 0.2, dissolved oxygen 8.2 ± 0.5 mg/l, $N-NO_2$ 0.04 ± 0.02 mg/l, $N-NH_4$ 0.14 ± 0.05 mg/l. Larvae were reared in triplicate (initial density = 90 larvae/l) under 0L : 24D photoperiod, fed six times a day from 4 to 17 dpf with non-enriched *Artemia* spp. *nauplii* in slight excess (0.4 to 17 nauplii/ml) considering the larval density, the weight increase of the larvae and the daily food ration (Baras *et al*., 2011) and weaned onto a

commercial inert diet (BioMar®, Nersac, France; proximate composition: 58% proteins, 15% lipids, 11% ash; particle size: 0.5 mm) within 4 days. Once weaned (22 dpf, 23.7 mm TL), larvae were fed five times a day at 5% of the larval wet weight, changing at 33 dpf (29.9 mm TL) to another commercial diet (Aquaxcel®, Cargill Animal Nutrition, Franklinton, LA, USA; proximate composition: 45% proteins, 12% lipids, 10% ash; particle size: 0.8 mm) until the end of the trial (41 dpf, 58.1 mm TL) (Figure 1).

Groups of 15 to 30 larvae were sampled from each tank at 0, 2, 4, 6, 7, 10, 13, 16, 18, 24, 32 and 41 dpf and anaesthetized using Eugenol (0.05 µl/ml; Moyco®, Moyco, Lima, Peru) for TL measurements. Larvae were placed in a Petri dish and photographed using a scale bar. Total length was measured on the pictures using ImageJ software (Rasband, 1997–2012). For histological purposes, larvae were sampled at the following sampling points: 20, 33, 41, 49, 57, 65, 81, 87 and 97 h post-fertilization (hpf) and 4, 5, 6, 7, 8, 9, 10, 12, 13, 15, 18, 22, 24, 29, 32 and 41 dpf. Five larvae per sampling point were dehydrated with graded series of ethanol and embedded in paraffin with an automatic tissue processor Histolab ZX-60Myr (Especialidades Médicas MYR SL, Tarragona, Spain). Then, paraffin blocks were prepared in AP280-2Myr station and then cut into serial sagittal sections (3 µm thick) with an automatic microtome Microm HM (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Paraffin larvae cuts were kept at 40°C overnight. After that, samples were deparafined with graded series of xylene and stained by means of hematoxylin and eosin and the trichromic VOF (light green, orange g and acid fucshin) stain (Sarasquete and Gutiérrez, 2005) for general histomorphological observations. Periodic Acid Schiff (PAS) and Alcian Blue (AB) at pH 2.5, 1.0 and 0.5 were used to detect neutral and carboxyl-rich and sulphated glycoconjugates in mucous cells (Pearse, 1985). Histological preparations were observed in a microscopy Leica DMLB equipped with a digital camera Olympus DP70 (Olympus España, S.A.U., Barcelona, Spain). Lipid droplet size was calculated as an average between the maximum and minimum diameters measured on a total of 40 lipid droplets from five fish per sampling point (Boglino *et al.*, 2012). Measurements on histological slides were performed with an image analysis software package (ANALYSIS™, Soft Imaging Systems GmbH, Münster, Germany) on five fish and data expressed as the ranged comprised between the minimum and maximum recorded values. The number of cannibals was counted on each tank twice a day (at 08:00 and 19:00 h) and the incidence of cannibalism expressed as the percentage of fish displaying cannibalistic behaviour at each feeding period. Survival was evaluated by counting the individuals surviving at 17, 32 and 41 dpf with respect to the number of individuals at the beginning of each feeding period. Differences in the percentage of cannibalism and survival rates (data arcsine-transformed) were evaluated by one-way ANOVA followed by the Holm – Sidak method for all pairwise comparisons ($P < 0.05$).

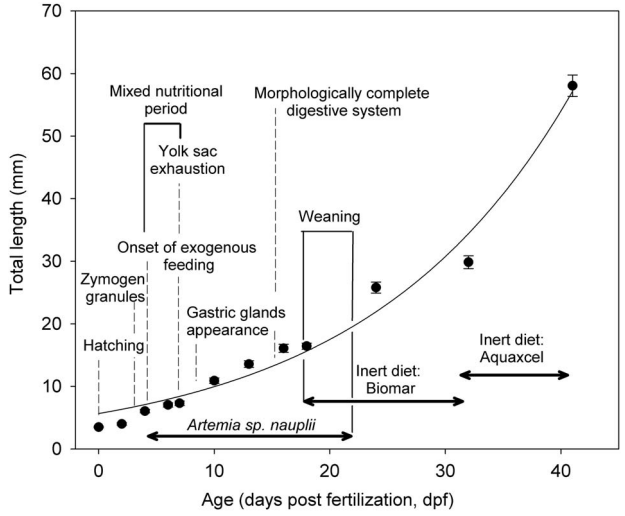

Figure 1 Larval growth in total length (TL, mm) of *Pseudoplatystoma punctifer* from hatching to 41 days post-fertilization. Dotted lines represent some of the main events with regards to the morphogenesis of the digestive system. The sequence of food supply (*Artemia* spp. *nauplii*, Biomar® and Aquaxcel® inert diets) is indicated by horizontal arrows under the growth curve.

Results

Larval growth in terms of TL of *P. punctifer* larvae is shown in Figure 1. Larval growth (TL) in age (*T*, dpf) under present rearing conditions followed an exponential curve represented by the following regression equation: TL (mm) = 4.181 $e^{0.23 \times T}$ ($r^2 = 0.97$, $P < 0.05$). In this study, larval growth was in the normal range of growth values known for this species (Nuñez *et al.*, 2008). Survival rate and the incidence of cannibalism (in %) at each feeding period are shown in Table 1. Survival rate was high (ca. 95%) and the incidence of cannibalism almost insignificant at the end of the *Artemia*-feeding phase (17 dpf), during which the ontogeny of the digestive system took place. However, cannibalism significantly increased at weaning and remained at around 27% during the second feeding period using inert diets (18 to 32 dpf). Survival rate at the end of this phase decreased almost two times with respect to the *Artemia*-feeding phase. The incidence of cannibalism decreased again when diet switched to another inert diet, reaching a similar percentage of cannibalism as found during the *Artemia*-feeding period. Survival rate at the end of this third feeding period continued to decrease to around 16% (Table 1).

At hatching (3.3 to 3.7 mm TL; 20 hpf), the digestive system of *P. punctifer* consisted of a straight tube lying

Table 1 *Survival rate and percentage of cannibalism evaluated at each feeding periods*

Feeding periods	Survival (%)	Cannibalism (%)
Live prey: *Artemia* (4 to 17 dpf)	94.7 ± 1.5^a	0.1 ± 0.1^b
Inert diet: Biomar® (18 to 32 dpf)	49.4 ± 13.6^b	27.7 ± 7.7^a
Inert diet: Aquaxcel® (33 to 41 dpf)	16.6 ± 7.1^c	7.6 ± 5.7^b

dpf = days post-fertilization.
Different letters denote significant differences between each feeding period ($P < 0.05$).

dorsally to the large eosinophilic yolk sac (0.14 to 0.20 mm^3) (Figure 2a). The yolk sac was surrounded by a thin basophilic syncytial layer and consisted of a large accumulation of ovoid eosinophilic and PAS-positive yolk platelets with scattered spherical non-stained vacuoles throughout the yolk-sac matrix. These vacuoles corresponded to lipids that were washed out during the paraffin embedding process of the samples. Yolk platelets were also slightly stained in light blue with AB (pH 0.5, 1 and 2.5), which indicated that they also contained a small quantity of acidic (carboxylated and sulphated) glycoproteins. At this stage, the mouth and anal pore were still closed and did not open to the exterior until 33 hpf (3.3 to 3.6 mm TL) (Figure 2a). During the endogenous feeding period comprised between 20 and 96 hpf (3.5 to 6.1 mm TL), the larval digestive system experienced a dramatic and fast transformation with the almost complete development and differentiation of all digestive organs, with the exception of the gastric stomach that completed its differentiation later on, between 10 and 15 dpf (10.9 to 15.8 mm TL). Yolk reserves were observed in histological slides until the age of 6 and 7 dpf (6.8 to 7.3 mm TL), indicating a short period of mixed nutrition that only lasted for 2 or 3 days (Figure 2d).

When the mouth opened at 33 hpf, the buccopharyngeal cavity was short (500 to 600 μm in length) and lined by a simple and flat epithelium with scattered (0.2 to 0.5 goblet cells in 100 μm of epithelium) round goblet cells (35 to 45 μm in diameter) protruding into the buccopharyngeal lumen (Figure 2a and b). These secretory cells stained blue and purple with PAS and AB pH 2.5, 1.0 and 0.5, indicating the presence of a combination of neutral and acidic (carboxylated and sulphated) mucins. The histochemical properties of buccopharyngeal goblet cells remained constant throughout the studied period. As a consequence of larval development and the acquisition of streamlined shape with a pointed head ending in a terminal mouth between 7 and 10 dpf (7.3 to 10.9 mm TL), the mouth cavity grew in length and the density of goblet cells increased (1 to 2 goblet cells in 100 μm of epithelium) (Figure 3d). The first taste buds were not observed until 57 hpf, at the same time that teeth were observed in both jaws (dentary and premaxilla). Taste buds were located in the lower oral valve and along the buccopharyngeal epithelium and were positively stained with the AB dye, indicating that sensory cells were rich in acid glycoproteins. Several canine-like teeth were visible in the connective tissue underlying the pharyngeal submucosa close to the oesophageal opening, but they did not protrude into the pharyngeal lumen until the age of 6 to 7 dpf (Figure 3a to c). Oral valves started to form in both jaws as a single layer of epithelial cells at 6 dpf (6.8 to 7.4 mm TL), whereas they were completely formed at 11 dpf (11.2 to 11.6 mm TL). According to the histological slides, oral valves resembled to the crescentic type ones according to the nomenclature of Mitchell (1904), with rows of setiform papillae in their surface (Figure 3c and d).

At 57 hpf (5.2 mm TL), the first oesophageal goblet cells (50 to 75 μm in diameter) were detected scattered along the unfolded short oesophageal epithelium (1 goblet cell in 100 μm of epithelium). Although goblet cell size did not vary, goblet cell density increased rapidly and almost the entire anterior region of the oesophagus was covered by this type of secretory cells (2 to 3 goblet cells in 100 μm of epithelium) at 6 dpf (Figure 2b and c). Goblet cells stained mainly in purple and light blue when they were stained with PAS and AB pH 2.5, 1.0 and 0.5, indicating their content in neutral and acidic (carboxylated and sulphated) mucins, respectively. Between 6 and 7 dpf, the oesophagus grew in length and two layers of circular and longitudinal muscle fibres were clearly distinguishable forming part of the oesophageal mucosa, as well as a thin layer of connective tissue surrounding them. As the oesophagus grew in length, the oesophageal mucosa also grew in depth and folded, resulting in an increase in the height of epithelial lining the oesophagus, and a thickening of the circular and longitudinal layers composing the mucosa. In addition, goblet cell density increased and the entire oesophageal epithelium was covered by them (8 to 10 goblet cells in 100 μm of epithelium; 13 to 17 μm in diameter) at the age of 10 dpf (10.5 to 11.3 mm TL). The above-mentioned increase in goblet cell density was inversely related to their size. At latter stages of development, there were no further important histological changes in the oesophagus.

Between 2 and 3 dpf (57 to 72 hpf, 5.2 mm TL), a dilatation in the region that connected the oesophagus with the anterior intestine indicated where the future stomach would develop in *P. punctifer* at later stages. At 5 dpf (6.5 to 6.8 mm TL), a mucosal fold separating the oesophagus (cubic epithelium) from the region of the anterior intestine (columnar epithelium) was visible in that point. At this age, this region of the intestine dramatically dilated in order to accumulate the ingested preys (Figure 2d and e). First gastric glands started to differentiate at 8 dpf (8.4 mm TL), 4 days later than the onset of exogenous feeding, as clusters of undifferentiated cubic cells close to the mucosal fold that separated the oesophagus from the anterior intestine (future cardiac stomach) (Figure 2f). These clusters of cuboidal cells would develop into gastric glands arranged along numerous longitudinal folds and surrounded by a thin layer of circular musculature and connective tissue before 10 dpf. At 15 dpf (15.0 to 15.8 mm TL), the pyloric sphincter, which started to differentiate at 81 hpf, achieved its definitive histological organization. This sphincter developed at the posterior end of the stomach, separating this region from the anterior intestine and was formed by thickened layers of connective tissue and circular bundles of smooth muscle cells surrounding the digestive epithelium. In parallel, the stomach became differentiated into three different regions: the cardiac (anterior), fundic and pyloric (posterior) portions surrounded by a thin submucosa with connective fibres and a thick layer of circular muscle fibres (Figure 5a and b). The cardiac region was short with several longitudinal mucosal folds lined by a simple short, ciliated columnar epithelium with basal nuclei. The fundic region occupied most part of the pouch-shaped stomach. This part was lined by a simple tall, ciliated columnar epithelium and contained a large number of simple

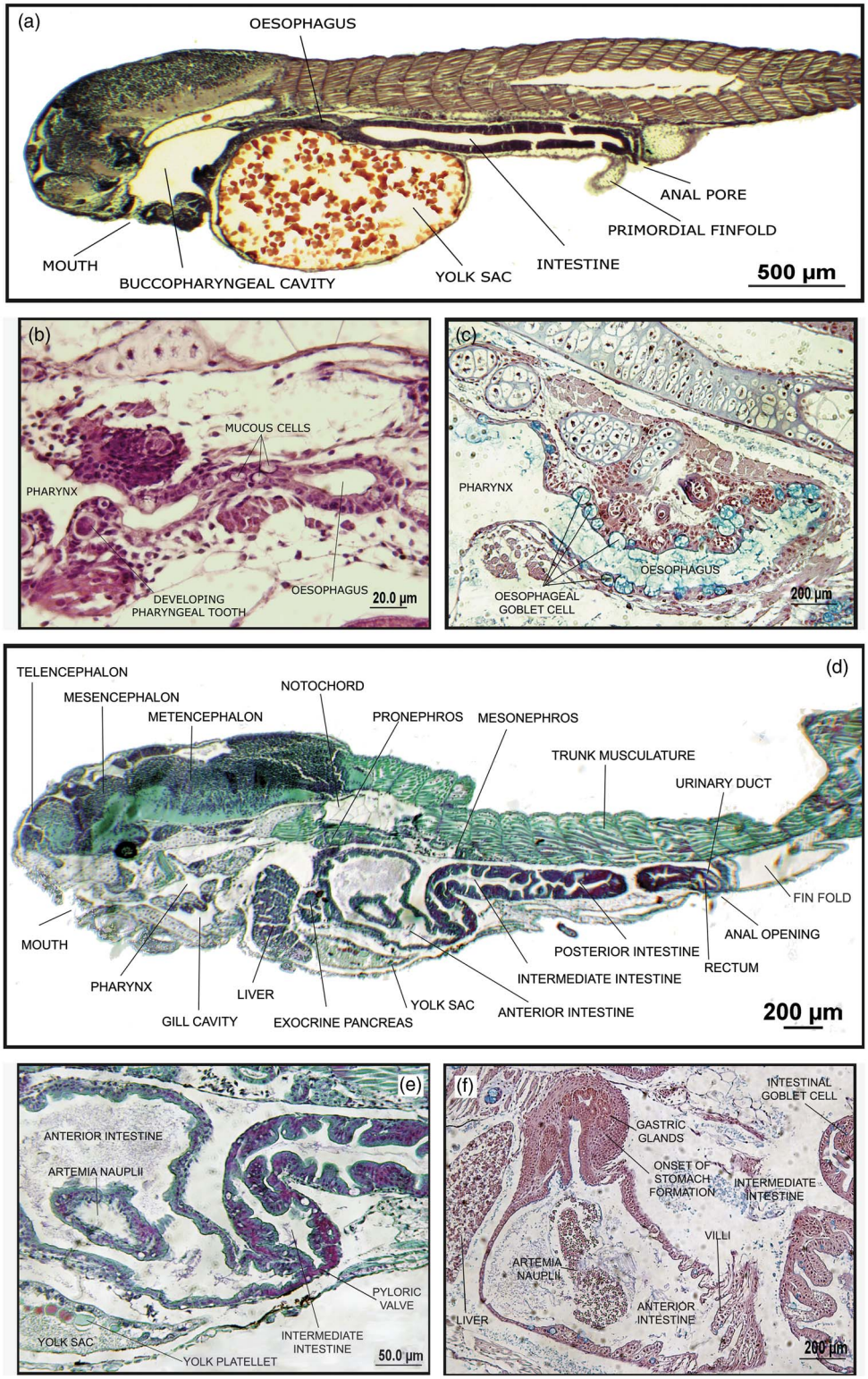

Figure 2 Histological sections of the early development of the digestive system in *Pseudoplatystoma punctifer*. (a) General view of a larva at 33 h post-fertilization (hpf) showing the mouth and anus opened, a short buccopharyngeal cavity, an oesophagus in differentiation and a rectilinear undifferentiated intestine. Note the large size of the yolk sac filled with yolk platelets and lipid droplet (unstained regions). Stain: hematoxylin–eosin (HE). (b) Detail of the first mucous cells appearing in the anterior region of the oesophagus close to the pharynx in a larva aged 57 hpf. Stain: HE. (c) Development of oesophageal mucous cells containing a mixture of acidic mucins in a larva aged 7 days post-fertilization (dpf). Stain: Alcian Blue (AB) pH 2.5, counterstained with hematoxylin. (d) General view of a 7 dpf larva showing the formation of the intestinal loop, the apparition of the spiral valve and the presence of yolk remnants ventrally to the anterior intestine. Stain: trichromic VOF (light green, orange g and acid fucshin). (e) Detail of the anterior intestine of a 7 dpf larva. Note the presence of a partially digested *Artemia nauplius*. Stain: trichromic VOF. (f) Detail of the anterior intestine in a larva aged 8 dpf showing the formation of first gastric glands. Staining: AB pH 2.5, counterstained with hematoxylin.

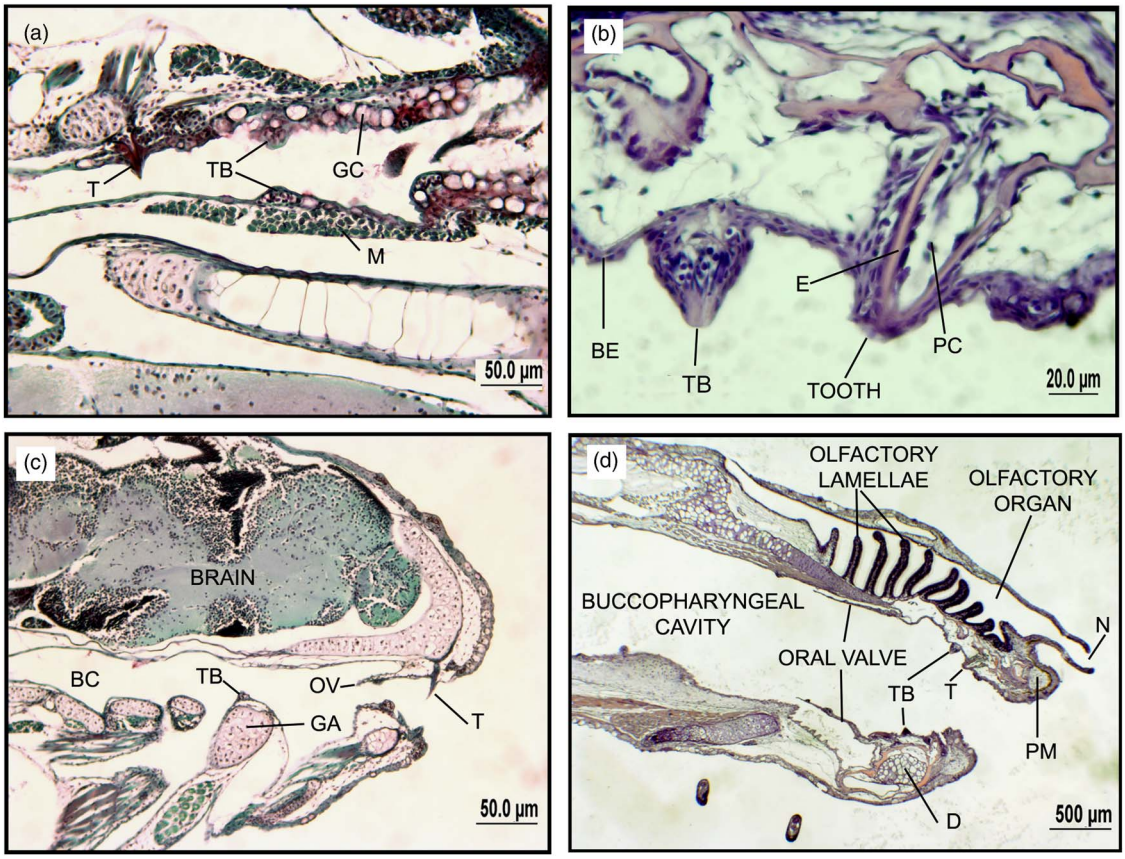

Figure 3 General view of the buccopharyngeal cavity in *Pseudoplatystoma punctifer*. (a) Detail of the posterior region of the pharynx in contact with the anterior intestine. Note the presence of a pharyngeal canine-like tooth protruding into the pharyngeal lumen and taste buds close to the transition to the oesophagus covered by round mucous cells. Stain: trichromic VOF (light green, orange g and acid fucshin). (b) Detail of a taste bud and tooth in the pharynx of a larva aged 6 dpf. Note the flat epithelium covering the pharyngeal cavity (BE). Stain: hematoxylin–eosin (HE). (c) General view of the short head of a larva aged 6 days post-fertilization (dpf). Stain: trichromic VOF. (d) General view of a streamline-shaped head in a larva aged 12 dpf. Note the large size of oral valves and large development of the olfactory organ. Stain: HE. BC = buccopharyngeal cavity; D = dentary bone; E = enamel; GA = gill arch; GC = goblet cell; M = muscular layer; N = nare; OV = oral valve; PC = pulp cavity; PM = premaxillar; T = tooth; TB = taste bud.

tubular gastric glands surrounded by a thin layer of connective tissue. Mucin-producing cells (PAS-positive) were found along the epithelium lining the fundic region of the stomach, as soon as first gastric glands were detected in the gastric mucosa. The pyloric region of the stomach was relatively short, devoid of gastric glands and lined by a short ciliated columnar epithelium.

The intestine appeared as a rectilinear undifferentiated tube lined by a simple layer of a columnar epithelium until the age of 57 hpf (Figure 2a), when first villi were observed in the posterior intestine. At this age, the anterior intestine showed no signs of mucosal folding and the rectum was already visible. Goblet cells did not appear in the intestine until 81 hpf and they were more abundant in the posterior and mid intestine (2.5 to 3.0 goblet cells in 100 µm of epithelium) than in its anterior region (0.5 to 1.0 goblet cell in 100 µm of epithelium). When first goblet cells were observed, they were mainly stained with AB pH 2.5, 1.0 and 0.5, which indicated their content in acidic (carboxylated and sulphated) mucins. Goblet cells were also positively stained with PAS (neutral mucins) between 1 and 2 days later (4 to 5 dpf) (Figure 4e and f). The intestine was rectilinear from hatching to 7 dpf when an intestinal loop was formed to accommodate the increasing length of the intestine inside the abdominal cavity (Figure 2d). At this age, the intestinal mucosa was mostly rectilinear with several short folds (30 to 40 µm in height) and first signs of fat accumulation (lipid droplets) in the intermediate and posterior intestinal regions were detected (Figure 4a and b). Intestinal lipid accumulation was observed until the end of the study, although examined animals at 41 dpf showed a large deposition of lipid droplets (7 to 9 µm in diameter). The level of folding of the intestinal mucosa increased along larval development, as well as the size of intestinal folds and the number of goblet cells. In this sense, intestinal folds were longer (325 to 500 µm in height at 41 dpf, 58.1 mm TL) in the anterior intestine in comparison to those from the mid and posterior intestinal regions (300 to 400 µm in height at 41 dpf), and goblet cells were more abundant in mid and posterior intestine (3.5 to 4.5 goblet cells in 100 µm of epithelium) than in the anterior intestinal mucosa (1.5 to 2.0 goblet cells in 10 µm of epithelium) (Figure 4c). No differences in the histochemical properties of mucin content in intestinal goblet cells were detected among different intestinal regions.

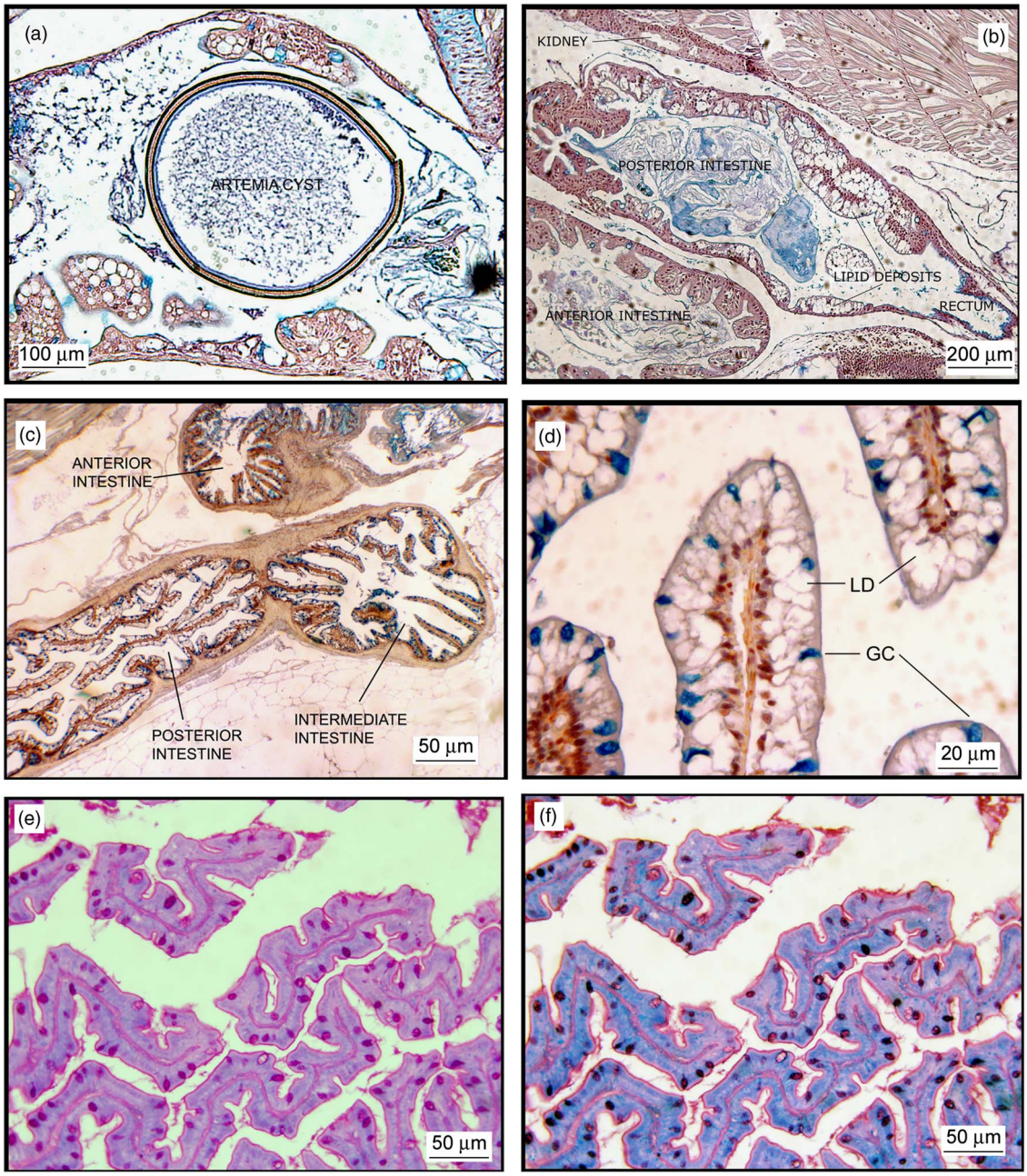

Figure 4 Different histological sections of the intestine in *Pseudoplatystoma punctifer*. (a) Detail of the mid intestine in a 7 days post-fertilization (dpf) larva with an undigested *Artemia* cyst. Note the small size of the villi and the presence of small lipid droplets inside enterocytes (unstained vacuoles). Stain: Alcian Blue (AB) pH 2.5, counterstained with hematoxylin. (b) General view of different regions of the intestine in a larva aged 11 dpf showing the abundant lipid deposits in the posterior intestine. Stain: AB pH 2.5, counterstained with hematoxylin. (c) Detail of the mid and posterior intestine in a larva aged 13 dpf showing the abundant presence of goblet cells containing acidic mucins. Note the higher density in the mid and posterior intestinal regions with regards to the anterior one. Stain: AB pH 2.5, counterstained with hematoxylin. (d) Detail of an intestinal villi showing large lipid deposits and goblet cells containing sulphated acid mucins (AB pH 1.0). Staining: AB pH 1.0, counterstained with hematoxylin. (e) General view of the posterior intestine in a specimen aged 18 dpf showing the large number of goblet cells containing neutral mucins (magenta). Stain: Periodic Acid Schiff (PAS). (f) General view of the posterior intestine in a specimen aged 18 dpf showing the large number of goblet cells containing a mixture of neutral and acidic mucins (dark purple). Stain: PAS and AB pH 2.5.

The liver started to differentiate as a cluster of basophilic undifferentiated cells located anteriorly to the yolk sac at 20 to 33 hpf. During the endogenous feeding phase, hepatocytes become spherical with centrally located basophilic nuclei and slight eosinophilic homogeneous cytoplasm, achieving a polyhedral shape as the larva developed. After the onset of exogenous feeding, hepatocytes increased in size and number, and were tightly packed between sinusoids, often around a central vein. As larval development proceeded, nutrient inclusions increased in the hepatic tissue. In this sense, between 6 and 7 dpf, the first signs of fat accumulation were observed in the liver of larvae; the presence of fat deposits

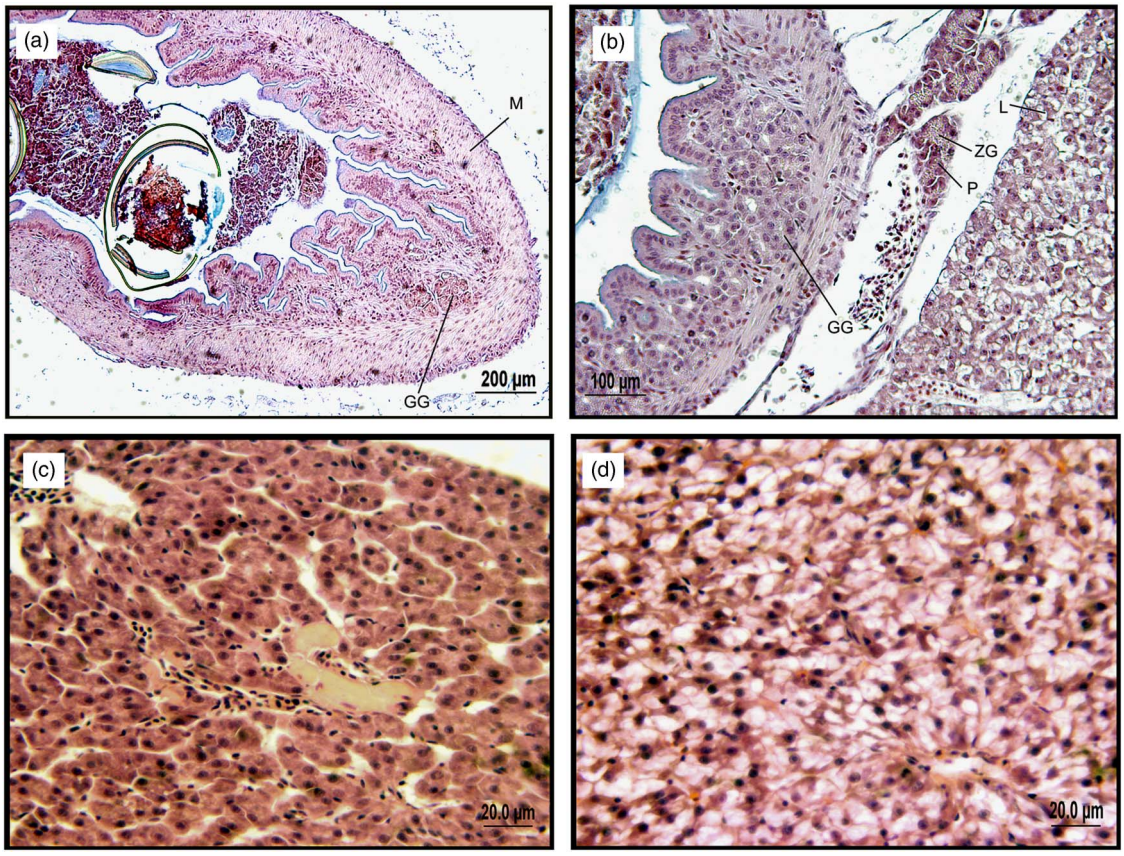

Figure 5 General view of the stomach formation in the anterior region of the digestive tract in a larva aged 10 days post-fertilization (dpf) (a). Note the formation of gastric glands arranged in clusters, as well as the development of a prominent layer of musculature surrounding the stomach. Stain: Alcian Blue (AB) pH 2.5, counterstained with hematoxylin. (b) Detail of the stomach, liver and exocrine pancreas in a larva aged 15 dpf. Stain: AB pH 2.5, counterstained with hematoxylin. (c) Detail of the liver in an early juvenile aged 41 dpf showing no lipid deposits within hepatocytes. Note the presence of centrally located nuclei within hepatocytes and the presence of an eosinophilic cytoplasm. Stain: hematoxylin–eosin (HE). (d) Detail of the liver in an early juvenile aged 41 dpf showing a large accumulation of lipids within hepatocytes denoting the large variability in fat accumulation among specimens of the same stage of development. Note the displacement of the nuclei towards the periphery of the hepatocyte and the large size of lipid vacuoles (unstained) within hepatic cells. Stain: HE. GG = gastric gland; L = liver; M = muscular layer; P = exocrine pancreas; ZG = zymogen granules.

within hepatocytes were concomitant with an increase in the accumulation of lipidic supranuclear vacuoles in the mid and posterior intestine. The position of the nucleus in the hepatocyte depended on the degree of accumulation of lipid reserves in the cytoplasm, as the higher content of fat deposits in the hepatocyte, the more peripheral disposition of the nucleus within the cell. Large and central nuclei were observed in livers containing few lipid inclusions at younger ages (7 to 10 dpf), while peripheral nuclei were detected in livers of larvae showing high levels of lipid deposition (12 to 41 dpf). However, the levels of fat accumulation greatly varied among specimens of the same age and size (Figure 5c and d).

At 57 hpf, the exocrine pancreas was already differentiated and contained zymogen acidophilic granules. The pancreas was organized in polyhedral basophilic cells arranged in acini grouped in rosette patterns, containing round-shaped eosinophilic and PAS-positive eosinophilic zymogen granules. At 81 hpf, the PAS-positive staining intensity of zymogen granules contained in acinar cells increased, denoting an increase in the synthesis of the precursors of digestive pancreatic enzymes. The quantitative growth after differentiation of the endocrine and exocrine pancreas included an increase in tissue size, as well as an increase in the content of zymogen granules, while no new structural elements developed at latter stages.

Discussion

Among freshwater species, catfishes are one of the groups that have received most attention regarding the histological development of their digestive system (Gisbert *et al.*, 2013), which may probably be linked to their fast growth potential, good quality meat and adaptability to culture conditions. Different species of catfish belonging to the *Siluridae* (*Ompok bimaculatus*, *Silurus glanis*), *Claridae* (*Clarias gariepinus*, *Clarias nieuhofii*), *Bagridae* (*Pelteobagrus fulvidraco*) and *Heptapteridae* (*Rhamdia quelen*) families have been described so far (Table 2; Verreth *et al.*, 1992; Kozarić *et al.*, 2008; de Amorim *et al.*, 2009; Yang *et al.*, 2010; Saelee *et al.*, 2011; Pradhan *et al.*, 2012), whereas this is the first study on a

Table 2 *Comparison of major developmental events of the digestive system ontogeny in different Siluriform species*

	Pseudoplatystoma punctifer[1] *Pimelodidae*		*Ompok bimaculatus*[2] *Siluridae*		*Silurus glanis*[3] *Siluridae*		*Pelteobagrus fulvidraco*[4] *Bagridae*		*Clarias nieuhofii*[5] *Claridae*		*Clarias gariepinus*[6] *Claridae*		*Rhamdia quelen*[7] *Heptapteridae*	
Developmental events	ADD	DAH	ADD	DAH	ADD	DAH	ADD	DAH	ADD	DAH	ADD	DAH	ADD	DAH
Appearance of intestine	28 to 42	1 to 2	27	1	69	3	24	1	53	2	27.5	1	na	na
Appearance of incipient liver and pancreas	14 to 28	0 to 1	27	1	69 to 115	3 to 5	48	2	40 to 53	1.5 to 2	27.5	1	na	na
Appearance of zymogen granules in the pancreas	42	1 to 2	27	1	69 to 115	3 to 5	96	4	na	na	55	2	na	na
Mouth opening	14 to 28	0 to 1	27 to 54	1 to 2	na	na	24 to 48	1 to 2	53	2	55	2	96	4
Onset of exogenous feeding	112	3	54	2	92	4	72	3	106	4	55	2	120	5
Intestine differentiation	112	3	54	2	92 to 138	4 to 6	72	3	106	4	27	1	72	3
Duration of mixed nutritional period	54	2	56	2	69	3	0	0	79	3	5	0 to 1	na	na
Oesophagus differentiation	140	5	54 to 81	2 to 3	92 to 138	4 to 6	96	4	106	2	na	na	na	na
Vacuolization of hepatocyte cytoplasm	140 to 168	5 to 6	81 to 108	3 to 4	115 to 161	5 to 7	264 to 312	11 to 13	53 to 265	2 to 10	na	na	na	na
Appearance of lipid droplets in the intestine	140	5	108	4	207	9	72	3	106	4	82.5	3	na	na
Yolk-sac exhaustion	168	6	135	5	161	7	na	na	185	7	60	2	96	4
Gastric glands appearance	196	7	216	8	115 to 161	5 to 7	72	3	106	4	110 to 138	4 to 5	na	na
Morphologically complete digestive system	392	14	324	12	161	7	600	25	133	324	193	7	na	na
Average rearing temperature (°C)	28		27		23.5		24.0		26.5		27.5		24.0	

na = data not available.
For comparative purposes with the rest of the literature, larval development is shown in accumulated degree days (ADD) and days after hatching (DAH).
[1]Present study.
[2]Pradhan *et al.* (2013).
[3]Kozarić *et al.* (2008).
[4]Yang *et al.* (2010).
[5]Saelee *et al.* (2011).
[6]Verreth *et al.* (1992).
[7]de Amorim *et al.* (2009).

representation of the family *Pimelodidae*. Although the morphogenesis of the digestive tract and accessory glands in *P. punctifer* was similar to that of other catfish and freshwater species, several differences in the timing of organ differentiation and development were observed between *P. punctifer* and other members of the Siluriformes order, as shown in Table 2. In this context, authors have decided to focus this section on the ontogenetic changes in the histomorphological organization of digestive organs and their link to rearing practices that could be of use for improving actual larval rearing procedures in *P. punctifer*, as well as the interspecific comparison of the histological organization and development with other catfish species. As different studies on several Siluriform species (Table 2) were conducted at different rearing temperatures, data were compared by means of degree day units, whenever it was possible.

As an altricial species, the digestive system of *P. punctifer* was not developed at hatching, but as expected in a fast-growing Neotropical catfish species (Nuñez *et al*., 2011), larval development and transformation to juvenile occurred quickly (Table 2). Thus, the morphogenesis of the digestive tract from an undifferentiated canal at hatching to a complex and regionalized juvenile-like digestive tract in *P. punctifer* occurred in just 2 weeks at 28°C. During this 2-week period, larvae were fed *Artemia* and they showed a good growth performance and survival rate (95%). Therefore, the ontogeny of the digestive tract presented here was not affected by feeding conditions and can be considered as the standard histological development of the *P. punctifer* digestive system using live prey. The alimentary canal opened during the first hours after hatching (0 to 1 dpf) and the accessory digestive glands (liver and pancreas) were already formed. Zymogen granules (precursors of pancreatic digestive enzymes) were detected in the exocrine pancreas (1 to 2 dpf) before the onset of exogenous feeding. At the onset of exogenous feeding (4 dpf), fish had already a well-differentiated mouth with structures for capturing, seizing and tasting preys, a developed intestinal mucosa and differentiated accessory digestive glands for nutrient storage (liver) and enzyme production (pancreas). At this stage, histological data indicated that the digestive system was functional and able to digest food, as first signs of lipid accumulation in the anterior and intermediate regions of the intestine and liver were noticed soon after the onset of exogenous feeding and at the end of the mixed nutritional phase.

The transition from endogenous to exogenous feeding is a critical stage of larval development and may result in high mortality rates if food is not properly administered to larvae once their yolk-sac reserves are exhausted (Sarasquete *et al*., 1995). In most studied catfish species, with the exception of *R. quelen* (de Amorim *et al*., 2009), there is a mixed feeding phase during which an overlap of endogenous nutrition and exogenous feeding occurs. The length of this period varies among species, ranging from just 5 degree days in *C. gariepinus* (Verreth *et al*., 1992), 54 and 56 degree days in *O. bimaculatus* (Pradhan *et al*., 2012) and *P. punctifer* (present study), up to 69 and 79 degree days in *S. glanis* (Kozarić *et al*., 2008) and *C. nieuhofii* (Saelee *et al*., 2011). The above-mentioned differences may be linked to different larval and yolk-sac sizes (Kamler, 1992; Gisbert *et al*., 2000), but as well as different rearing conditions that might have affected the rate of yolk consumption and the onset of exogenous feeding. This period of mixed nutrition is of relevance with regard to larval performance as it may neutralize any potential deficit in nutrient provision before completion of yolk reserves, as well as serve as a temporary reserve of nutrients for the larva to withstand short periods of food deprivation (Treviño *et al*., 2011). The presence of a mixed nutrition stage might be an advantage for rearing *P. punctifer* larvae in ponds, as it would guarantee the successful transition to exogenous feeding of larvae or minimize the potential negative effects of a delayed time of first feeding (Gisbert and Williot, 1997). The transition to exogenous food in the presence of yolk reserves generally implies that the alimentary canal is functional, although structural and functional development still continues from the larval to the juvenile and adult forms (Jaroszewska and Dabrowski, 2011).

Although the stomach anlagen in *P. punctifer* appeared as a dilatation between the oesophagus and anterior intestine before the onset of exogenous feeding (2 to 3 dpf), gastric glands did not appear until 8 dpf, whereas the complete morphoanatomical differentiation of this organ (cardiac, fundic and pyloric regions) was not completely achieved until 15 dpf. The histochemical properties of mucous cells (PAS-positive: secretion of neutral mucins) lining the fundic gastric regions at 8 dpf may be considered as an indirect sign of stomach functionality (Gisbert *et al*., 2013), as neutral mucosubstances may protect the stomach from autodigestion processes caused by HCl and enzymes produced by gastric glands (Chen *et al*., 2006). The appearance of gastric glands normally indicates the formation of a functional stomach (Stroband and Kroon, 1981), which is also a histological criterion to differentiate larvae from juveniles (Sarasquete *et al*., 1995). Similar results were reported for most part of Siluriformes described so far (Verreth *et al*., 1992; Kozarić *et al*., 2008; Yang *et al*., 2010; Saelee *et al*., 2011; Pradhan *et al*., 2012), although species-specific differences in terms of stomach morphogenesis and the putative transition from alkaline to acid digestion were observed among species (Table 2). These results indicated that *P. punctifer* might be weaned onto microdiets after 10 dpf, although further research on digestive system functionality (quantification of digestive enzyme activities) and weaning strategies are needed, as the physiology and morphogenesis of larval digestive tract might be stimulated or impaired, depending on how co-feeding is performed (Cahu and Zambonino-Infante, 2001; Pradhan *et al*., 2013). Besides, cannibalism appears around this transition from larval to juvenile stage, suggesting that nutritional needs might be changing and that feeding protocols could influence the incidence of such behaviour. Indeed, cannibalistic behaviour began to appear coinciding with the formation of the oral valves and the gastric glands of the stomach (from 11 dpf). This could indicate that *Artemia* might not be

completely covering the nutritional needs of *P. punctifer* larvae. At that time, oral valves are already equipped with taste buds believed to serve for screening the quality of food before it is passed onto the mouth cavity (Yashpal *et al.*, 2006; Gamal *et al.*, 2012). Moreover, the incidence of cannibalism clearly increased at weaning, especially at the end of the co-feeding, when the amount of *Artemia* offered decreased up to 75% of the ration. This cannibalistic behaviour persisted until larvae began to be fed with the second inert diet. Although the inert diet used to wean larvae allowed them to grow normally (Nuñez *et al.*, 2008) and that histological results showed that the digestive system at 18 dpf was ready to process inert diets, the correlation observed between cannibalism and the feeding protocol suggested that larvae fed the first inert diet were not fully exploiting their potential for growth. Whether the quality (i.e. texture) and composition of the diet could reduce such a behaviour needs to be evaluated. Besides, survival rate at the end of the third feeding phase using inert diets continued to decrease, although cannibalism seemed to be reduced. However, no dead individuals were observed in the tanks, they only disappeared. The reason for the lower incidence of cannibalism at this rearing period (33 to 41 dpf) could be that juveniles of *P. punctifer* were able to cannibalize bigger specimens and, together with the more efficient digestive machinery, their nutritional needs might be covered with a lower rate of cannibalism. This might indicate again that the inert diet used to feed *P. punctifer* juveniles during that period could be also inappropriate, as suggested below.

Digestive tissues and organs are particularly sensitive to non-optimal feeding conditions or nutritional stress during larval development, because they are under progressive and intensive morphogenesis, and consequently, they respond rapidly and sensitively to nutritional disorders (Gisbert *et al.*, 2008). In this sense, changes in the histological organization of the liver or the intestine have been used as histological targets to analyse the nutritional condition of fish larvae and elucidate the effects of different dietary regimes or nutrients on larvae (Papadakis *et al.*, 2009; Boglino *et al.*, 2012; Pradhan *et al.*, 2013). In this study, the accumulation of lipids in the intestinal mucosa soon after the onset of exogenous feeding might be interpreted as an indicator of luminal digestion and absorption, and temporal storage of lipids, reflecting the functional development of the intestine (Gisbert *et al.*, 2008). In addition, the moderate accumulation of lipids in the intestine was positively correlated with changes in the degree of lipid deposits in the liver along most part of the studied period, which indicated that the lipid content of feed did not exceed the fatty acid absorption and exporting capacities of enterocytes, whereas the large accumulation of lipid deposits (droplets) in the intestine and liver at 41 dpf might be attributed to a change in the early juvenile capacity to absorb and export lipids through the circulatory system towards the liver to be stored and mobilized for growth when needed (Tso, 1994) and/or a nutritional imbalance with regard to protein and lipid content of the administered inert diet. In any case, the accumulation of lipids in the intestine or liver did not result in a potential pathological situation that might have affected cell functionality and ultimately the larval performance, as no signs of epithelial abrasion, cellular necrosis and/or inflammatory reactions were detected as a consequence of large lipid deposits (Gisbert *et al.*, 2008). However, these results seemed to indicate that inert diets for *P. punctifer* early juveniles might be refined in order to match the specific nutritional requirements of the species (e.g. dietary protein : lipid levels) and improve fingerling performance.

In conclusion, the ontogeny of the digestive system of *P. punctifer* followed the same general pattern that most Siluriform and other teleost species described to date, although species-specific differences regarding tissue and organ development were noted among species. Findings on the development of the digestive system in *P. punctifer* coupled with those on its functionality could lead to a better understanding of the digestive physiology of this fast-growing Neotropical species. These results on the organogenesis of larvae are a useful tool for establishing the functional capabilities and physiological requirements of larvae to ensure optimal welfare and growth under aquaculture conditions, which might be useful for improving current larval rearing practices for this species. Future research must be focused on the ontogeny of enzymatic secretions to provide precise information about the functionality of the digestive tract and to evaluate the effect of different feeding and weaning strategies on digestive tract maturation.

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Influence of dietary protein and lipid levels on growth performance and the incidence of cannibalism in *Pseudoplatystoma punctifer* (Castelnau, 1855) larvae and early juveniles

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Resumen

El objetivo del estudio fue evaluar la influencia de diferentes niveles y proporciones de proteínas y lípidos en la dieta en el crecimiento larvario, la supervivencia y la incidencia de canibalismo en *Pseudoplatystoma punctifer*. Las larvas fueron cultivadas en un sistema de recirculación de 3 a 26 días post-fertilización (dpf) (2 a 25 días post-eclosión, dph) a una densidad inicial de 40 larvas L^{-1}, 27,8 ± 0,65 °C y un fotoperiodo de 0L:24O. Las larvas fueron alimentadas de 4 a 12 dpf con nauplios de *Artemia* y fueron destetadas a 13 dpf durante 3 días con cuatro dietas balanceadas diferentes y luego alimentadas exclusivamente con estas dietas hasta 26 dpf. Estas dietas contenían niveles de proteínas:lípidos (P:L) (en % de materia seca) de 30:15, 30:10, 45:15 o 45:10. Un grupo de control fue alimentado con nauplios de *Artemia* hasta los 17 dpf y luego fue destetado con la dieta balanceada 45:10. El experimento se llevó a cabo por triplicado. Los resultados mostraron una mayor tasa de crecimiento y supervivencia y una menor incidencia de canibalismo en el grupo alimentado con la dieta 45:15 que en los otros tratamientos. Las diferencias en la supervivencia de las larvas y el rendimiento del crecimiento se asociaron con un mayor contenido de proteínas y lípidos en lugar de la proporción proteína:lípidos de esta dieta. Al comparar dietas con el mismo nivel de proteína, el aumento de lípidos en la dieta condujo a una mejora en el crecimiento, lo que sugiere que la energía de los lípidos ahorra proteínas para el crecimiento en los juveniles de *P. punctifer*. Un período de alimentación de *Artemia* superior a 12 dpf no mejoró el crecimiento o la supervivencia de las larvas.

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Influence of dietary protein and lipid levels on growth performance and the incidence of cannibalism in *Pseudoplatystoma punctifer* (Castelnau, 1855) larvae and early juveniles

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Summary

The aim of the study was to evaluate the influence of different dietary protein and lipid levels and their ratios on larval growth, survival and the incidence of cannibalism in *Pseudoplatystoma punctifer*. Larvae were raised in a recirculation system from 3 to 26 days post-fertilization (dpf) (2–25 days post hatching, dph) at an initial density of 40 larvae L^{-1}, 27.8 ± 0.65°C and 0L : 24D photoperiod. Larvae were fed from 4 to 12 dpf with *Artemia* nauplii and weaned onto four different compound diets from 13 dpf within 3 days, then fed exclusively with these diets until 26 dpf. These diets contained 30 : 15, 30 : 10, 45 : 15 or 45 : 10 protein : lipid (P : L) (in % of dry matter) levels. A control group was fed *Artemia* nauplii until 17 dpf and weaned thereafter with the 45P : 10L compound diet. The experiment was carried out in triplicate. Results showed higher growth and survival rates and lower incidence of cannibalism in the group fed the 45P : 15L diet than in the other treatments. Differences in larval survival and growth performance were associated with the higher protein and lipid content rather than the protein : lipid ratio of this diet. When comparing diets with the same protein level, the increase in dietary lipid led to an improvement in growth, suggesting that energy from lipids spares protein for growth in *P. punctifer* fingerlings. An *Artemia* feeding period longer than 12 dpf did not improve larval growth or survival.

Introduction

Fish represent one of the main sources of protein for human consumption in the Peruvian Amazonia; the rapid demographic growth during the last decades has led to an increased exploitation of fisheries resources (Garcia et al., 2009). In this context, the development of a sustainable aquaculture has become essential to satisfy increasing demands. *Pseudoplatystoma punctifer* is a catfish species that suffers from high fishing pressures but has been considered to have high potential for aquaculture diversification in South America for almost 20 years (Kossowski, 1996). However, although research efforts have been made to control the complete life cycle in captivity (Padilla et al., 2001; Nuñez et al., 2008; Baras et al., 2011; Núñez et al., 2011), low survival at the end of the larval and juvenile stages continues to be the principal hindrance in its culture. Mortality is attributed to the high incidence of cannibalism and the low acceptability of compound diets at weaning (Baras et al., 2011; Núñez et al., 2011; Gisbert et al., 2014). However, there is no information on the nutritional needs of this species during the early life stages.

Establishing an adequate feeding protocol adapted to the digestive capacities and nutritional needs during early development while also addressing options to reduce cannibalism is of primary importance to improve survival and growth. We have already described the ontogeny of the digestive system of *P. punctifer* in order to synchronize the stage of development and maturation of their digestive organs with the feeding protocol and rearing practices (Gisbert et al., 2014). We found a clear correlation between the feeding protocol and the incidence of cannibalism, suggesting that improving feeding strategies and nutrition could reduce cannibalistic behaviour. In this context, the first signs of cannibalism were observed at 11 dpf (10 days post-hatching, dph) and coincided with the formation of the gastric glands of the stomach and the oral valves, fully equipped with taste buds for screening the quality of food, suggesting that *Artemia* might not be covering the nutritional needs of the larvae (Gisbert et al., 2014). Moreover, the incidence of cannibalism clearly increased at weaning, especially at the end of co-feeding, also showing the nutritional inadequacy of the diet used in that study. These results highlighted the need to study the nutritional requirements of larvae and juveniles as this may reduce cannibalism.

During the larval and early juvenile stages, proteins constitute the main macronutrient for growth (Rønnestad et al.,

1999) and lipids represent the main energy source during morphogenesis (Sargent et al., 1999). There is little information on nutritional requirements of *Pseudoplatystoma* species, and most refers to the juvenile stage (Martino et al., 2002a, b; Lundstedt et al., 2004; Campos et al., 2006; Arslan et al., 2009, 2013; Bicudo et al., 2012; Silva, 2013; Cornélio et al., 2014; Gonçalves, 2014). The goal of the present study was to evaluate the influence of different dietary lipid and protein levels and their ratios on growth, survival and the incidence of cannibalism of *P. punctifer*. Since carbohydrates were used to obtain the desired dietary protein : lipid proportions, the variation of carbohydrate content was also considered in the interpretation of the results.

Materials and methods

Spawning and larval and early juvenile rearing

Larvae were obtained by hormonally-induced spawning of a sexually mature pair of *P. punctifer* (♀: 3.6 kg; ♂: 1.85 kg body weight, BW) from a broodstock maintained in captivity at the Instituto de Investigaciones de la Amazonía Peruana (IIAP, Iquitos, Peru). A pair of mature females and males was transferred from the pond to a 500-L indoor tank where they were kept at 27.5 ± 0.5°C and 12L : 12D photoperiod during the induction process. Females and males were injected intramuscularly with Carp Pituitary Extract (Argent Chemical Laboratories, Inc., Redmond, WA) at 5 mg kg^{-1} and 1 mg kg^{-1} BW, respectively. Hormone injections were administered in one dose for the male and two doses for the female: the first at 10% of the total dose and the second 12 h later at 90% of the total dose. Stripping of the female, sperm collection and the fertilization procedure were performed following the protocol described by Nuñez et al. (2008). Spawned eggs (fertilization rate = 99.9%) were incubated at 27.7 ± 0.6°C in four 60-L tanks (50-L water volume; temperature measured daily at 07.00 h in each tank) connected to a clear water recirculating system, whereby hatching occurred 18 ± 2 h later (hatching rate = 96%). Larvae were transferred at 4 days post-fertilization – dpf (3 days post-hatching – dph; 5.6 ± 0.7 mm total length – TL, n = 30) into 40-L tanks (30-L water volume) connected to a water recirculation system provided with mechanical and biological filters. Water conditions throughout the experiment were: 27.8 ± 0.7°C, pH 7.0 ± 0.5, dissolved oxygen 7.4 ± 0.2 mg L^{-1}, $N–NO_2$ 0.38 ± 0.27 mg L^{-1}, $N–NH_4$ 0.26 ± 0.13 mg L^{-1}. Water temperature, pH and dissolved oxygen were measured daily and $N\text{-}NO_2$ and $N\text{-}NH_4$ weekly at 07.00 h in six tanks. Water supply was adjusted in each tank to assure a water flow rate of 0.2 L min^{-1}. In this study we considered the previously used feeding protocol for *P. punctifer* (Gisbert et al., 2014) for the control group, in which weaning took place at 18 dpf (17 dph at 28°C). However, we decided to advance the weaning age for the remainder of the treatments to 13 dpf in order to coincide with the gastric gland formation of the stomach (Gisbert et al., 2014) and improve the use of the experimental compound diets. Thus, larvae were reared in triplicate (initial density = 40 larvae L^{-1}, n = 1200 larvae per replicate) from 4 to 26 dpf under 0L : 24D photoperiod (<0.001Lx at the water surface) and fed five times per day with *Artemia* spp. nauplii in slight excess from 4 to 17 dpf (0.6–12.2 nauplii ml^{-1}) in the control group and from 4 to 12 dpf in the other treatments (0.6–9 nauplii ml^{-1}) considering larval density, weight increase and the daily food ration (Baras et al., 2011). At 13 dpf, larvae were weaned within 3 days onto four compound diets containing different protein and lipid levels (Fig. 1), the control group onto the 45 : 10 protein : lipid (P : L) diet from 17 dpf, and all treatments fed up to 26 dpf exclusively with these diets.

Proximate composition and lipid class analyses

Four experimental diets were prepared at the Ifremer (Plouzané, France) as described in Cahu et al. (2003) including different percentages of proteins and lipids in their formulation (Table 1). Total lipids of the compound diets were extracted in chloroform : methanol (2 : 1, v : v) according to the method of Folch et al. (1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow

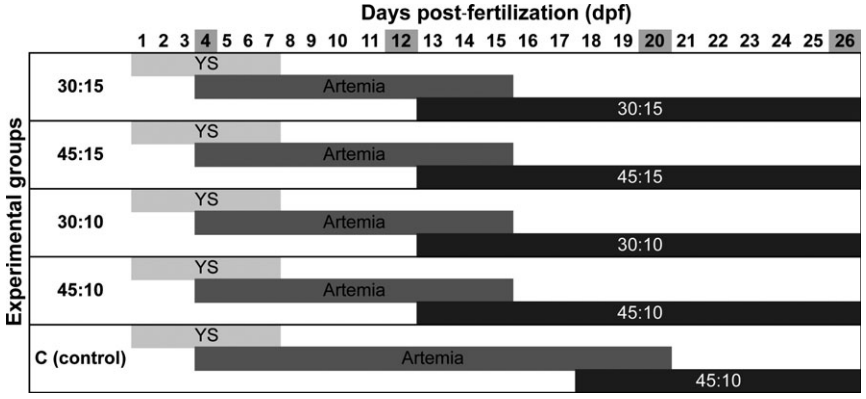

Fig. 1. Experimental nutrition design, larval and early juvenile stage, *Pseudoplatystoma punctifer*. Four experimental groups were weaned from 13 days post fertilization (dpf) within 3 days with compound diets containing different levels of proteins and lipids. Dietary treatment code corresponds to protein : lipid (P : L) level included in tested diets. The control group was weaned onto 45P : 10L compound diet at 18 dpf. YS, yolk-sac stage. Days marked in grey = sampling points.

followed by vacuum desiccation overnight. Analysis of lipid classes was performed according to Olsen and Henderson (1989). Protein and carbohydrate contents were determined following the Lowry et al. (1951) and Dubois et al. (1956) methods, respectively.

Larval and early juvenile performance

Groups of larvae and early juveniles were sampled from each tank at 4 (n = 30), 12 (n = 15), 20 (n = 15) and 26 (n = 15) dpf and anaesthetized using Eugenol (0.05 μl ml^{-1}; Moyco®, Moyco, Lima, Peru) for growth measurements. Individual wet weight (WW) was determined using an analytic microbalance (Sartorius BP 211 D, Data Weighing Systems, Inc., Elk Grove, IL, ±0.01 mg). Specific growth rate (SGR, in % day^{-1}) was calculated as SGR = (ln WW_f − ln WW_i)/(t_f − t_i) x 100; where WW_f, WW_i, t_f and t_i represented final and initial WW and time of the experiment, respectively. For total length (TL) measurements, specimens were placed on a Petri dish and photographed using a scale bar. TL was measured on the pictures using ImageJ software (Rasband, 1997–2012).

The number of cannibals was counted in each tank twice a day (08.00 h and 17.00 h) and the incidence of cannibalism expressed as the percentage of fish displaying cannibalistic behaviour at each feeding period. Two types of cannibalism were recorded: type I, when larvae were partially damaged (pectoral fins and/or stomach bitten), and type II, when individuals were completely ingested by their siblings (Fig. 2). Survival was evaluated by counting the individuals surviving at 12 and 26 dpf with respect to the number of individuals at the beginning of each feeding period and calculated considering the number of individuals sampled at each feeding period.

Table 1
Composition of experimental diets. Dietary treatment code corresponds to protein : lipid level included in tested diets. DM, dry matter

Dietary treatments	30 : 15	30 : 10	45 : 15	45 : 10
Ingredients[1] (in % DM)				
Fishmeal	36	36	53	53
Hydrolysed fishmeal (CPSP)	9	9	14	14
Lipids	14	8	12	7
Marine lecithin	3	8	3	7
Soybean lecithin	11	0	9	0
Gelatin	15	15	15	15
Wheat starch	20	26	0	5
Vitamin mix[2] (×4)	2	2	2	2
Mineral mix[3]	3	3	3	3
Betain	1	1	1	1
Analyses of the diets (% DM)				
Proteins	30.07	30.90	43.13	42.86
Total lipids	12.50	7.43	12.46	10.36
Neutral lipids	6.99	5.42	7.29	6.93
Phospholipids	6.35	2.20	4.80	2.60
Carbohydrates	24.87	31.31	2.34	7.58
Moisture	18.89	22.35	17.48	15.25

[1]All dietary ingredients obtained commercially. Fishmeal hydrolysate CPSP 90 : 10% lipids; Soluble Fish Protein Concentrate (SopropÊche, Boulogne sur Mer, France); soy lecithin (Ets Louis François, St Maur des Fossés, France); marine lecithin LC 60 (Phosphotech, St Herblain, France).
[2]Composition per kilogram of vitamin mixture: choline chloride 60%, 333 g; vitamin A acetate, (4000 IU g^{-1}) 2 g; vit. D_3 (1920 IU g^{-1}) 0.96 g; vit. E (40 IU g^{-1}) 20 g; vit. B_3 2 g, vit. B_5 4 g; vit. B_1 200 mg; vit. B_2 80%, 1 g; vit. B_6 600 mg; vit. B_9 80%, 250 mg; vit. concentrate B_{12} (10 g kg^{-1}), 0.2 g; biotin, 1.5 g; vit. K_3 51%, 3.92 g; meso-inositol 60 g; cellulose, 543.3 g.
[3]Composition per kilogram of mineral mixture: 90 g KCl, 40 mg KIO_3, 500 g $CaHPO_4$ $2H_2O$, 40 g NaCl, 3 g $CuSO_4$ $5H_2O$, 4 g $ZnSO_4$ $7H_2O$, 20 mg $CoSO_4$ $7H_2O$, 20 g $FeSO_4$ $7H_2O$, 3 g $MnSO_4$ H_2O, 215 g $CaCO_3$, 124 g $MgSO_4$ $7H_2O$, and 1 g NaF.

Statistical analysis

Results were expressed as mean ± SD. Statistical tests were conducted using SIGMASTAT 3.0 (Systat Software Inc., Richmond, VA). All data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett's test) and evaluated by one-way ANOVA followed by

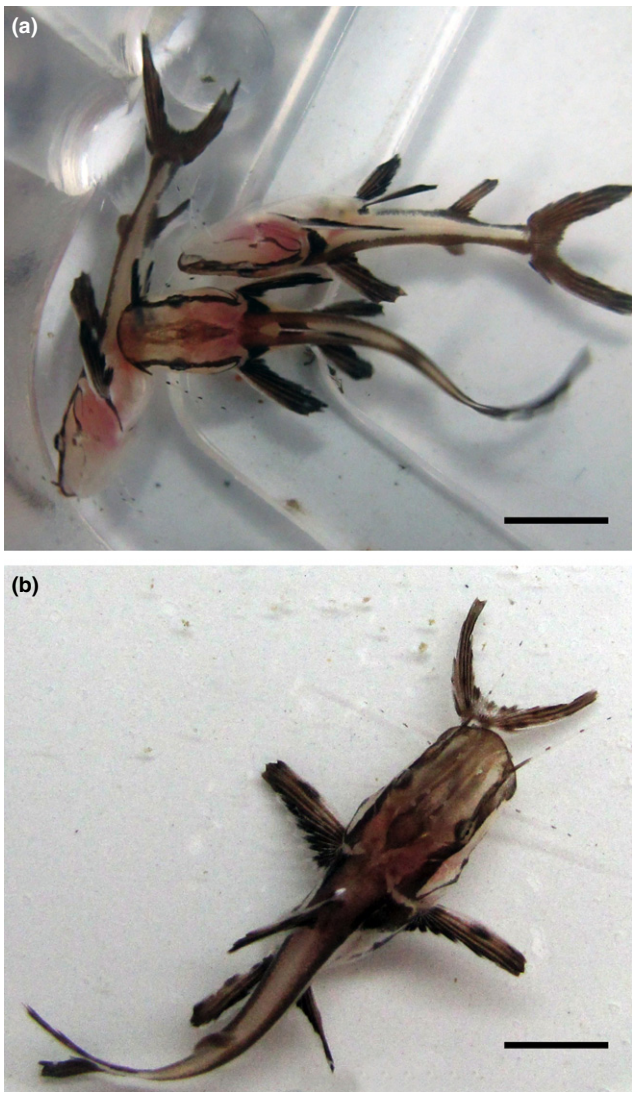

Fig. 2. Images showing type I (a) and type II (b) cannibalism in reared *Pseudoplatystoma punctifer* juveniles. Scale bar = 5 mm.

the Holm–Sidak method for all pairwise comparisons ($P < 0.05$). Data were previously arcsine-transformed for survival and cannibalism variables. The incidence of type I and type II cannibalism within the same dietary group was compared using a *t*-test ($P < 0.05$).

Results

Proximate composition and lipid class analyses

Analyses of proximate composition and lipid classes of the compound diets are shown in Tables 1 and 2, respectively. Diets were formulated for designing two feeds with high protein levels (about 45%) and two feeds with low protein levels (about 30%), crossed with high lipid levels (around 15%) or low lipid levels (around 10%). The four diets exhibited similar neutral lipid (NL) levels, when phospholipid (PL) was higher in diets with higher lipid levels. Phosphatidylcoline was the major component of PL and together with phosphatidylserine and phosphatidylinositol accounted for most of the differences. Triacilglicerids (TAG) were the most predominant NL, followed by cholesterol and free fatty acids. TAG levels were higher in the 45P : 15L and 45P : 10L diets (Table 2). Carbohydrates were added as wheat starch in order to maintain the four diets with a similar energy level, around 1350 KJ g^{-1} wet weight.

Growth performance and incidence of cannibalism

P. punctifer larvae from the five dietary treatments did not show significant differences in TL, WW and survival during the *Artemia* feeding phase, being on average 12.0 ± 0.13 mm TL (n = 45), 7.3 ± 0.55 mg WW (n = 45) and 87.5 ± 6.6% survival (initial n = 3600) ($P > 0.05$, Figs 3a, 4a and 5a). However, differences in growth were observed at 20 dpf, 5 days after weaning was completed ($P < 0.001$). Larvae fed the 45P : 15L diet presented significantly higher TL and WW values (two times higher) than the rest of the treatments ($P < 0.001$, Figs 3b and 4b).

At 26 dpf, early juveniles fed the 45P : 15L diet had the highest TL ($P < 0.05$), followed by those fed the 45P : 10L, 30P : 15L and C diets, which presented similar TL ($P > 0.05$), and those fed the 30P : 10L, which were the smallest ones ($P < 0.05$, Fig. 3c). Regarding WW, larvae from the 45P : 15L group were also heavier, followed by those fed the 45P : 10L diet, by the 30P : 15L and C groups that had similar WW values ($P > 0.05$), and finally by the 30P : 10L group ($P < 0.05$, Fig. 4c).

No significant differences in SGR between treatments were observed during the *Artemia* feeding period. However, SGR of larvae fed the 45P : 15L diet was significantly higher than in the other treatments, larvae from the 30P : 10L and C groups showing the lowest SGR values ($P < 0.001$, Table 3). These results indicate that SGR was preferentially affected by the protein level and then by the lipid level.

Survival at the end of the experiment (26 dpf) was significantly higher in larvae fed the 45P : 15L diet, followed by those fed the 45P : 10L, 30P : 15L, C and 30P : 10L diets ($P < 0.05$, Fig. 5b).

No cannibalism was observed during the *Artemia* feeding period. Incidence of cannibalism during the compound diet feeding period (13–26 dpf) is shown in Fig. 6. Larvae from the 30P : 10L group showed a significantly higher incidence of type I cannibalism than the 30P : 15L, 45P : 15L and C groups ($P < 0.05$), whereas the 45P : 10L group presented intermediate values. Regarding type II cannibalism, the 30P : 10L and 30P : 15L groups showed higher incidence of cannibalism than the rest of treatments ($P < 0.05$). There was higher incidence of type I cannibalism than type II ($P < 0.05$) in the 30P : 10L group, whereas values of type I

Table 2
Lipid classes (in % of dry matter) analysed in the prepared experimental diets. Data expressed as mean ± SD (n = 3). Different superscript letters denote differences statistically significant between dietary treatments (one-way ANOVA, $P < 0.05$). Dietary treatment code corresponds to the protein : lipid level included in the tested diets

	Dietary treatments			
	30 : 15	30 : 10	45 : 15	45 : 10
SM	0.28 ± 0.0^{c}	0.61 ± 0.25^{b}	0.00 ± 0.00^{d}	0.92 ± 0.00^{a}
Lyso PC	1.37 ± 0.02^{a}	0.90 ± 0.13^{b}	1.31 ± 0.10^{a}	1.41 ± 0.07^{a}
PC	21.18 ± 0.15^{a}	16.63 ± 0.34^{c}	18.04 ± 0.24^{b}	16.67 ± 0.52^{c}
PS/PI	6.94 ± 1.86^{a}	3.54 ± 0.13^{b}	5.45 ± 1.04^{a}	2.94 ± 0.15^{b}
Lyso PE	2.70 ± 0.62^{a}	0.00 ± 0.00^{c}	1.81 ± 0.13^{b}	0.00 ± 0.00^{c}
PE	9.73 ± 1.61^{a}	0.00 ± 0.00^{b}	8.05 ± 0.45^{a}	0.00 ± 0.00^{b}
Uk1	5.06 ± 0.89^{c}	7.55 ± 0.28^{a}	3.78 ± 0.49^{d}	6.49 ± 0.04^{b}
Uk2+3	2.69 ± 0.13^{a}	0.00 ± 0.00^{c}	2.15 ± 0.29^{b}	0.00 ± 0.00^{c}
Total PL	50.12 ± 3.56^{a}	29.23 ± 0.46^{c}	40.78 ± 1.46^{b}	27.97 ± 0.99^{c}
CHOL	14.41 ± 1.64^{b}	23.83 ± 1.63^{a}	15.32 ± 0.71^{b}	22.81 ± 1.09^{a}
FFA	8.63 ± 1.45^{b}	14.59 ± 1.66^{a}	11.82 ± 0.08^{a}	14.09 ± 2.14^{a}
TAG	16.86 ± 0.39^{b}	18.20 ± 1.31^{b}	21.29 ± 0.58^{a}	22.51 ± 1.73^{a}
SE+W	9.98 ± 2.98	14.15 ± 1.80	10.79 ± 1.26	12.61 ± 3.77
Total NL	49.88 ± 3.56^{c}	70.77 ± 0.46^{a}	59.22 ± 1.46^{b}	72.03 ± 0.99^{a}

CHOL, Cholesterol; FFA, Free Fatty Acids; Lyso PC, LysoPhosphatidylColine; NL, Neutral Lipids; PC, PhosphatidylColine; PE, PhosphatidylEthanolamine; PI, PhosphatidylInositol; PL, Phospholipids; PS, PhosphatidylSerine; SE, Sterolesters; SM, Sphingomieline; TAG, Triacilglicerids; Uk, Unknown; W, wax.

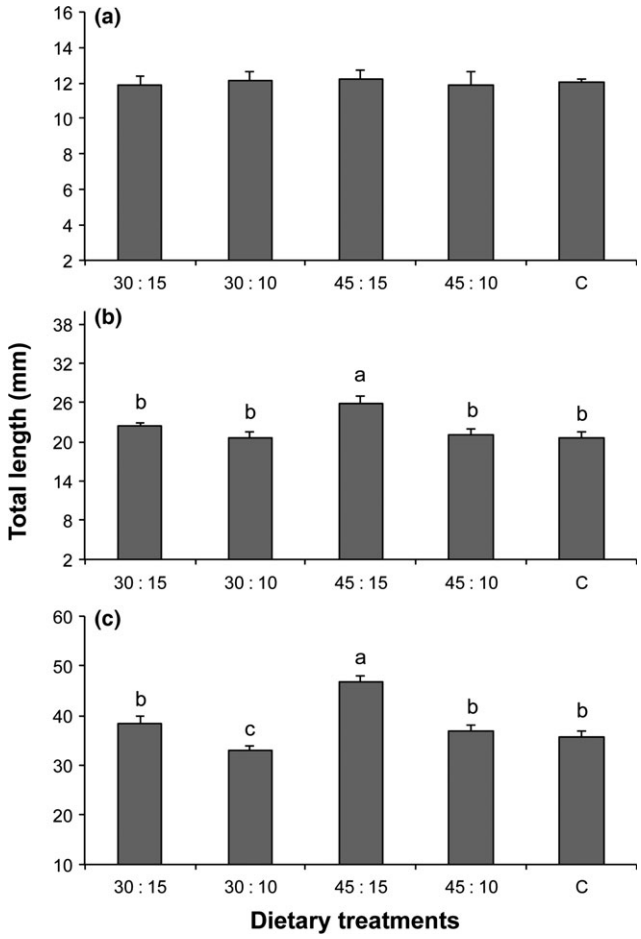

Fig. 3. Total length (TL), *Pseudoplatystoma punctifer* at (a) 12 days post fertilization – dpf (n = 45), (b) 20 dpf (n = 45) and (c) 26 dpf (n = 45) reared at 27.8 ± 0.7°C and in complete darkness. Data expressed as mean ± SD. Different superscript letters = statistically significant differences between dietary treatments (one-way ANOVA, $P < 0.05$). Dietary treatment code corresponds to the protein : lipid level included in the tested diets.

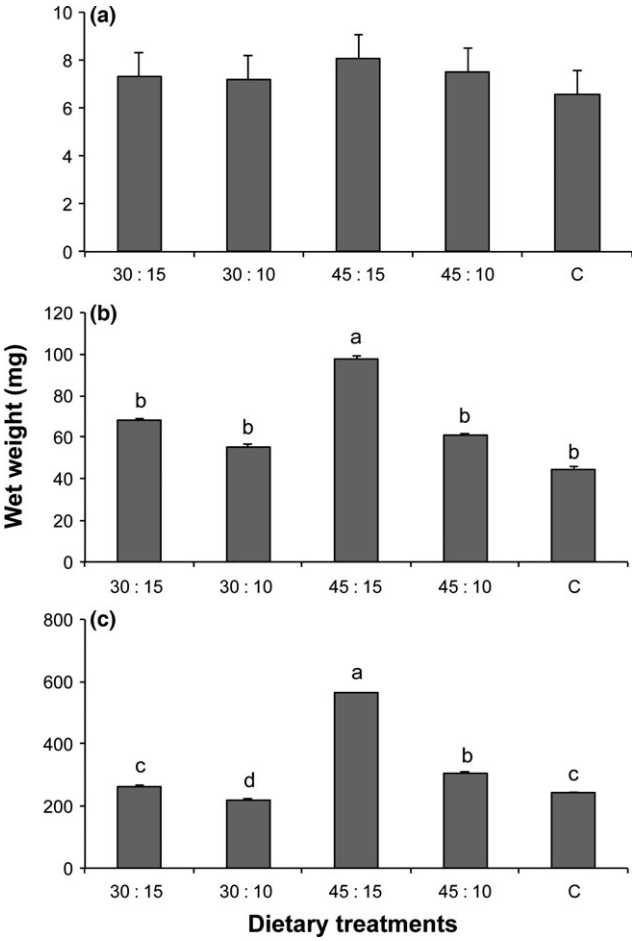

Fig. 4. Wet weight (WW), *Pseudoplatystoma punctifer* at (a) 12 days post fertilization – dpf (n = 45), (b) 20 dpf (n = 45) and (c) 26 dpf (n = 45) reared at 27.8 ± 0.7°C and in complete darkness. Data expressed as mean ± SD. Different superscript letters = statistically significant differences between dietary treatments (one-way ANOVA, $P < 0.05$). Dietary treatment code corresponds to the protein : lipid level included in the tested diets.

and type II cannibalism were similar among the rest of the diets ($P > 0.05$).

Discussion

Growth performance

Extending the *Artemia* feeding phase did not provide any advantage in terms of larval growth and survival in view of the results observed in the C and 45P : 10L groups, despite being fed the same compound diet. As larvae grew, the differences in growth between both groups increased (see WW at 20 and 27 dpf), group C showing no signs of compensatory growth. Whether this developmental delay generated during the early stages would be irreversible remains to be elucidated. This finding is of special relevance considering the high cost of *Artemia* and the associated costs of nauplii production. The results are also consistent with our hypothesis that *Artemia* nauplii do not cover the nutritional needs of *P. punctifer* larvae (Gisbert et al., 2014), as survival and growth performance of specimens from this group (C) were similar to those of the 30P : 15L group and only higher than the group fed the 30P : 10L diet. These results indicate that the feeding protocol used previously (Gisbert et al., 2014) allowed larvae to grow normally, but did not let them fully exploit their growth potential. In addition, the large accumulation of lipid deposits found in the intestine and liver suggested a nutritional imbalance with regard to protein and lipid content of the administered compound diet (Gisbert et al., 2014). The 45P : 15L diet soon showed an evident positive effect on growth (1 week after weaning) and at the end of the experiment allowed the amelioration of larval growth performance (six times in terms of WW and two times in terms of TL) and survival (two times) compared to preceding protocols under similar rearing conditions (Gisbert et al., 2014; M.J. Darias, unpublished data). Nevertheless, parental origin, which significantly affects growth during the early development of this species (Núñez et al., 2011), might also account for such differences.

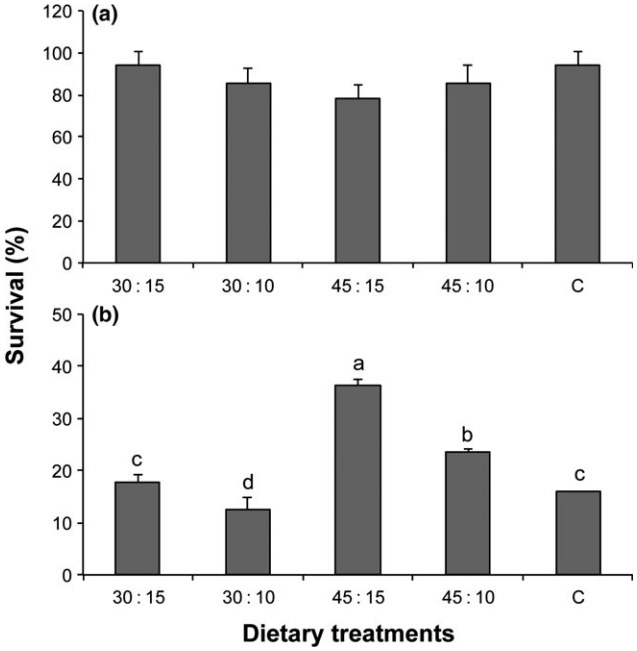

Fig. 5. Survival (%) of *Pseudoplatystoma punctifer* at (a) 12 days post fertilization – dpf and (b) 26 dpf (initial n = 3600 per treatment) reared at 27.8 ± 0.7°C and in complete darkness. Data were expressed as mean ± SD. Different superscript letters denote statistically significant differences between dietary treatments (one-way ANOVA, $P < 0.05$). Dietary treatment code corresponds to the protein : lipid level included in tested diets.

Table 3
SGR (in % day^{-1}) of *Pseudoplatystoma punctifer* larvae during *Artemia* and compound diet feeding periods in each dietary treatment (rearing temp. 27.8 ± 0.7°C, photoperiod 0L : 24D). Data expressed as mean ± SD (n = 45). Different superscript letters denote differences statistically significant between dietary treatments (one-way ANOVA, $P < 0.05$). Dietary treatment code corresponds to the protein : lipid level included in the tested diets

	Feeding periods	
Dietary treatments	*Artemia* (4–12 dpf)	Compound diets (13–26 dpf)
30 : 15	0.20 ± 0.01	0.53 ± 0.02^{bc}
30 : 10	0.20 ± 0.00	0.52 ± 0.01^{c}
45 : 15	0.21 ± 0.00	0.61 ± 0.01^{a}
45 : 10	0.20 ± 0.02	0.55 ± 0.01^{b}
C	0.19 ± 0.00	0.53 ± 0.01^{c}

Incidence of cannibalism

A variable incidence of cannibalism during the early juvenile stage was observed between treatments (3.6–6.4%), although this was considerably reduced compared to previous studies (16–30%, Arslan et al., 2009; Baras et al., 2011; Gisbert et al., 2014). In the present study, type I cannibalism was predominant, but not exclusive, at early stages; type II cannibalism was more important from 15 dpf onwards, coinciding with the formation of the gastric glands and the end of weaning. Two peaks of cannibalism were observed: the first day of weaning (13 dpf) and 2 days after the end of weaning

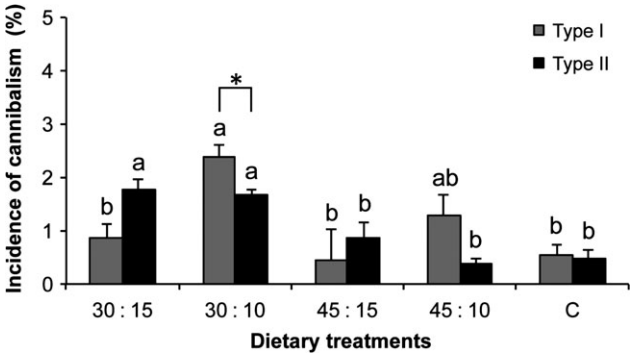

Fig. 6. Incidence of cannibalism (%) in *Pseudoplatystoma punctifer* during compound diet feeding period (13–26 days post fertilization – dpf; rearing temperature 27.8 ± 0.7°C; photoperiod 0L : 24D; initial n = 400). Data expressed as mean ± SD. Different superscript letters and asterisks = statistically significant differences between dietary treatments within each type of cannibalism and between the two types of cannibalism, respectively (one-way ANOVA, $P < 0.05$). Dietary treatment code corresponds to protein : lipid level included in tested diets.

(17 dpf). Cannibalism then gradually decreased until disappearing at 21 dpf (data not shown). Although weaning had previously been a critical stage promoting cannibalistic behaviour (Gisbert et al., 2014), the lower incidence of cannibalism noted in the present study and, in particular, its gradual disappearance after weaning, showed that a better-suited diet could greatly reduce this behaviour. Cannibalism was not consistently related to growth or survival, the smaller specimens (30P : 10L group) presenting the higher incidence of cannibalism and groups showing low incidence of cannibalism displaying low survival rates (30P : 15L and C groups). Although higher size distribution was generally found in groups displaying higher cannibalism in preceding experiments, no effect of cannibalism on size distribution of the cohort among the experimental groups was observed in the present study (data not shown). In any case, its influence in those parameters might have been very limited due to the low registered incidence of cannibalism. Indeed, a beneficial effect of all tested diets in the behaviour of *P. punctifer* was observed, fish being calmer and less aggressive than in previous experience. This suggests that, besides the overall nutritional value of the diets, the inclusion of specific nutrients inducing relaxing effects, such as PL, could participate to attenuate cannibalism. In fact, dietary PL have been shown to reduce the locomotor activity, stress and aggressiveness in humans and rats when provided in sufficient amounts (Chalon et al., 1998; DeMar et al., 2006; Hamazaki and Hamazaki, 2008), while its deficiency promotes an anxious behavioural profile in fish (Lund et al., 2014). Research on the nutritional effect on behaviour would be useful for understanding the incidence of cannibalism in this species. Besides being essential for growth and performance in many fish species, including juveniles of *P. fasciatum* (Arslan et al., 2009; Cahu et al., 2009), PL could also account for improving the palatability of the diets (Hadas et al., 2003; Tocher et al., 2008; Gong et al., 2014). This is particularly important for *P. punctifer* since taste and smell seem to have a key

role in feeding, as suggested by the presence of large size oral valves equipped with taste buds believed to serve for screening the food quality (Yashpal et al., 2006; Gamal et al., 2012), and large development of the olfactory organ (Gisbert et al., 2014). Indeed, larvae and early juveniles of *P. punctifer* demonstrated a sensitivity to food texture, being more attracted by humid than by dry compound diets (Fernández-Méndez et al., 2015), this being likely linked to the palatability and/or smell associated to the attractants released.

Macronutrient requirements in larvae and early juveniles

To our knowledge, this is the first report on the nutritional requirements during the larval and early juvenile stage of the genus *Pseudoplatystoma*. Reports in the literature show a notable difference in protein, lipid and carbohydrate requirements for older juveniles (initial wet weight ranging from 1 to 120 g) of several *Pseudoplatystoma* species. Thus, optimal levels range from 36 to 49% for proteins (Campos et al., 2006; Zanardi et al., 2008; Silva, 2013; Cornélio et al., 2014; Gonçalves, 2014), from 8 to 19% for lipids (Martino et al., 2005; Campos et al., 2006; Arslan et al., 2013; Silva, 2013) and from 13% to 25% for carbohydrates (Lundstedt et al., 2004; Okamura, 2009; Gonçalves, 2014) depending on the quality of ingredients and their relative proportions. In particular, Gonçalves (2014) working with *P. reticulatum* found that protein content could be reduced up to 36% protein when other energetic nutrients were balanced (15% carbohydrate and 8% lipids). Our study showed that a very good larval growth can be obtained with a diet containing 45% protein, brought as fishmeal (native and hydrolysed), and 15% lipids, brought as phospholipid (marine and soybean lecithin) and neutral lipid (oil included in fishmeal). Independently of the energy level that was the same in all diets, protein and/or lipid levels were insufficient in the other three diets for promoting similar growth. Besides the apparent insufficient protein and lipid content of the 30P : 10L diet, its high carbohydrate level (30%) could also be responsible for the impaired growth observed in this group. Indeed, Gonçalves (2014) did not find a protein-sparing effect by carbohydrates in juveniles of *P. reticulatum* fed two dietary carbohydrate (15 and 25%) and three protein (44, 40 and 36%) levels. Okamura (2009) found that juveniles of *Pseudoplatystoma* spp. hybrid (*P. corruscans* × *P. fasciatum*) presented persistent hyperglycemia when fed 20% cornstarch and concluded that the optimum level would be around 15%. The capacity of *P. punctifer* larvae and early juveniles to digest carbohydrates needs to be determined. Meanwhile, results from this study indicate that there is room for optimizing the balance of energetic compounds for *P. punctifer* larvae and early juveniles to spare protein for growth.

Indeed, one of the goals in fish nutrition science is to reduce the dietary protein content through incorporation of other energy sources, such as lipids and carbohydrates, allowing improvement of protein utilization for growth. The protein-sparing effect of lipids has been reported in many fish species, including in fingerling stages (Vergara et al., 1996; Li et al., 2012). However, a lipid level higher than 19% did not improve growth, nor had protein-sparing effects in *P. corruscans* juveniles, but resulted in increased visceral lipid content (Martino et al., 2005). Differences in larval and early juvenile survival and growth observed in the present study seemed to be associated with the higher protein and lipid content rather than the protein : lipid ratio. Comparing diets with the same protein level, the increase in dietary lipids led to an improved growth, suggesting that energy from lipids spares protein in *P. punctifer* fingerlings. These results indicate that the growth potential for this species might not have been fully exploited. Thus, further research to optimize the balance of proteins and energetic compounds (lipids and carbohydrates) is needed in *P. punctifer,* especially during the larval stage.

Conclusions

In conclusion, *P. punctifer* larvae were successfully weaned at 13 dpf but *Artemia* spp. did not satisfy the nutritional requirements of individuals, at least after the completion of the digestive system development. Among the tested diets, the 45P : 15L diet greatly improved growth and survival of *P. punctifer* larvae and early juveniles and reduced the incidence of cannibalism compared to previous feeding protocols used for this species. Differences in larval survival and growth were associated with the higher protein and lipid content of the diet rather than its protein : lipid ratio. Further studies on the digestive enzyme activity response to these dietary treatments will allow determination of a suitable macronutrient composition of the diet during the early development of this species.

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