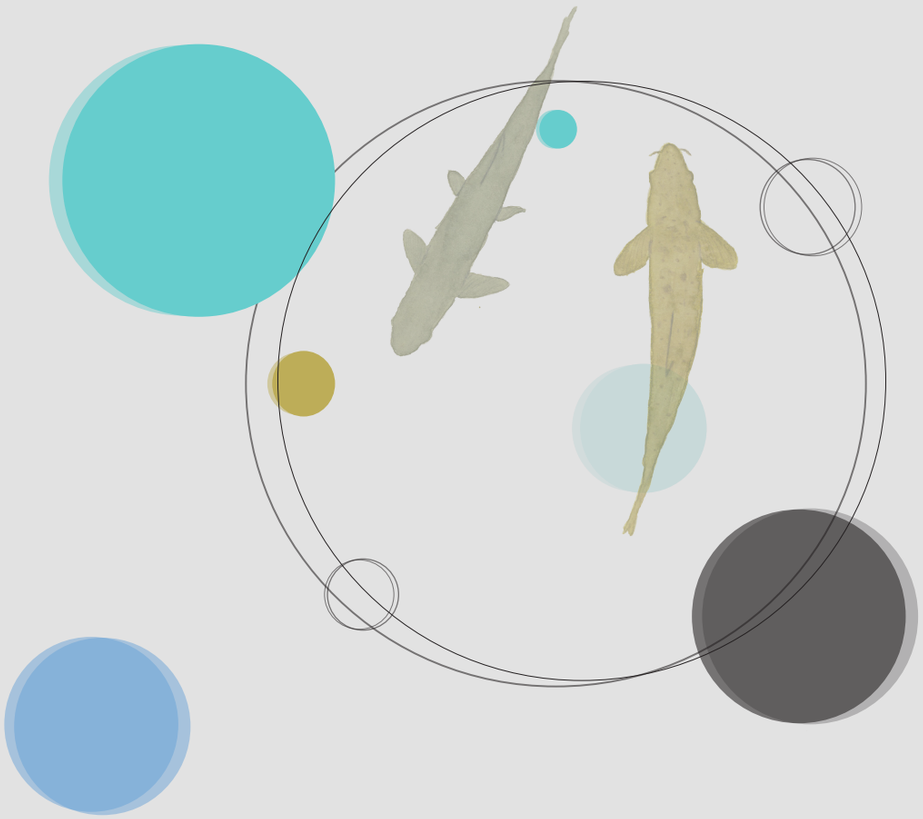
UNIVERSITAT DE BARCELONA

Efectos de la contaminación sobre la biología y el comportamiento de dos ciprínidos autóctonos de la Península Ibérica

Patricia M. Soler Vilaplana

Los ríos mediterráneos están sometidos a una intensa contaminación de origen antrópico. Entre los contaminantes más frecuentes están el amonio y los compuestos emergentes como los disruptores del sistema endocrino (EDC). La presente tesis ha analizado los efectos de parte de estos contaminantes sobre la reproducción y el estrés fisiológico del bagre *(Squalius laietanus)* y sobre el comportamiento del barbo de montaña *(Barbus meridionalis)*, dos ciprínidos autóctonos de la península ibérica. En los estudios de campo realizados en *S. laietanus* (cuenca del río Besòs), EDC como el benzotriazol y el benzotiazol (procedentes de una industria textil) pudieron ser responsables tanto de una disminución en el índice gonadosomático (en ambos sexos) como de una menor fecundidad y una desincronización de la puesta (en las hembras), así como de un menor diámetro en los túbulos seminíferos de los machos. Unos mayores niveles de estrés (cortisol) encontrados tanto en sangre como en el mucus epidérmico, así como las anomalías detectadas en glóbulos rojos y blancos de *S. laietanus*, fueron atribuibles a contaminación urbana procedente de estaciones depuradoras de aguas residuales. En *B. meridionalis*, mediante estudios experimentales de laboratorio, se demostró que el amonio (a concentraciones subletales) alteraba el comportamiento alimentario (voracidad y saciedad), pero no la actividad de natación. No obstante, en peces que habían estado pre-expuestos a amonio (en el ambiente natural), dichas alteraciones se presentaron en ausencia de este compuesto. En otro experimento, realizado también en *B. meridionalis* y dirigido a investigar el efecto del amonio sobre la cognición, se encontró que los peces expuestos tuvieron una peor retención de memoria que los no expuestos. Después de una experiencia de aprendizaje, los individuos de esta especie fueron capaces de retener memoria por al menos 16 días, lo que es considerado como una memoria a largo plazo.



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EFECTOS DE LA CONTAMINACIÓN SOBRE LA BIOLOGÍA Y EL COMPORTAMIENTO DE DOS CIPRÍNIDOS AUTÓCTONOS DE LA PENÍNSULA IBÉRICA

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TESIS DOCTORAL 2020

Departamento de Biología Evolutiva, Ecología y Ciencias Ambientales

Programa de Doctorado en Biodiversidad HDK04

Efectos de la contaminación sobre la biología y el comportamiento de dos ciprínidos autóctonos de la península ibérica

Memoria presentada por Patricia M. Soler Vilaplana para optar al grado de doctora por la Universitat de Barcelona

Barcelona, diciembre de 2020

La doctoranda

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La directora y tutora de tesis

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Patricia M. Soler Vilaplana (2020). Efectos de la contaminación sobre la biología y el comportamiento de dos ciprínidos autóctonos de la península ibérica.
Tesis doctoral, Universitat de Barcelona, 175 pp.
Ilustración y diseño: Patricia M. Soler Vilaplana y Roberto Otero

A la meua família i amics

“Qui escolta als peixos quan ploren?”

H. D. Thoreau

AGRADECIMIENTOS

Quien me iba a decir hace 6 años que estaría escribiendo estas palabras. Y es que hace unos 6 años el destino cruzo mi camino con el de mi directora de tesis Dolors Vinyoles en aquella primera clase de Biología de la Conservación. Fue esa manera que tenia de dar la clase, la pasión con la que trasmitía todos sus conocimientos, la que me impulso a acercarme y pedirle que me dejara realizar el TFM con ella. Y después de aquel día aquí estamos, con una tesis doctoral acabada de salir del horno. Aquel día empezó un largo, bonito y, en algunos momentos, difícil camino en el que he aprendido muchísimo, no solo de peces, ríos y técnicas diversas, sino de la vida misma. Así que no puedo estar más agradecida a Dolors por haberme ofrecido la oportunidad de transitar este camino, por todo lo enseñado y por haber confiado en mi durante todo este tiempo.

A lo largo de toda esta aventura son muchas personas las que han contribuido de diferentes maneras a la conclusión de este trabajo, y voy a tratar de agradecer a todas y cada una de ellas. Dar las gracias en primer lugar a la profesora Mercé Durfort, por su paciencia y amabilidad a la hora de enseñarme y aconsejar sobre las diferentes técnicas histológicas. Y por supuesto por dejarme ocupar su laboratorio. A los profesores Domingo Rodríguez-Teijerio y Humbert Salvadó, por su orientación y ayuda técnica. Al personal del estabulario húmedo de la facultad de Biología, que han sido imprescindibles para llevar a cabo toda la experimentación con los peces, y en especial agradecer a Jordi Guinea, por su asistencia, ayuda y consejos varios durante las largas jornadas pasadas en las salas de estabulación (que después de tantas horas es como si fueran parte de mi casa). También agradecer la ayuda de los técnicos de laboratorio Jordi Correas, Jessica Borrego y Daniel Díaz, y al personal de la Unitat de Medi Ambient dels CCiTUB.

También he tenido la oportunidad de conocer a gente maravillosa tanto de dentro y como de fuera del departamento. Como mi compañera de departamento y despacho Carolina Quirós, los compañeros de Ecología, Pau Fortuño, Núria Cid y Marc Marimon ("Equip Sant Llorenç"), y, como no a Annaïs Carbajal, al profesor Narcís Prat y Carlos Barata. Con todos ellos he podido

compartir largas y divertidas jornadas de muestreo en los ríos, luchando muchas veces contra los elementos y el clima extremo. Echaré de menos a la selvática riera de Castelló y su sustrato arcilloso. A Noëlle Fabre por compartir conmigo su entusiasmo por los habitantes de los ríos y por su ayuda. A Eduardo García-Galea, por su infinita paciencia conmigo y con el R. A los estudiantes Raquel Bañón y Philip Manning por su dedicación y trabajo, imprescindibles para el desarrollo de esta tesis. Y no puedo olvidarme de la familia del CRAI de Biología de la UB, a Miriam (mi compi y futura copropietaria de VillaBiblio), Jaume, Blanca, Joan, Raquel y a la súper jefa Montse, por los consejos y los ánimos trasmitidos todo este tiempo.

A todos mis amigos, por todo apoyo y comprensión. A Bea, Eli, Tere y Maddi, sin vuestro apoyo y cariño no lo habría conseguido, muchas gracias por todos los consejos, la ayuda, las aventuras varias y las risas, sobre todo las risas. A mi eterno compañero de piso Juanra, por aguantarme, sobretodo en esta etapa final, que la risa y el surrealismo nos acompañen siempre. A Mery, Isis y a mis queridísimos amigos de València, a mi María, Majo, Paula, Elena, Sara, Eli, Javi, Tomás, Andrés y Carles, Vicen y Nati, porque, aunque habéis estado lejos os he sentido muy cerca y por conseguir sacarme siempre una sonrisa. A Álvaro porque no todo iba a ser entrenar la mente, *mens sana in corpore sano* A la familia de Bosc de Llum, por ponérmelo todo tan fácil y cuidarme tanto, muchas gracias. A Roberto, por la ayuda en el diseño y maquetación de esta tesis y, por toda la dedicación e ilusión que pone en lo que hace.

A mis padres y a mi hermano, por estar siempre a mi lado, por todo el apoyo y la paciencia, que no siempre es fácil de mantener. Muchas gracias por haberme alentado a seguir este camino y por no solo respetar mi pasión sino fomentarla y darle alas. A la Iaia por su amor incondicional. A Geno, al Iaio y al Abuelito que siempre están conmigo. Y, por último, a toda mi gran familia, me ha tocado teneros lejos sobretodo este último año, pero ha sido una distancia afortunadamente solo física, vos vull moltíssim.

La última parte de la tesis ha sido realizada durante el confinamiento de la pandemia del coronavirus que nos ha hecho ver la vulnerabilidad del ser humano y su condición fugaz, que tantas veces recordo el tópico latino, *Dum vivimus, vivamus.*

ABSTRACT

Mediterranean rivers are subject to intense pollution of anthropic origin. Among the most frequent pollutants are ammonia and emerging compounds such as endocrine system disruptors (EDCs). This thesis has analyzed the effects of some of these pollutants on the reproduction and physiological stress of the Catalan chub *(Squalius laietanus)* and on the behavior of the Mediterranean barbel *(Barbus meridionalis)*, two cyprinids native to the Iberian Peninsula. In field studies carried out in *S. laietanus* (Besòs river basin), EDCs such as benzotriazole and benzothiazole (coming from a textile industry) could be responsible for both a decrease in the gonadosomatic index (in both sexes) and a lower fecundity and spawn desynchronization (in females), as well as a smaller diameter in the seminiferous tubules of males. Higher levels of stress (cortisol) found in both blood and epidermal mucus, as well as the abnormalities detected in red and white blood cells of *S. laietanus,* were attributable to urban pollution from sewage treatment plants. *In B. meridionalis*, through experimental laboratory studies, it was shown that ammonia (at sublethal concentrations) altered feeding behavior (voracity and satiety), but not swimming activity. However, in fish that had been pre-exposed to ammonia (in the natural environment), these alterations occurred in absence of this compound. In another experiment, also carried out in *B. meridionalis* and aimed at investigating the effect of ammonia on the cognition, exposed fish were found to have poorer memory retention than non exposed ones. After a learning experience, individuals of this species were able to retain memory for at least 16 days, which is considered long-term memory.

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INTRODUCCIÓN GENERAL

La contaminación del agua se presenta cuando ingresan sustancias tóxicas en las masas de agua (arroyos, ríos, lagos, acuíferos, océanos, etc.). Estas sustancias se pueden disolver, quedar suspendidas en la superficie o depositarse en el lecho, deteriorando en consecuencia la calidad de los ecosistemas y perjudicando a seres humanos y otros organismos. El agua es conocida como el disolvente universal ya que es capaz de disolver más sustancias que cualquier otro líquido del mundo. A pesar de encontrarse en movimiento y de disolver rápidamente los contaminantes, la mayoría de los ríos y arroyos del mundo se encuentran gravemente contaminados. El agua es crucial para la vida, pero pese a ello el 80% de las aguas residuales del mundo se vierten sin tratar al medio ambiente (Laws, 2018). Además de aguas residuales urbanas, se vierten desechos industriales y residuos agrícolas, estos últimos normalmente llegan por la acción de la escorrentía superficial (Lloyd, 1992). Por lo tanto, los tipos de contaminantes que se pueden encontrar en el agua tienen una gran variedad, incluyendo desde contaminantes orgánicos a inorgánicos, radioactivos, etc. Los sistemas acuáticos son altamente vulnerables, ya que tienen la tendencia de acumular altas concentraciones de químicos que entran desde los sistemas terrestres que los rodean (**Fig. 1**) (Erickson et al., 2008). En el caso de los ríos el problema se acentúa, ya que históricamente la mayoría de las industrias y ciudades se han ubicado a lo largo de ellos al proporcionar un acceso cercano y fácil al agua dulce, transporte y lugar conveniente para verter residuos. También las actividades agrícolas han tendido a concentrarse cerca de los ríos, al ser sus llanuras aluviales excepcionalmente fértiles debido a la gran cantidad de nutrientes que se depositan en el suelo cuando los ríos se desbordan.

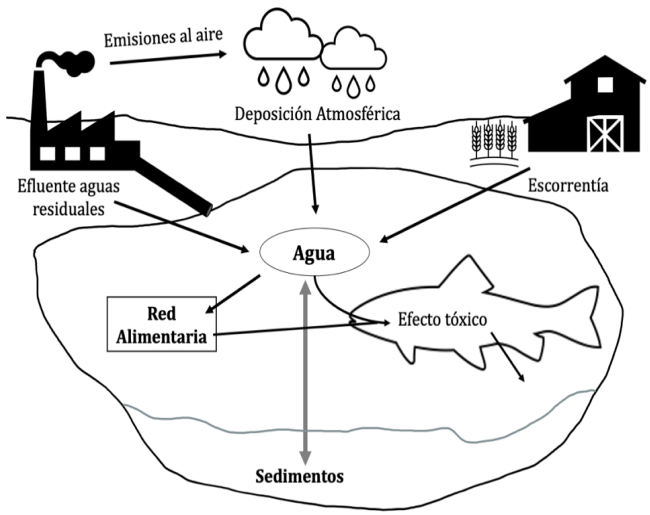

Fig. 1. Entradas químicas y distribución dentro de los sistemas acuáticos, enfatizando la acumulación en los peces (Adaptado de Erickson et al., 2008).

Los ríos mediterráneos y su ictiofauna

Los ríos mediterráneos, es decir, los que están caracterizados por un tipo de clima mediterráneo, sufren acusadas oscilaciones de caudal a lo largo del año ya que se alternan períodos de sequía estival con fuertes crecidas en otoño (menos acusadas en primavera). Durante el verano algunas partes de estos ríos pueden quedar totalmente secas, únicamente con la presencia de unas pocas pozas sin conexión entre ellas. Estos ríos están considerados puntos calientes o "hotspots" en términos de biodiversidad y se encuentran entre los ecosistemas más amenazados del mundo (Cuttelod et al., 2008). Lamentablemente, los ríos mediterráneos tienen una larga historia de impactos antropogénicos, entre los que se incluyen la contaminación, la introducción de especies exóticas y la degradación del hábitat, lo que resulta en una disminución alarmante de las poblaciones de peces nativas (Clavero et al., 2004).

Cuando se habla de peces continentales se está hablando del 25% de las especies de vertebrados de la Tierra (Darwall & Freyhof, 2015). Este grupo se encuentran dentro de los más amenazados del planeta, siendo sus principales

amenazas la sobrepesca, la contaminación, la introducción de especies exóticas y la degradación de los hábitats (Aparicio et al., 2016). Dentro de los diferentes grupos de peces de agua dulce, la familia Cyprinidae es una de las que cuenta con un mayor número de especies. Dentro de esta familia se presenta una gran variabilidad en cuanto a tamaño y forma corporal. Se encuentra distribuida por todo el mundo, pero en Europa se la considera la familia dominante, hablando en términos de riqueza de especies. En cuanto a la reproducción, se suele presentar sincronía en la época de freza, de modo que machos y hembras de las diferentes especies coinciden en la liberación de los gametos durante el ciclo reproductor. Su alimentación es muy variada y puede basarse en invertebrados, organismos planctónicos, elementos vegetales (plantas acuáticas y algas) e incluso alevines de peces. Los ciprínidos constituyen una fuente importante de proteína en muchos países y, son objeto de pesca y cría en cautividad. Entre las principales amenazas para la conservación se encuentran la destrucción de hábitat, la alteración del caudal y la presencia de barreras artificiales en los ríos. Por esta razón, para la realización de la presente tesis se seleccionaron dos especies ampliamente distribuidas en los ríos del NE de la península ibérica y del sur de Francia con algunas poblaciones en regresión: el bagre (*Squalius laietanus*) (**Ficha 1**) y el barbo de montaña (*Barbus meridionalis*) (**Ficha 2**).

La mezcla de contaminantes, una amenaza para la conservación de los peces de agua dulce

La conservación de los peces de agua dulce ha alcanzado un punto crítico en todo el mundo. En Europa, alrededor del 37% de las especies están incluidas en alguna categoría de amenaza (Freyhof & Brooks, 2011). La situación es aún peor en la península ibérica, donde aproximadamente el 70% de las especies son endémicas (Doadrio et al., 2011). En Cataluña, al NE de la península ibérica, se estima que hay 29 especies de peces autóctonos, de los cuales dos se consideran extintos regionalmente y otros 22 (75%) se encuentran bajo alguna categoría de amenaza según la UICN (2012). A pesar de los esfuerzos para implementar la Directiva Marco Europea del Agua (2000/60/EC) (2000) con prácticas para el tratamiento de aguas residuales, la calidad de las aguas dulces sigue siendo preocupante. Se siguen produciendo vertidos de aguas residuales no tratadas adecuadamente y que, invariablemente, conducen a un deterioro

de la calidad del agua (Maceda-Veiga et al., 2013). Además, como se ha descrito anteriormente, los ríos de tipo mediterráneo están sujetos a sequías estivales, hecho que puede agravar los efectos nocivos de la contaminación sobre la biota acuática al disminuir la capacidad de dilución de estos contaminantes (Colin et al., 2016; 2017). Es probable que esta situación empeore debido a la mayor demanda de agua para uso humano causada por el cambio climático, lo que aumentaría el estrés hídrico en los ríos (Mekonnen & Hoekstra, 2016).

Entre los contaminantes descritos en los ríos mediterráneos se pueden encontrar metales pesados, compuestos nitrogenados (nitratos, nitritos y amonio), pesticidas, herbicidas, hormonas sintéticas y drogas (Figuerola et al., 2012; Lavado et al., 2006; Lavado et al., 2004; Maceda-Veiga & De Sostoa, 2011). Algunos de estos contaminantes se encuentran clasificados dentro de los denominados contaminantes de preocupación emergente o CEC, por sus siglas en inglés ("Contaminants of Emergin Concern"). Dentro de estos compuestos se considera cualquier sustancia química descubierta en el agua o en el medio ambiente que habían sido detectados previamente o que solo se presentaban a niveles insignificantes (Diamond et al., 2011). Los CEC abarcan desde productos farmacéuticos, y de cuidado personal, hasta contaminantes orgánicos persistentes utilizados en muchos procesos industriales (Sauvé & Desrosiers, 2014). Cada vez hay más evidencias de que muchos de estos CEC, cada vez más frecuentes en la península ibérica, actúan como disruptores endocrinos (EDC, por sus siglas en inglés "Endocrine Disrupting Chemicals") (Gorga et al., 2015; Osorio et al., 2016). Se define a un EDC como un agente exógeno capaz de interferir con la síntesis, secreción, transporte, metabolismo, acción de unión o eliminación de las hormonas naturales transmitidas por la sangre y, que son responsables de la homeostasis, la reproducción y los procesos de desarrollo en los animales (Diamanti-Kandarakis, et al., 2009). Son químicos que tienen la capacidad de imitar, bloquear o interferir con las hormonas del sistema endocrino (Diamanti-Kandarakis, et al., 2009). Tienen efecto a bajas concentraciones y pueden llegar a afectar a los caracteres sexuales como es el caso de las gónadas de los peces (Leino et al., 2005). Dentro de los EDC, destacan los de origen farmacéutico como el ibuprofeno y el ácido clofíbrico, los esteroides y las hormonas (estradiol, estriol, etc.), los compuestos tensioactivos metabólicos (nonifenol, octifenol, etc) y los compuestos retardantes de llama

(bisfenol A (BPA), tris (1, 3-diclor-2-propil) fosfato (TDCPP), etc.) (Banjac et al., 2015; Barceló, 2003; Céspedes et al., 2005).

En general, entre los efectos de los EDC sobre la biología reproductiva de los peces se encuentran la aparición de intersexualidad (ovocitos femeninos en un tejido testicular masculino), alteración de la ovogénesis en las hembras y una disminución de la fecundidad, factores que llevan a una reducción de las poblaciones (Jobling et al., 1998; Kidd et al., 2014) y, en última instancia, a un impacto sobre el estado de conservación de las especies nativas. Aunque existen abundantes estudios sobre los efectos adversos de los EDC sobre los peces en condiciones experimentales de laboratorio, no está tan claro cómo estos compuestos pueden afectar en condiciones naturales a las poblaciones silvestres (Mintram et al., 2018). El principal inconveniente de la mayoría de los estudios de laboratorio es que no pueden reproducir las condiciones naturales y no consideran la potencial sinergia de la mezcla de contaminantes. De hecho, los resultados de la exposición a sustancias químicas mixtas pueden diferir significativamente de aquellos que analizan individualmente los efectos de los EDC (Mintram et al., 2018). Por otra parte, la mayor parte de los estudios de laboratorio no investigan el efecto de los contaminantes sobre las especies autóctonas, sino que suelen hacerlo sobre especies modelo.

Otro de los contaminantes que más abundan en los ambientes acuáticos es el amonio, una de las formas de los compuestos nitrogenados. Este compuesto se puede encontrar naturalmente en ambientes acuáticos como producto final del proceso metabólico de los animales (Randall et al., 1989) y es un componente importante en el ciclo del nitrógeno (Raven & Johnson, 1989) (**Fig. 2**). Además del amonio que se puede encontrar de forma natural, hay que añadir el procedente de efluentes de aguas residuales, residuos industriales y escorrentías agrícolas (**Fig. 2**). La presencia de amonio en las aguas dulces se asocia con la acidificación de ríos y lagos, con fenómenos de eutrofización y con una toxicidad directa para los organismos acuáticos (Baker et al., 1991; Camargo & Alonso, 2006; CEPA, 2001).

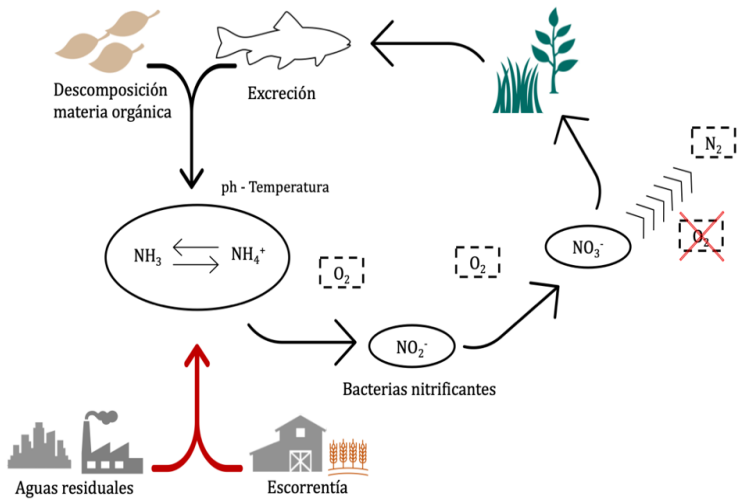

Fig. 2. Ciclo simplificado del nitrógeno en el agua dulce con las entradas y salidas naturales, y los aportes adicionales de fuentes antrópicas.

La toxicidad de este compuesto sobre los organismos acuáticos depende de la forma química del amonio, el pH, la temperatura y el tiempo de exposición (Francis-Floyd et al., 2009). Una vez disuelto en el agua, se puede encontrar en dos formas: amonio no ionizado o amoníaco (NH_3) y amonio ionizado (NH_4^+) (Francis-Floyd et al., 2009). El NH_4^+ no atraviesa fácilmente las branquias de los peces y se encuentra menos biodisponible, pero una vez que está dentro del organismo, puede causar daño celular (EPA, 2013; Francis-Floyd, 2009). El NH_3 es la forma más tóxica para la vida acuática ya que puede pasar fácilmente del agua al interior del pez y, una vez dentro, transformarse en NH_4^+ (EPA, 2013; McKenzie et al., 2009). Por esta razón, la toxicidad del amonio se expresa como la suma de ambas formas o amonio total (TAN, por sus siglas en inglés "Total Ammonia Nitrogen") (**Fig. 3**). El equilibrio entre NH_3 y NH_4^+ está influenciado por el pH y la temperatura (CEPA, 2001; EPA, 2013; Thurston et al., 1979). Se ha demostrado que este compuesto puede dañar las branquias, el hígado, los riñones, el bazo y los tejidos de otros órganos en los peces, lo cual conlleva dificultades respiratorias (Benli et al., 2008; Schram et al., 2010), alteraciones fisiológicas y, eventualmente, lleva al agotamiento o a la muerte (Schram et al., 2010). El amonio puede causar daño celular e incluso llegar a afectar al sistema de defensa antioxidante alterando así los niveles de estrés

oxidativo en los peces (EPA, 2013; Sinha et al., 2014). Este compuesto también puede alterar el comportamiento de los peces, se ha visto que su exposición a concentraciones subletales puede reducir la actividad de natación (Wicks et al., 2002), el comportamiento de búsqueda de alimento (Tudorache et al., 2008) y la capacidad de huida frente a los depredadores (McKenzie et al., 2009; Tudorache et al., 2008).

Estudios previos demuestran que ejemplares de peces expuestos a concentraciones elevadas de amonio experimentan dificultades para eliminar este metabolito del cuerpo (Sinha et al., 2014) y también que las exposiciones prolongadas a este compuesto provocan su acumulación en el organismo (McKenzie et al., 2009). Por otro lado, hay varios estudios que indican que los peces pre-expuestos a episodios de contaminación por compuestos nitrogenados (Boyd, 2013; Shrivastava et al., 2016) y metales pesados (Adeyemi & Klerks, 2013; McGeer et al., 2007; Zheng et al., 2016) podrían ser más tolerantes a estos contaminantes por aclimatación. En estos estudios se observó que los individuos pre-expuestos a concentraciones subletales de un contaminante exhibían una mayor tolerancia a la exposición a altas concentraciones del mismo. En el caso concreto del amonio pasa algo parecido, ya que los especímenes pre-expuestos a concentraciones subletales podrían tolerar altas concentraciones de este compuesto al aumentar la tasa de excreción de amonio, así como al favorecer la evolución de los mecanismos de adaptación (Boyd, 2013; Shrivastava et al., 2016). También se ha demostrado que estos mecanismos funcionan con otros tipos de factores estresantes como la hipoxia (Rees et al., 2001), la salinidad (Al-Amoudi, 1987) y los cambios de temperatura (Long et al., 2013). Hay que señalar que todos estos estudios analizan el efecto de la pre-exposición de peces desde un punto de vista bioquímico y fisiológico.

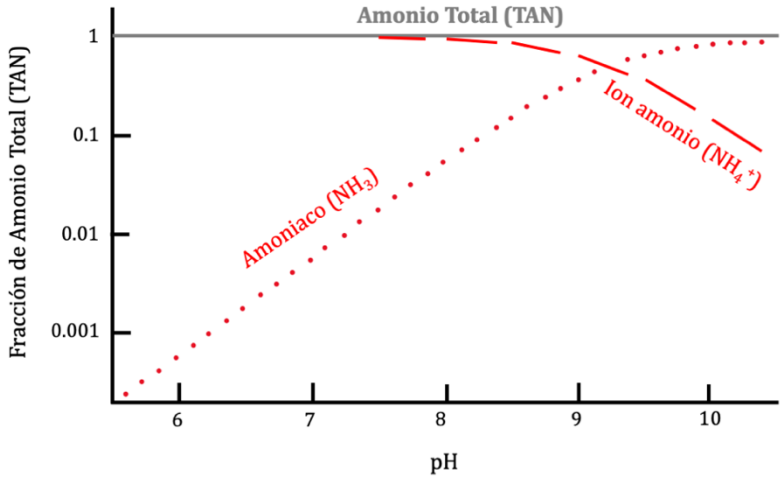

Fig. 3. Especiación química del amonio en un rango de valores de pH (Adaptado de EPA, 2013).

Como ha quedado patente, en la naturaleza algunos productos químicos pueden expresar interacciones sinérgicas o antagónicas con otros, y esto debe tenerse en cuenta en los estudios de campo, ya que los efectos resultantes de las exposiciones mixtas podrían subestimarse o sobreestimarse (Ginebreda et al., 2014). La contaminación del agua es una de las principales causas de la regresión de las especies de peces de agua dulce en todo el mundo, pero se sabe poco sobre cómo las mezclas de compuestos en el agua pueden interactuar entre sí agravando sus efectos.

Evaluando los efectos de la contaminación en los peces

Reproducción y estrés fisiológico

Cualquiera de los contaminantes clasificados como EDC presentes en el agua, en concentraciones subletales, puede interferir en el correcto funcionamiento del sistema reproductor de los peces y, en consecuencia, reducir su eficacia biológica. El estudio de las posibles interacciones entre el efecto de los contaminantes y la reproducción es un punto crucial para mejorar las estrategias de conservación (Grandcourt et al., 2009). Otro de los aspectos

donde se pueden observar los efectos de la contaminación sería sobre el estrés fisiológico. La exposición a diferentes estresores en el medio natural, dependiendo de su magnitud y duración, pueden causar respuestas de estrés agudas o crónicas (Schreck et al., 2016; Sheriff et al., 2011). Las respuestas crónicas al estrés, como las provocadas por la exposición durante un largo periodo de tiempo a la contaminación ambiental, están asociadas con una amplia gama de efectos sobre la fisiología de los peces (Scott & Sloman, 2004) que pueden, en última instancia, conducir a una pérdida de biodiversidad (Dantzer et al., 2014; Hooper et al., 2012). Cuando los organismos responden a estos estresores lo hacen tanto desde el punto de vista fisiológico como comportamental. Tanto las consecuencias de la contaminación sobre la reproducción como sobre el estrés fisiológico pueden analizarse desde diferentes metodologías, algunas más invasivas que otras. Esta tesis se ha centrado en diferentes métodos histológicos y en el análisis de biomarcadores.

Dentro de los métodos histológicos, la histopatología se utiliza para el diagnostico de enfermedades y se ha convertido en una herramienta clave para analizar los efectos potenciales de los disruptores endocrinos sobre los peces (Vos et al., 2000) y las consecuencias del estrés fisiológico provocado por la contaminación (Maceda-Veiga et al., 2015). Esta metodología es muy fiable ya que están totalmente estandarizada y favorece, al mismo tiempo, la comparabilidad entre diferentes estudios (Dietrich & Krieger, 2009). Como se ha remarcado anteriormente, los efectos de los disruptores endocrinos sobre la reproducción de los peces a nivel individual han sido ampliamente descritos. Los EDC pueden tener efecto tanto a nivel celular como tisular, y se ha demostrado que pueden inducir cambios histopatológicos sobre las gónadas de los peces (Vos et al., 2000). Mediante el estudio histológico, se pueden identificar lesiones en las gónadas que pueden afectar a su correcto funcionamiento y, por tanto, que tendrán un efecto sobre la fecundidad y la fertilidad de los individuos. Entre las lesiones o patologías más frecuentes provocadas por los EDC y que se pueden observar en cortes histológicos estarían: la intersexualidad, la atresia, la necrosis, la apoptosis, la fibrosis, la hipertrofia y la hiperplasia de las células de Sertoli y las de Leydig, así como deformaciones en los conductos gonadales (Dietrich & Krieger, 2009).

Otros de los estudios frecuentes para diagnosticar el estado de salud general de los peces es el análisis de la sangre periférica. Son métodos bastante económicos, prácticos y poco invasivos ya que, a diferencia de los estudios sobre las patologías de las gónadas, no implican el sacrificio de los animales. Con una simple gota de sangre se puede obtener información detallada sobre su estado de salud. Hay que tener en cuenta que estos animales en sus ambientes naturales se enfrentan a una gran cantidad de desafíos, algunos de ellos son los derivados de la propia dinámica de los ecosistemas, pero otros derivan de los impactos antropogénicos que provocan un aumento considerable del estrés y, por lo tanto, una reducción del bienestar (Pankhurst, 2011). La sangre de los peces tiene prácticamente la misma composición que la de los grandes vertebrados, contiene glóbulos rojos o eritrocitos, glóbulos blancos y plaquetas o trombocitos (Douglas et al., 2010). Cada componente aporta una información diferente que puede contribuir a la monitorización de la vida salvaje. La detección de daños y alteraciones del ADN se puede realizar mediante la evaluación de anomalías nucleares en los glóbulos rojos o eritrocitos, así como por la presencia de micronúcleos circulantes y de glóbulos rojos o eritrocitos senescentes e inmaduros (Pacheco & Santos, 1996; Tavares-Dias, 2006). De hecho, la detección de anomalías en los glóbulos rojos se ha utilizado ampliamente como indicador de exposición a contaminantes genotóxicos y mutagénicos (Braham et al., 2017; Castaño et al., 2000; Colin et al., 2017; Hussain et al., 2018; Ivanova et al., 2016). Con el recuento relativo de los glóbulos blancos, por otra parte, se puede obtener una medida muy común de estrés y respuesta inmune innata (Davis et al., 2008), de hecho, la proporción relativa de neutrófilos y linfocitos se ha aplicado con éxito como medida de exposición prolongada a contaminantes (Hedayati & Jahanbakhshi, 2012; Johnstone et al., 2012; Witeska, 2005).

Fuera de los métodos histológicos, tanto los efectos de los disruptores endocrinos como del estrés se puede detectar analizando los niveles hormonales en muestras de sangre (Busch & Hayward, 2009; Crespo & Solé, 2016; Homyack, 2010; Solé et al., 2003). En el caso de concreto de las evaluaciones de la respuesta al estrés, el método más utilizado es el análisis de los niveles de cortisol (el principal glucocorticoide en los peces teleósteos) circulantes en el organismo (Mommsen et al., 1999; Schreck et al., 2016). Pero esta metodología

tiene como inconveniente la dificultad de obtener muestras de sangre de los animales, ya que, aunque no sea necesaria la muerte del animal no deja de ser un método invasivo. Por lo tanto, y dado el creciente interés de los estudios sobre la fisiología de la conservación en evaluar los aumentos crónicos de cortisol (Danzter et al., 2014), se hace imperativo el desarrollo de nuevas técnicas menos invasivas o directamente no invasivas, y mediante las cuales reducir al máximo la manipulación de los ejemplares de estudio. Recientemente, se han implementado nuevas técnicas como la obtención muestras de cortisol a partir de la recolección del moco epidérmico (De Mercado et al., 2018; Guardiola et al., 2016; Simontacchi et al., 2008) y también de las escamas de los peces (Carbajal et al., 2019). La implementación de este tipo de técnicas proporciona grandes ventajas, ya que se reduce la manipulación y, al mismo tiempo, no se provoca un estrés adicional que podría afectar al grado de bienestar de los animales (King et al., 2016; Pottinger et al., 2016). Aunque todas estas técnicas necesitan de investigaciones futuras para saber si pueden ser implementadas, especialmente en estudios de peces silvestres en ambientes no controlados, los resultados son prometedores.

El comportamiento de los peces

Siempre se había considerado que los peces actuaban de manera automática y que su comportamiento no era más que una serie de pautas de acciones fijas en respuesta a los estímulos del medio. Afortunadamente esta visión ha cambiado en las últimas décadas y cada vez son más los estudios que demuestran la complejidad de comportamientos que rigen la vida de los peces e incluso se puede hablar de personalidad. La personalidad animal viene definida como aquel conjunto de respuestas conductuales (un síndrome conductual o conjunto de rasgos de comportamiento) que son diferentes entre individuos de la misma especie y que son consistentes a lo largo del tiempo y en diferentes contextos (Sih et al., 2004). Los estudios de estos rasgos del comportamiento animal han ido cogiendo relevancia en los últimos años (Roche et al., 2016), ya que son capaces de proporcionar información a diferentes niveles, incluyendo el bienestar animal, la mejora de la productividad y, en última instancia, la conservación de las especies al permitir predecir su capacidad de adaptación frente al cambio global (Sih et al., 2012). La personalidad se puede dividir en

cinco categorías: actividad, timidez–osadía, exploración–evitación, agresividad y sociabilidad (Réale et al., 2007). La actividad generalmente se define como un movimiento espontáneo en un entorno seguro (Conrad et al., 2011; Réale et al., 2007). La osadía se define como la reacción de un individuo frente a una situación novedosa o peligrosa, como la presencia de un depredador (Réale et al., 2007). Se entiende como exploración a la latencia que tiene un individuo en investigar ambientes y objetos nuevos (Conrad, et al., 2011; Réale et al., 2007). En la categoría de agresión entran todos aquellos comportamientos que tienen como objetivo ganar o defender recursos (alimentos, territorio o pareja reproductora) (Silva, et al., 2013). Por último, la sociabilidad se define como la relación de un individuo con otros sin interacciones agresivas (Conrad et al., 2011). A estos rasgos de comportamiento hay que añadir la capacidad de aprendizaje y de memoria. El estudio de esta capacidad es importante desde diferentes puntos de vista. Desde un punto de vista evolutivo, el aprendizaje y la memoria facilitan, de alguna manera, el proceso de adaptación por selección natural de los animales a su entorno (Gerlai, 2017). La existencia de esta plasticidad neuronal provee a los individuos de un mecanismo que les permite cambios rápidos de comportamiento en respuesta a experiencias previas (Gerlai, 2017). La capacidad de aprender proporciona a los peces una flexibilidad para poder ajustar, en base a una experiencia, su comportamiento en un entorno cambiante (Dodson, 1988). El aprendizaje ha demostrado ser una herramienta útil a la hora, por ejemplo, de reeducar a los peces que han sido criados en cautividad o que han pasado un largo periodo en cautividad antes de ser liberados, para que recuperen el reconocimiento del depredador (Chivers & Smith, 1994) y para que adquieran hábitos "naturales" por imitación (Brown & Laland, 2003).

Los análisis de comportamiento se utilizan comúnmente en ecotoxicología de animales acuáticos como indicadores de toxicidad subletal y se ha demostrado la efectividad de este enfoque en una amplia gama de escenarios de exposición (Bae & Park, 2014; Melvin & Wilson, 2013). Además, este tipo de estudios proporcionan información sobre la variabilidad individual, lo que a su vez tiene implicaciones en diferentes campos como son los de la ecología del comportamiento (Budaev & Zworykin, 2002; Réale et al., 2010; Sih et al., 2004), la neurociencia (Johansen et al., 2012), la acuacultura (Huntingford &

Adams, 2005; Martins et al., 2011), el bienestar animal (Martins et al., 2012), la susceptibilidad a enfermedades y el estado de salud tanto a nivel individual como poblacional (Fevolden et al., 1992), así como en la interpretación de datos moleculares (Alves et al., 2010). En general, el comportamiento de los peces es un indicador sensible que permite detectar los efectos de ciertos contaminantes a bajas concentraciones y que pueden tener lugar antes de que sean evidentes otros efectos subletales (Atchison et al., 1987; Scott & Sloman, 2004). Entre los que se han estudiado con más frecuencia están el comportamiento alimentario, el comportamiento de natación (o actividad de natación) y la osadía. El comportamiento alimentario abarca diferentes respuestas conductuales entre las que se incluyen los hábitos alimentarios, los modos de alimentarse, las preferencias tróficas y los mecanismos de detección (Volkoff & Peter, 2006). A su vez, la ingesta de alimento está relacionada con dos variables más el apetito y la saciedad. En cuanto al comportamiento de natación, se puede evaluar tanto el modo en cómo los peces se mueven (es decir, si se producen comportamientos aberrantes) como el grado de actividad que presentan (cantidad de tiempo que pasan nadando). El grado de actividad natatoria es una de las medidas más sensibles para detectar los efectos de la contaminación. Se han detectado alteraciones en este comportamiento durante la exposición a varios contaminantes a concentraciones tan bajas como 0,7 - 5% de sus valores de LC_{50} (Little & Finger, 1990). Por lo tanto, la evaluación de la actividad de natación de los peces se puede incorporar fácilmente en los protocolos de los test de toxicidad estándar (Little & Finger, 1990). En el caso de comportamientos como el de osadía, o propensión de un individuo a asumir riesgos, se ha relacionado con el grado de expansión de las poblaciones debido a que es más probable que individuos más osados exploren con más frecuencia nuevos hábitats y se dispersen más (Cote et al., 2010). También se ha relacionado con la habilidad de responder mejor frente a los depredadores ya que los individuos más atrevidos tienen más probabilidades de inspeccionar a un depredador (Dugatkin, 1992; Huntingford, 1976; Pitcher et al., 1986) y recibir información más precisa sobre este (Brown & Magnavacca, 2003; Dugatkin & Godin, 1992). Todo esto, sin embargo, puede ser una desventaja porque aquellos individuos más osados también son los que tienen un mayor riesgo de sufrir daños o morir (Dugatkin, 1992). Alteraciones tanto en el aprendizaje como en la capacidad para retener la memoria producidas

por la exposición a contaminantes pueden tener consecuencias directas sobre la eficacia biológica ("fitness") de los peces (Jacquin et al., 2020). Contaminantes como el aluminio y contaminantes orgánicos, pueden afectar gravemente la capacidad de memoria y el aprendizaje espacial de los peces, lo que puede traer como consecuencia una disminución de su habilidad para procesar información y para hacer frente a nuevos entornos (Grassie et al., 2013; Hong & Zha, 2019). En resumen, la exposición a la contaminación tiene unas consecuencias severas sobre la habilidad de los peces para aprender y memorizar información que es necesaria para escapar, encontrar comida y pareja, y para evitar áreas contaminadas (Jacquin et al., 2020). Es clave añadir estudios de comportamiento a los de biología de la conservación para mejorar la recuperación, gestión y conservación de las especies de peces autóctonas.

Ficha 1: *Squalius laietanus*

Nombres comunes: Bagre (Castellano) / Bagra catalana (Català) / Ebro Chub (Inglés) / Chevaine catalan (Francés)

Categoría de conservación: VU (Categoría IUCN regional y "*Catàleg de fauna amenaçada de Catalunya*") y debería de incluirse dentro del listado de especies protegidas (Decreto legislativo 2/2008, del 15 de abril).

Taxonomía: Clase: Actinopterygii, Orden: Cypriniformes, Familia: Cyprinidae.

Distribución: Es una especie autóctona del NE de España y SE de Francia, recientemente descrita por Doadrio et al. (2007). Está presente en la mayoría de las cuencas catalanas (NE península ibérica) y se la puede encontrar en los siguientes ríos: Port Bou, Francolí, Muga, Fluvià, Ter, Daró, Tordera, Besòs, Llobregat, Gaià, Segre (en la cuenca del Ebro) y Ebro.

Descripción: Presenta un cuerpo alargado recubierto de unas escamas con los márgenes de color negruzco, lo que las hace muy visibles. Tienen cabeza y ojos grandes, con boca en posición terminal, ligeramente subterminal, con el labio inferior incluido dentro del superior. Las aletas pélvicas y anales pueden presentar una coloración anaranjada, más acusada en la época reproductora, y la aleta anal tiene un margen posterior convexo. La coloración del cuerpo es principalmente parduzca, con tonalidades metalizadas doradas, más oscura en la zona dorsal y aclarándose hasta ser blanca en la zona ventral.

Biología y ecología: No suele superar los 20-30 cm de longitud furcal (normalmente las hembras son más grandes que los machos), aunque hay

ejemplares que pueden alcanzar los 40 cm. Tiene una longevidad de entre 9 y 12 años, y alcanza la madurez sexual alrededor de los 2 años. La época de reproducción es a finales de primavera principios de verano, entre los meses de abril y julio, cuando la temperatura del agua alcanza los 15 ºC. Las hembras pueden realizar una o varias puestas a lo largo de este periodo. La puesta se realiza sobre grava o piedras en zonas de poca corriente, y su fecundidad es muy alta. Esta especie puede vivir en ambientes de diferentes características, pero prefiere tramos fluviales del curso medio de los ríos, en aguas claras, con fondos de grava y con corriente moderada. Sin embargo, puede tolerar temperaturas altas (de hasta 30ºC) y aguas ligeramente eutrofizadas. Tiene costumbres gregarias y su dieta es omnívora, alimentándose principalmente de macroinvertebrados bentónicos, plantas acuáticas y algas, e incluso alevines o juveniles de otras especies de peces

Amenazas: Sus poblaciones se encuentran fragmentadas y en regresión, con la disminución de éstas en algunos tramos de los ríos más importantes (Ter, Llobregat, Segre y Ebro). Los principales factores de amenaza son la contaminación, la alteración del hábitat y la introducción de especies exóticas.

Adaptado de Aparicio et al. (2016)

Ficha 2: *Barbus meridionalis*

Nombres comunes: Barbo de montaña (Castellano) / Barb de muntanya (Català) / Mediterranean Barbel (Inglés) / Barbeau meridional (Francés)

Categoría de conservación: VU (Categoría IUCN regional y "*Catàleg de fauna amenaçada de Catalunya*"), incluida en los anexos II y V de la Directiva de Hábitats (UE) y debería de incluirse dentro del listado de especies protegidas (Decreto legislativo 2/2008, del 15 de abril).

Taxonomía: Clase: Actinopterygii, Orden: Cypriniformes, Familia: Cyprinidae.

Distribución: Presente en Europa, autóctona de las cuencas del NE de la península ibérica y el SE de Francia. En la península ibérica se encuentra en las cuencas del Muga, Fluvià, Ter, Daró, Ridaura, Tordera y Besòs (Cataluña). También se puede encontrar presencia de esta especie en ríos costeros pequeños como la Riera de Calonge y la Riera de la Valleta.

Descripción: Tiene un cuerpo alargado, con una cabeza relativamente grande y con un hocico alargado. En la boca presenta dos pares de barbillones cortos y los labios son anchos con un lóbulo medio visible pero poco desarrollado. Las aletas dorsal, anal y caudal tienen numerosas manchas negras. La aleta anal, en las hembras es más larga que en los machos. Presenta dientes faríngeos dispuestos en tres hileras a cada lado de la faringe. La base de las escamas está pigmentada. El barbo de montaña tiene una coloración marrón amarillenta con tonalidades doradas, que tiende a ser más oscura hacia el dorso y más clara en la parte ventral y, por todo el cuerpo presentan manchas oscuras.

Biología y ecología: Los ejemplares de esta especie rara vez alcanzan los 30 cm de longitud furcal, siendo las hembras las que alcanzan un mayor tamaño (25 cm de media). Tienen una longevidad de entre 7-10 años, y alcanzan la madurez sexual entre los 2 primeros años de vida, en el caso de los machos, y a los 2-3 años en el caso de las hembras. Se reproducen en primavera y a principios de verano, entre los meses de abril y julio. Durante todo este periodo las hembras pueden realizar puestas múltiples y fijan los huevos en el sustrato, al ser éstos ligeramente adhesivos. Los valores de fecundidad oscilan entre 2,000 y 20,000 huevos dependiendo del tamaño de la hembra. En cuanto al hábitat, esta especie tiene preferencia por los ríos entre pequeños y medianos con corriente de aguas frías y oxigenadas, y con sustratos de rocas, piedras o grava. Es una especie de hábitos bentónicos, y como tal, busca refugio debajo de las rocas, la vegetación, cuevas, etc. Su alimentación se basa en macroinvertebrados bentónicos como larvas de insectos, crustáceos, moluscos, etc.

Amenazas: Es una especie sensible a las alteraciones del hábitat, a los cambios de caudal, a la extracción de agua y a la competencia derivada de la introducción de especies exóticas. Aunque tiene una cierta tolerancia a la contaminación de carácter orgánico, sus poblaciones se encuentran en regresión. Ha reducido su abundancia en algunos tramos de ríos, como es el caso de los tramos medios y bajos del río Ter, y en algunos tramos de la cuenca del Besòs, aunque en este último se esta observando una recuperación de la especie.

Adaptado de Aparicio et al. (2016)

OBJETIVOS

El objetivo general de esta tesis ha sido evaluar los efectos de determinados contaminantes sobre la reproducción, el estrés fisiológico y el comportamiento dos especies de ciprínidos autóctonas del NE de Cataluña y el SE de Francia. Esta es una tesis que ha buscado analizar, desde un punto de vista multidisciplinar, y combinando trabajos de campo con experimentos de laboratorio, los efectos de la contaminación sobre las poblaciones de peces. Con este propósito general se establecieron los siguientes objetivos específicos:

- Evaluar, en un ambiente natural, los posibles efectos de la contaminación de origen industrial, frecuente en los ríos mediterráneos, sobre la biología reproductiva del bagre (*Squalius laietanus)*. A tal efecto se planteó describir el ciclo gonadal, calcular la fecundidad y analizar la posible presencia de histopatologías en ovarios y testículos para determinar cómo la presencia de contaminantes considerados disruptores endocrinos podía alterar la biología reproductiva de este ciprínido autóctono. El estudio fue complementado mediante un análisis por biomarcadores.

- Analizar el efecto de la contaminación urbana sobre el estrés fisiológico de *S. laietanus* mediante el análisis de cortisol en moco epidérmico. El objetivo fue validar una *técnica de análisi*s del cortisol por métodos no invasivos (moco epidérmico) y utilizarla como método para determinar el grado de bienestar de esta especie bajo los efectos de la mencionada contaminación.

- Evaluar el efecto de la contaminación por amonio sobre el comportamiento en el barbo de montaña (*Barbus meridionalis*) en condiciones

experimentales de laboratorio. En particular, se analizaría también si la exposición previa de los peces a este contaminante en un ambiente natural podría predisponerlos a una mejor tolerancia después de haber sido detoxificados en el laboratorio. Los peces serían sometidos a diferentes concentraciones subletales de amonio y se analizaría tanto su comportamiento alimentario (voracidad y saciedad) como su actividad de natación.

- Determinar el efecto de la contaminación por amonio sobre la personalidad de *B. meridionalis* en condiciones experimentales de laboratorio. En particular, se investigó sobre su capacidad de retención de memoria tras haber pasado por una experiencia de aprendizaje apetitivo basada en un test de osadía (en ausencia de amonio) y tras haber sido expuesto a diferentes concentraciones subletales de este contaminante en posteriores test.

INFORME DE LA DIRECTORA

La Dra. Dolors Vinyoles Cartanyà como directora de la tesis doctoral titulada "Efectos de la contaminación sobre la biología y el comportamiento de dos ciprínidos autóctonos de la Península Ibérica" realizada por Patricia M. Soler Vilaplana, presenta el siguiente informe sobre la contribución de la doctoranda en las publicaciones en coautoría que componen la tesis:

Capítulo 1. Sección 1.1. P. Soler, M. Solé, R. Bañón, E. García-Galea, M. Durfort, V. Matamoros, J.M. Bayona y D. Vinyoles (2020). Effects of industrial pollution on the reproductive biology of *Squalius laietanus* (Actinopterygii, Cyprinidae) in a Mediterranean stream (NE Iberian Peninsula). *Fish Physiology and Biochemistry* 46:247-264. doi:10.1007/s10695-019-00713-7

Contribución de la doctoranda: Procesamiento de muestras en el laboratorio, elaboración de la base de datos, análisis de los resultados y redacción del manuscrito.

Acerca de la revista: *Fish Physiology and Biochemistry* tiene un índice de impacto de 2.242 según el Journal Citation Reports (JRC) de 2019. Se encuentra en el número 15 de 53 en el área de "*Fisheries Sciences*" (Q2), en el número 47 de 81 en el área de "*Physiology Sciences*" (Q3) y en el número 210 de 216 en el área de "*Biochemistry & Molecular Biology Sciences*" (Q4).

Capítulo 1. Sección 1.2. A. Carbajal, P. Soler, O. Tallo-Parra, M. Isasa, C. Echevarria, M. López- Bejar y D. Vinyoles (2019). Towards non-invasive methods in measuring fish welfare: the measurement of cortisol concentrations in fish skin mucus as a biomarker of habitat quality. *Animals* 9:939. doi:10.3390/ani9110939

Contribución de la doctoranda: Participación en la recogida de muestras y toma de datos en el campo, procesamiento de muestras en el laboratorio y participación en la redacción del manuscrito.

Acerca de la revista: *Animals* tiene un índice de impacto de 2.323 según el Journal Citation Reports (JRC) de 2019. Se encuentra en el número 14 de 141 en el área de "*Veterinary Sciences*" (Q1) y en el número 10 de 63 en el área de "*Agricultural, Dairy & Animal Science*" (Q1).

Capítulo 2. Sección 2.1. P. Soler, M. Faria, C. Barata, E. García-Galea, B. Lorente y D. Vinyoles. Improving water quality does not guarantee fish health: effects of ammonia pollution on the behaviour of wild-caught pre-exposed fish. *PLOS ONE* (En revisión).

Contribución de la doctoranda: Participación en el diseño del experimento y en el trabajo de campo, desarrollo del experimento en el laboratorio, análisis de los resultados y redacción del manuscrito.

Acerca de la revista: *PLOS ONE* tiene un índice de impacto de 2.740 según el Journal Citation Reports (JRC) de 2019. Se encuentra en el número 27 de 71 en el área de "*Multidisciplinary Sciences*" (Q2).

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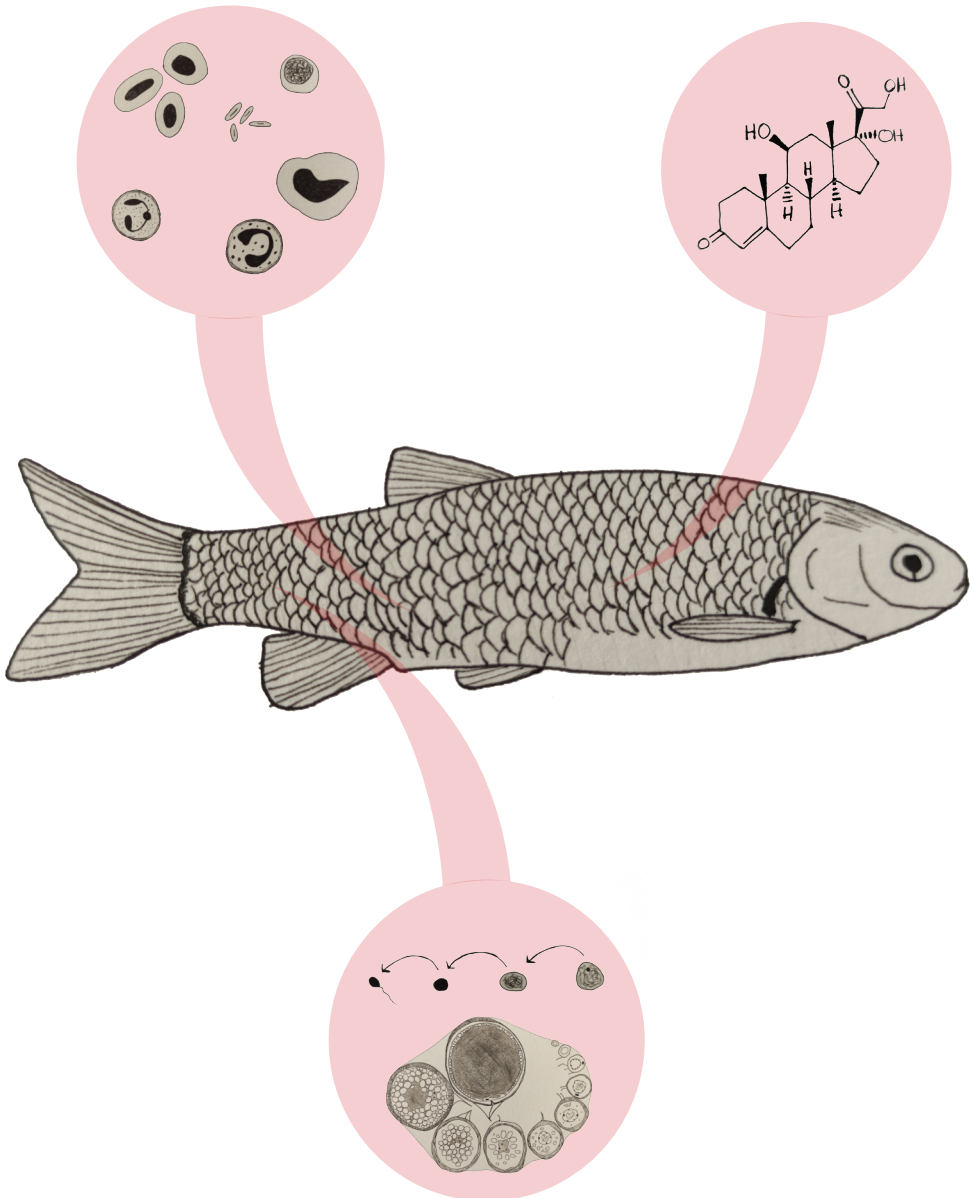
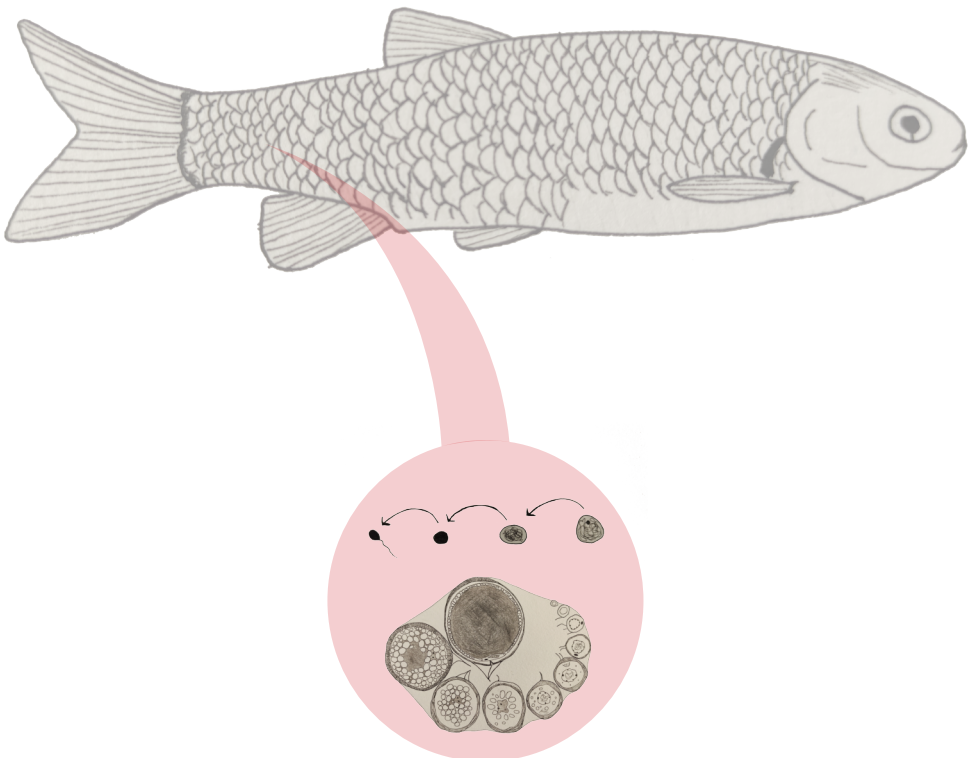
CAPÍTULO 1

EFECTOS DE LA CONTAMINACIÓN SOBRE LA REPRODUCCIÓN Y EL ESTRÉS FISIOLÓGICO DEL BAGRE



Sección 1.1.

Efectos de la contaminación industrial sobre la biología de la reproducción de *Squalius laietanus* (Actinopterygii, Cyprinidae) en un río Mediterráneo (NE península ibérica)



Soler et al. (2020). Effects of industrial pollution on the reproductive biology of *Squalius laietanu*s (Actinopterygii, Cyprinidae) in a Mediterranean stream (NE Iberian Peninsula). Fish physiology and Biochemistry, 46, 247-264. http://doi.org/10.1007/s10695-019-00713-7

Fish Physiol Biochem (2020) 46:247–264
https://doi.org/10.1007/s10695-019-00713-7

Effects of industrial pollution on the reproductive biology of *Squalius laietanus* (Actinopterygii, Cyprinidae) in a Mediterranean stream (NE Iberian Peninsula)

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Received: 13 May 2019 / Accepted: 29 September 2019 / Published online: 17 October 2019

Abstract Mediterranean rivers are severely affected by pollutants from industry, agriculture and urban activities. In this study, we examined how industrial pollutants, many of them known to act as endocrine disruptors (EDCs), could disturb the reproduction of the Catalan chub (*Squalius laietanus*). The survey was conducted throughout the reproductive period of *S. laietanus* (from March to July 2014) downstream an industrial WWTP located in the River Ripoll (NE Iberian Peninsula). Eighty fish (28 females and 52 males) were caught by electrofishing upstream and 77 fish (33 females and 44 males) downstream a WWTP. For both sexes, the gonadosomatic index (GSI) and gonadal histology were examined and related to water chemical analysis and fish biomarkers. Female fecundity was assessed using the gravimetric method. Fish from the polluted site showed enhanced biomarker responses involved in detoxification. Also, in the polluted site, lower GSI values were attained in both sexes and females displayed lower numbers of vitellogenic oocytes. Gonadal histology showed that all maturation stages of testicles and ovaries were present at the two study sites but fish males from the polluted site had smaller diameter seminiferous tubules. Water chemical analysis confirmed greater presence of EDCs in the river downstream the industrial WWTP. The chemicals benzotriazole and benzothiazole could be partially responsible for the observed alterations in the reproductive biology of *S. laietanus*.

Keywords Endocrine disruption · Benzotriazole · Benzothiazole · Gonadal histology · Female fecundity · Desynchronised spawning

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Introduction

Conservation of freshwater fish has reached a critical point worldwide. In Europe, about 37% of freshwater fish species are included under some threat category of extinction (Freyhof and Brooks 2011). The situation is even worse in the Iberian Peninsula, where roughly 70% of its fish species are endemic (Doadrio et al. 2011). Mediterranean rivers are considered *hotspots* in terms of biodiversity despite being among the most endangered ecosystems worldwide (Cuttelod et al. 2008); with a

long-history of anthropogenic insults, including pollution, introduction of exotic species and habitat degradation resulting in an alarming decline of fish populations (Clavero et al. 2004). In Catalonia (NE Iberian Peninsula), 2 out of the 29 species of native fish are regionally extinct and further 22 (75%) are under threat according to the IUCN (2012). Despite the efforts to implementing the European Water Framework Directive (2000/60/EC) (2000) with wastewater treatment practices, the quality of freshwaters remains worrying. Spillages of wastewater that have not been appropriately treated are still occurring and they invariably lead to the deterioration of water quality downstream (Maceda-Veiga et al. 2013). Moreover, as it is the case of streams in other semi-arid regions, Mediterranean-type climate streams are subjected to summer droughts that can worsen the harmful effects of pollution on the aquatic biota (Colin et al. 2016, 2017). This situation is likely to worsen due to the higher water demand for human use because of climate change (Mekonnen and Hoekstra 2016).

There is a strong body of evidence that an increasing number of chemicals, frequently found in rivers of the Iberian Peninsula, act as endocrine disruptors (EDCs) (Gorga et al. 2015; Kuster et al. 2008; Matamoros et al. 2010a; Osorio et al. 2016). Overall, among the EDCs' effects on the reproductive biology of fish are the occurrence of intersex condition (female oocytes in male testicular tissue), altered oogenesis in females, decreases in fecundity and population recruitment failure (Jobling et al. 1998; Kidd et al. 2014). Some EDCs can interact, even at very low environmental doses (ng/L), with the genesis of fish steroid and the processes of sexual maturation and differentiation (Vos et al. 2000). Fish reproductive output may be altered by the presence of EDCs in the environment (Nash et al. 2004) and this, in natural environments, may have a strong impact on the ecology and conservation status of native species.

EDCs are widespread in freshwater environments and both laboratory and field-based studies have shown reproductive alterations in fish at environmentally relevant exposures. However, it is unclear how these effects may affect fish populations in the wild (Mintram et al. 2018). The main inconvenient of most laboratory studies is that they do not fully reproduce natural conditions and/or do not consider the potential synergy of mixtures of contaminants. Indeed, mixed chemical exposure outcomes can differ significantly from those for single classes of EDCs (Mintram et al. 2018). Moreover, laboratory experiments may not account adequately for vital ecological processes and environmental variations (Galic et al. 2010). The assessment of reproductive disturbances, including histological aspects, is well documented for chemicals with well-known endocrine disruption abilities but the effects may vary depending on concentration, species and sex (Dietrich and Krieger 2009; Sumpter and Johnson 2005). There is a lack of knowledge on how pollutants discharged into Mediterranean rivers may be capable of modifying the reproductive biology of native fish, where most ecotoxicological studies have focused on exotic fish species such as carp (Fernandes et al. 2002; Solé et al. 2003; Lavado et al. 2004). The main aim of this study was to investigate how the presence of EDCs in the water composition of a Mediterranean-type climate stream could alter the reproductive output in a species of native fish. This knowledge is a necessary tool for their conservation. Current approaches for the environmental risk assessment (ERA) of chemicals, including EDCs, lack certainty for protecting wildlife because of differences in species sensitivity to pollutants (Hamilton et al. 2016). Typically, ERA relies on assessments extrapolated from animal laboratory studies at pollutant concentrations not observed in nature (Mintram et al. 2018).

The species selected was the Catalan chub, *Squalius laietanus* Doadrio et al. 2007, a freshwater fish endemic to the NE Spain and SE France. During the 1990s, this species suffered an important decline in the rivers of NE Spain due to pollution, habitat alterations and the introduction of exotic species (Doadrio et al. 2007). Both sexes reach the first sexual maturity when they are 2 years old (females at 10 cm and males at 7 cm long) and the breeding period extends from April to July (Casals 2005). Since the *S. laietanus* population is decreasing, it has been listed as *vulnerable* in both the IUCN (2012) and the Spanish catalogues of threatened species (Royal Decree 139/2011 of 4th February). However, this species has currently a relatively wider distribution (Aparicio et al. 2016), so investigating the effects of pollution on its reproduction can help improve its management and conservation in the future. In this study, the concentration of chemicals potentially acting as EDCs was evaluated through water chemical analyses, and fish stress responses were complemented using biomarkers. The gonadal cycle and the gonadal histology of both sexes, as well as female fecundity, were examined upstream and downstream from the textile dye WWTP, throughout the species breeding period. The results of this study help to clarify the role of pollutants on the freshwater fish decline in semi-arid regions.

Methods

Study area

This study was conducted in the mid stretches of the River Ripoll, a 39.5 km tributary of the River Besòs basin located in the NE of the Iberian Peninsula. It is a river of Mediterranean-type climate with alternating periods of droughts and flooding. The polluted site was 1.6 km downstream from the discharge of a WWTP from a textile industry near Castellar del Vallès (41° 34′ 17.88″ N, 2° 06′ 01.40″ E). The control site, named Les Arenes (41° 38′ 45.05″ N, 2° 03′ 24.07″ E), was 2.7 km upstream from the WWTP of the textile dye plant (Fig. 1). In the polluted site (thereafter, *downstream WWTP*), the river width during the sampling period was 3–5 m and the average water depth was 20–40 cm. In the control site (thereafter, *upstream WWTP*), the river width was 4–6 m and the average water depth was 20–30 cm. A small dam located between the two sampling sites prevented the passage of fish from one point to another.

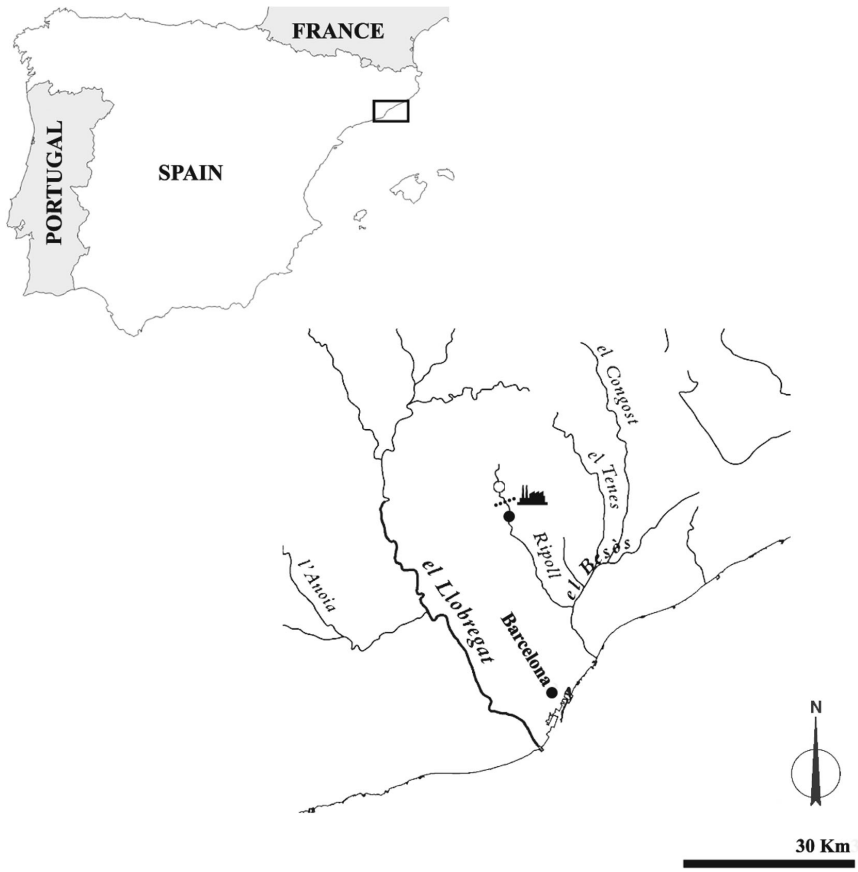

Fig. 1 Map of the sampling sites in the Ripoll River (Besòs basin, NE of the Iberian Peninsula). The black point indicates the location of the polluted site (1.6 km downstream from the WWTP of a textile dye plant) and the white point indicates the location of the control site (2.7 km upstream from the WWTP). A small dam (dashed line) located just above the textile dye plant (factory symbol) prevented the passage of fish from one site to the other

Site characterisation by water chemical analysis

Water chemical analysis of this study was carried out following the protocol optimized for freshwater Mediterranean watercourses by Matamoros and Bayona (2006). Water samples were collected at the two sites in March (spring sampling) and July (summer sampling) using pre-cleaned 1 L amber glass bottles. Four water samples were, therefore, collected for chemical analysis. Samples were kept at 4 °C, filtered within the next 24 h and processed as previously reported (Matamoros and Bayona 2006). A sample volume of 500 mL was percolated through a previously activated polymeric solid-phase extraction cartridge (200 mg Strata-X cartridge). Elution was performed with 10 mL of hexane/ethyl acetate (1:1). The eluted extract was evaporated to ca. 100 μL under a gentle nitrogen stream, and 186 ng of triphenylamine was added as an internal standard. Finally, the extract was reconstituted to 300 μL with ethyl acetate. Methylation of the acidic carboxyl group was performed in a hot gas chromatography (GC) injector (290 °C) by adding 10 μL of TMSH solution (0.25 mol/L in methanol) to a 50 μL sample before injection. Derivatized samples were analysed in a Bruker 450-GC gas chromatograph coupled to a Bruker 320-MS triple quadrupole mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA) in the electron impact mode (70 eV ionization energy) fitted with a chromatographic column TRB5-MS coated with 5% diphenyl 95% dimethylpolysiloxane (20 m × 0.18 mm i.d., 0.18 μm film thickness) from Teknokroma (Sant Cugat del Vallès, Spain). A 5 μL volume of sample was injected in the PTV mode. Chromatographic conditions, data processing and validation of the methodology have been described elsewhere (Matamoros et al. 2010a; Matamoros and Bayona 2006).

Fish sampling

Fish were sampled by electrofishing using a portable unit which generated up to 200 V and 3 A pulsed DC throughout the breeding period (March, April, June and July 2014). Samplings took place in the middle of the month. Samplings during April (which took place at the end of the month) were considered representative of the reproductive state of the fish between April and May (Casals 2005). The number of individuals used in each section of this study is specified in Table 1. The fish (except those used in the biomarker analysis) were sacrificed with an overdose of MS-222 and frozen at − 20 °C. All fish were sized (fork length, FL ± 0.1 mm) and weighed (eviscerated mass, W_E ± 0.01 g), and

Table 1 Number of fish used in each section of this study: site characterization by biomarkers (B), gonadal development and female fecundity (G and F) and histological analysis (H). Fork length (FL, mean ± S.D) is shown for each sample

		Total	FL (mm)	B	G and F	H
Upstream ♀♀ ($n = 27$)	March	5	143 ± 12.2	5	4	5
	April	7	170.3 ± 20.9	0	4	5
	June	9	205.9 ± 22.5	0	9	5
	July	6	151.8 ± 11.2	6	4	5
Downstream ♀♀ ($n = 36$)	March	9	178.3 ± 19.4	9	6	5
	April	5	164.2 ± 19.5	0	5	5
	June	7	154.7 ± 37.4	0	7	5
	July	15	86.4 ± 30.5	15	4	3
Upstream ♂♂ ($n = 35$)	March	11	157.4 ± 18.1	11	5	5
	April	5	156 ± 14.5	0	5	5
	June	5	154.4 ± 19.2	0	5	5
	July	14	145.6 ± 12.7	14	0	0
Downstream ♂♂ ($n = 22$)	March	11	152.7 ± 14.4	11	5	5
	April	5	152 ± 9.8	0	5	5
	June	5	116.6 ± 10.7	0	5	5
	July	1	–	1	0	0

gonads (gonad mass, $W_G \pm 0.01$ g) and liver (liver mass, $W_L \pm 0.01$ g) were removed and weighed. All fishes used in this study were adults. Fish collection was approved by the Regional Government of Catalonia (Ref. AP/007). All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures were conducted in accordance with the European Directive for animal experimentation (2010/63/EU). One of the co-authors holds a category C FELASA certificate that regulates the use of animals for experimental and other scientific purposes.

Site characterisation by the use of fish biomarkers

A subsample of 36 fish from upstream WWTP and 36 fish from downstream WWTP were transported alive in aerated tanks to the laboratory facilities (Table 1). Fish were sacrificed with an overdose of MS-222. A portion of muscle and the whole liver were frozen in dry ice and liquid nitrogen, respectively. Tissues were stored at − 80 °C until biochemical determinations.

A portion of muscle (≈ 0.2 g) was homogenised in ice-cold 50 mM, and the whole liver was homogenised in 100 mM buffer phosphate (pH 7.4) containing 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenanthroline, 0.1 mg/mL trypsin inhibitor and 1 mM ethylenediaminetetraacetic acid (EDTA) at a 1:4 (w:v) ratio using a Polytron® blender. The homogenates were centrifuged at 10,000 g × 30′ at 4 °C and the supernatants obtained (S10) were used for the enzymatic determinations in muscle and liver.

All assays were carried out in triplicate at 25 °C, except 7-ethoxyresorufin O-deethylase (EROD) and 7-benzyloxy-4-[trifluoromethyl]-coumarin-O-debenzyloxylase (BFCOD), which were at 30 °C, in 96-well plates using a TECAN Infinite M200 microplate reader (Salzburg, Austria). A detailed description of this multi-biomarker approach has been recently detailed elsewhere (Crespo and Solé 2016). Briefly, EROD and BFCOD activities were measured using 50 μL of undiluted liver homogenate samples (S10) with a reaction mixture containing 0.2 mM NADPH, 3.3 μM 7-ethoxyresorufin (ER) or 0.02 mM NADPH and 200 μM BFC in 100 mM phosphate buffer (pH 7.4). The reaction was followed over 10 min in 96-well plates using the fluorescence for hydroxyl metabolites formation at fluorometric conditions as described in the former reference. Carboxylesterase (CbE) activity was measured using 25 μL of the appropriately diluted S10 fraction of the liver using 200 μL of α-naphthyl acetate (αNA; 250 μM final concentration in well) or 1 mM final concentration of ρ-nitrophenyl acetate (ρNPA) as substrates. Glutathione S-transferase (GST) activity was measured in 25 μL of diluted S10 using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. The final reaction mixture contained 1 mM CDNB and 1 mM reduced glutathione (GSH). Catalase (CAT) activity was measured using 50 mM H_2O_2. Glutathione reductase (GR) activity was measured using 0.9 mM oxidized glutathione (GSSG) and 0.09 mM nicotinamide adenine dinucleotide phosphate (NADPH) and total glutathione-peroxidase (GPX) activity was measured using 2.5 mM reduced glutathione (GSH), 1 Unit glutathione reductase (GR), 0.625 mM cumene hydroperoxide (CHP) and 0.3 mM NADPH.

In muscle, acetylcholinesterase (AChE) and propionilcholinesterase (PrChE) activities were measured in fourfold diluted (AChE) or undiluted S10 (PrChE). In each microplate well, 25 μL of sample were mixed with 150 μL of 5,5′-dithio-bis-2-nitrobenzoate (DTNB; 270 μM), and the reaction was initiated by adding 50 μL of the respective thio-substrates, all of them at 1 mM final concentration. Lactate dehydrogenase (LDH) activity was also measured in muscle using 25 μL of diluted S10 and mixed with 150 μL NADH (300 μM) and 50 μL pyruvate (4.5 mM). The total protein contents of the samples for all of the assays were determined following Bradford (1976) adapted to microplate, using the Bradford Bio-Rad Protein Assay reagent and bovine serum albumin (BSA) as the standard (0.1–1 mg/mL).

Before completing the processing of the samples, the hepatosomatic index (HSI) was calculated according to the formula: $HSI = W_L/W_E \times 100$. This index can provide information related to the fitness of fish, but also on its energy reserves, potential diseases and exposure to exogenous stresses (Schmitt and Dethloff 2000).

Gonadal development and female fecundity

The gonadal cycle was described through the gonadosomatic index (GSI) calculated according to the formula: $GSI = W_G/W_E \times 100$. A subsample of 36 fish (21 females and 15 males) from upstream WWTP and a subsample of 37 fish from downstream WWTP (22 females and 15 males) were analysed over the breeding

period of *S. laietanus* (Table 1). Means and 95% CI of GSI were calculated for sex and site.

Oocyte development and female fecundity were examined by the gravimetric method (Bagenal 1978) using the same females that were used for the calculation of the GSI. A portion of one ovary from each female was weighted and transferred into Gilson's fluid and, once the gonads had been separated, oocytes were poured into a column of 18 sieves with a range of mesh-sizes (from 0.15 to 1.8 mm) and flushed with running water to facilitate their descent. Based on oocyte diameter frequency distributions, developmental stages corresponding to previtellogenic (immature) and vitellogenic (ripening, or in maturation process, and ripe) oocytes were identified. Fecundity, defined here as the total number of ripening or vitellogenic oocytes in the ovaries prior to spawning (Bagenal 1978), was determined by counting all vitellogenic oocytes in gravid females (i.e. in females with ripe oocytes). Mature females could be recognized by the presentation of amber-coloured oocytes (or yolk eggs) in the ovaries. Fecundity (F) was calculated as: ln $F = a + b$ ln FL (Bagenal 1978). Since females downstream WWTP were smaller in size, only females in the upstream site that were within a similar range of lengths (from 175 to 215 mm of FL) were taken into account to avoid an underestimation of fecundity in the polluted site. Only mature females prior to spawn (i.e. with amber-coloured oocytes in the ovaries) were considered for the calculation of fecundity. For females downstream WWTP, due to a lack of synchronization of the time of egg laying, females throughout the breeding period were considered for the calculation ($N = 3$ in March, $N = 3$ in April and $N = 1$ in June). Upstream WWTP females prior to spawn were found in June ($N = 6$).

Histological analyses

Gonads of 20 females (5 per month over the reproductive period of *S. laietanus*) and 15 males (5 per month except in July, which was not analysed) were used in the histological analyses from upstream WWTP. In the polluted site, gonads of 18 females and 15 males (no males sample in July) were analysed (Table 1). Ovaries and testes were fixed in a 10% Bouin's solution, dehydrated with increasing ethanol, cleared in xylene and finally embedded in paraffin wax (Dietrich and Krieger 2009). Gonad sections of 5 μm were cut on a PFM Rotary 3003 microtome (pfm medical, Germany) and stained with conventional Delafield haematoxylin and eosin. Stained gonad sections were examined in an Olympus CH-2 microscope and photographed with a Nikon DS-Ri1 digital camera.

Five oocyte stages were defined according to their maturation status: stage I, primary oocytes; stage II, large primary oocytes with Balbiani body and pleomorphic nucleoli-like bodies bordering the nuclear envelope; stage III, early vitellogenic stage; stage IV, advanced vitellogenic stage with many variably sized yolk vesicles; and stage V, mature oocyte with yolk mass occupying the entire oocyte. An additional, non-defined stage that did not fit the former criteria was scored: atretic oocyte, a set of small cells with strong staining and irregular shapes. Frequency and size of each stage of development were calculated following Dietrich and Krieger (2009). For males, four stages of maturation were defined: spermatogonia, spermatocytes, spermatids and spermatozoa. Quantitative methods proposed by Smith (1978) and Gimeno et al. (1998) were used to determine the percentage of testicular stages and to measure the diameter of seminiferous tubules.

A maturity index for each fish was calculated according to the standardized criteria for ovaries and testes established in Baumann et al. (2013). This is an enhancement to the OECD Histopathology Guidance Document (Johnson et al. 2010). The maturity index gives a fixed value (ranging from zero to one) for each fish according to its maturity stage and it increases as fish sexual maturity progresses.

Statistical analysis

In order to test whether biomarkers and HSI showed differences between sites, a generalized lineal model (GLM) was performed for each of them. The structure of these models was the same for both a biomarker as the dependent variable and *season* (two levels: *Spring* and *Summer*) and *site* (two levels: *upstream* and *downstream*) as factors together with their interaction. The gamma distribution and the identity link function were assumed for all biomarker GLMs. Due to the multiple testing, resulting p values where adjusted by the method of Benjamini and Hochberg (1995). GSI differences between sites were tested performing a GLM for each sex. Both male and female GLM's used GSI as the dependent variable, *month* (four levels in female GLM: March, April, June and July; three levels in male GLM: March, April and June) and *site* (two levels:

upstream and downstream) as factors together with their interaction. The gamma distribution and the identity link function were used.

For the analysis of the variability of the number of oocytes between sites, a GLM was performed per development stage (vitellogenic and non vitellogenic oocytes). Each model used the number of *vitellogenic* and *non vitellogenic* oocytes as the dependent variable. *Month* (four levels: March, April, June and July) and *site* (two levels: upstream and downstream) were added as fixed factors together with their interaction. The negative binomial distribution was assumed (Poisson distribution was discarded due to overdispersion). The effect of site on female fecundity was tested by means of a GLM with *number of vitellogenic oocytes* as the dependent variable. The *fork length* was added as a covariate, *site* (two levels: upstream and downstream) as factor as well as the interaction between them. The negative binomial distribution and the log link function for count data were assumed (Poisson distribution was discarded due to overdispersion).

In order to analyse how the degree of maturity was affected by the WWTP discharges, a generalized lineal mixed model (GLMM) was performed for each sex. Both male and female models had the same structure: *maturity index* was the dependent variable, *month* (four levels in female GLMM: March, April, June and July; three levels in male GLMM: *M*arch, April and June) and *site* (two levels: upstream and downstream) were added as fixed factors together with their interaction. Given that we obtained six maturity index values from each individual (each value from a different gonad section), *specimen* was added to the model as a random factor.

The effect of each oocyte development stage on oocyte size was tested performing a GLMM. Each model used cellular size of a stage as the dependent variable. *Month* and *site* (two levels: upstream and downstream) were added as fixed factors together with their interaction. Levels of the *month* factor were not the same for all models given that the development stages were not present during the same months. *Specimen* was also included as a random factor as we used several measures for each female. The gamma distribution was assumed. In order to test whether the diameter of the seminiferous tubules differed between sites, a GLMM was performed. *Tubule diameter* was the dependent variable and *month* (three levels: March, April and June) and *site* (two levels: upstream and downstream) were the fixed factors together with their interaction. Tubule diameter was measured from six different gonad sections for each specimen, thus *specimen* was added as a random factor. The gamma distribution and the identity link function were assumed.

All analyses were conducted in R 3.4.3 (R Core Team 2017). GLMs assuming a gamma distribution were performed with glm() function (package *stats*: R Core Team) and the negative binomial GLM (female fecundity model) with glm.nb()(package *MASS*: Venables and Ripley 2002). GLMM was performed with glmer() (package *lme4*: Bates et al. 2015). Non-significant interactions were removed from final models. Homogeneity and normality of residuals were visually checked for all models.

Results

Site characterisation by water chemical analysis and the use of fish biomarkers

The physico-chemical water parameters at the two sites at the spring and summer samplings are detailed in Table 2. These water quality indicators suggested poorer water conditions downstream WWTP, especially in summer. The chemical analysis of selected contaminants of emerging concern (CEC), including some EDCs, is presented in Table 3. Among the CEC detected, only benzotriazole, benzothiazole and their metabolites were found at high levels in water, particularly at the polluted site at both sampling times.

Results concerning liver and muscle biomarkers are summarized in Table 4. Results in HSI showed a significant effect of the *site* factor ($\chi^2_1 = 25.21,\ p < 0.001$), being the highest values downstream WWTP. For liver biomarkers, significant effects of the *season* factor for CAT ($\chi^2_1 = 14.29,\ p < 0.001$), GR ($\chi^2_1 = 32.71, p < 0.001$), ERod ($\chi^2_1 = 13.70, p < 0.001$) and BFCOD ($\chi^2_1 = 46.75, p < 0.001$) were found, which consisted in higher values in July (summer). The *site* factor showed a significant effect for GR ($\chi^2_1 = 94.08, p < 0.001$), GPX ($\chi^2_1 = 169.40, p < 0.001$), CbE-α NA ($\chi^2_1 = 23.42, p < 0.001$), CbE-ρ NPA) ($\chi^2_1 = 16.15, p < 0.001$), GST ($\chi^2_1 = 17.21, p < 0.001$), ERod ($\chi^2_1 = 19.82, p < 0.001$) and BFCOD

Table 2 Physico-chemical water parameters upstream and downstream WWTP where fish, *S. laietanus* were sampled during spring (March) and summer (July) 2014

	March		July	
	Upstream	Downstream	Upstream	Downstream
Flow (L/s)	239.5	241.2	128.4	124.0
Temperature (°C)	14.2	16.9	24.0	23.0
Oxygen (mg/L)	7.23	6.2	7.1	8.03
Conductivity (μS/cm)	728	3680	709	4777
pH	8.1	8.3	8.0	8.3
NH_3 (mg/L)	0.04	0.40	0.04	5.30
NO_2 (mg/L)	0.008	0.90	0.008	5.51
NO_3 (mg/L)	0.126	19.6	0.056	10.6
PO_4 (mg/L)	0.1	1.0	0.1	0.8
SO_4 (mg/L)	15.8	414.1	17.9	464.0
Cl (mg/L)	40.0	987.0	31.9	1088

($\chi^2_1 = 10.31, p = 0.002$). All of these biomarkers presented higher levels in the fish collected downstream WWTP. A significant interaction between *season* and *site* factors was only found for BFCOD ($\chi^2_1 = 44.88, p < 0.001$), which consisted of higher values in summer only downstream WWTP. In muscle, AChE and PrChE biomarkers showed a similar pattern: a significant interaction between *season* and *site* factors (AChE: $\chi^2_1 = 19.06, p < 0.001$; PrChE: $\chi^2_1 = 7.35, p = 0.010$) as well as a significant effect of the *season* (AChE: $\chi^2_1 = 116.11, p < 0.001$; PrChE: $\chi^2_1 = 126.59, p < 0.001$). For LDH, a significant effect of the *season* factor was detected ($\chi^2_1 = 57.11, p < 0.001$) which consisted of a higher value in summer. In summary, biomarkers showed a general tendency to show higher values downstream than upstream WWTP on the one hand, and in summer than in spring on the other.

Gonadal development and female fecundity

Female GSI model showed a significant interaction between *month* and *site* factors ($\chi^2_2 = 12.30, p = 0.006$) and a significant effect of the *month* factor ($\chi^2_3 = 24.39, p < 0.001$). Females upstream WWTP showed maximum GSI values in April and June while females downstream did not reach any peak (Table 5). In males, the *month* ($\chi^2_2 = 8.53, p = 0.014$) and the *site* ($\chi^2_1 = 12.79, p < 0.001$) factors were significant showing a maximum in April upstream WWTP (Table 5). In summary, both females and males displayed the highest GSI values upstream WWTP.

Size-frequency distributions of previtellogenic (≤ 0.35 mm) and vitellogenic oocytes in maturation process (> 0.35 mm) during the breeding period of *S. laietanus* at the two sampling sites are shown in Fig. 2. A different pattern in size-frequency distributions of vitellogenic oocytes was observed. Females upstream WWTP showed a maximum number of vitellogenic oocytes in June with a peak of mature amber-coloured oocytes (≥ 0.80 mm). By contrast, females downstream had lower amounts of vitellogenic oocytes (in maturation process) throughout the entire breeding period with no clear reproductive peak, and a small group of mature oocytes in April. The statistical analysis of vitellogenic oocytes showed a significant interaction between *month* and *site* factors $\left(\chi^2_3 = 18.487, p < 0.001\right)$ as well as a significant effect of the *month* $\left(\chi^2_3 = 44.256, p < 0.001\right)$ and *site* $\left(\chi^2_1 = 6.484, p = 0.011\right)$ factors. For previtellogenic oocytes, a significant interaction between *month* and *site* factors was found $\left(\chi^2_3 = 12.167, p = 0.01\right)$ as was a significant effect of the *month* factor $\left(\chi^2_3 = 35.685, p < 0.001\right)$. Females upstream WWTP had more vitellogenic oocytes than females downstream.

As the covariate *female length* did not affect in the female fecundity model, it was removed from the model. The final model showed a significant effect of the *site*

Table 3 Occurrence of CEC in river water samples (ng/L) during spring (March) and summer (July) 2014

	March		July	
Compound name	Upstream	Downstream	Upstream	Downstream
Azole derivatives (corrosion inhibitors)				
5-Methyl benzotriazole	< LOD	388	33	61
Benzothiazole*	< LOD	506	50	61
OH-Benzothiazole	27	143	17	265
Benzotriazole*	< LOD	438	92	1289
Herbicides				
2,4-D	5	4	< LOD	< LOD
Diazinon*	< LOD	135	< LOD	< LOD
Fragances				
Galaxolide*	8	18	20	18
Methyl dihydrojasmonate	37	160	99	38
Tonalide*	9	27	26	13
Cashmeran	5	8	< LOD	< LOD
Flame retardants				
Tri(2-chloroethyl) phosphate	5	34	9	15
Tributyl phosphate	11	25	19	54
Triphenyl phosphate	< LOD	17	< LOD	< LOD
PPCPs				
Bisphenol A*	10	< LOD	< LOD	29
Carbamazepine	< LOD	12	9	11
Ibuprofen*	10	< LOD	< LOD	134
Acetominophen	N.A.	N.A.	< LOD	104
Caffeine	14	51	59	234
Methylparaben*	< LOD	< LOD	23	104
Oxybenzone*	< LOD	11	< LOD	13

PPCP pharmaceuticals and personal care products. Limit of detection LOD = 3 ng/L. EDCs for which an effect on fish has been described are marked with an asterisk

factor ($\chi^2_1 = 13.93, p < 0.001$): upstream females had about 2.6 times (CI 1.5–4.6) more vitellogenic oocytes than downstream females.

Histological analyses

Females and males collected at both sites displayed all the defined stages of maturation. In Fig. 3, details of the different stages are presented. The female maturity index model showed a significant interaction between *month* and *site* factors ($\chi^2_3 = 8.609, p = 0.035$), a significant effect of the *month* factor ($\chi^2_3 = 33.44, p < 0.001$). Both upstream and downstream females showed a quite similar maturation pattern, but upstream females reached a maturation peak in June that was absent for downstream females (Fig. 4). For males, only the *month* factor was significant in the maturity index model ($\chi^2_2 = 16.98, p < 0.001$). In summary, upstream and downstream males showed an identical maturation pattern which consisted of higher maturity index values in April and June than in March.

Oocyte size expressed as area units (in μm^2) could only be accurately measured from the stage II of maturation onwards (Table 6). The *site* factor was significant for stages II ($\chi^2_1 = 9.57, p = 0.002$) and IV ($\chi^2_1 = 3.89, p = 0.05$), being cells of both stages larger for females downstream WWTP. Occurrence of atretic did not differ per site or season. In the tubule diameter model, only a significant effect of the *site* factor ($\chi^2_1 = 6.51$, $p = 0.011$) was found, with males upstream WWTP having a

Table 4 Biomarkers analysed in liver and muscle of *S. laietanus* at the two sampling sites (upstream and downstream WWTP) during spring (March) and summer (July) 2014. Number of replicates (*n*) and means ± SE are shown

	March		July	
	Upstream ($n = 16$)	Downstream ($n = 20$)	Upstream ($n = 20$)	Downstream ($n = 16$)
HSI	1.0 ± 0.1a	1.3 ± 0.1b	0.9 ± 0.1a	1.2 ± 0.1b
Biomarkers in liver				
EROD[1]	1.6 ± 0.2a	3.0 ± 0.3b	2.7 ± 0.3b	4.1 ± 0.4c
BFCOD[1]	1.5 ± 0.2a	2.7 ± 0.3b	1.4 ± 0.2a	10.9 ± 1.9c
CbE-aNA[2]	312.1 ± 11.2a	376.9 ± 12.0b	313.2 ± 10.3a	378.0 ± 12.6b
CbE-pNPA [2]	510.4 ± 41.4a	742.7 ± 51.3b	611.9 ± 43.8a	844.1 ± 57.0b
GST[2]	692.2 ± 35.7a	872.6 ± 38.8b	696.5 ± 32.9a	876.9 ± 41.4b
CAT[3]	1050.9 ± 55.6a	1143.4 ± 54.5a	1309.1 ± 60.0b	1401.6 ± 66.5b
GPX[2]	210.8 ± 9.0a	372.3 ± 12.6b	211.6 ± 8.2a	373.0 ± 13.0b
GR[2]	25.3 ± 1.0a	40.0 ± 1.3b	33.3 ± 1.1c	48.1 ± 1.5d
Biomarkers in muscle				
AChE[2]	45.8 ± 2.9a	46.0 ± 2.6a	74.3 ± 4.8b	115.0 ± 7.2c
PrChE[2]	12.7 ± 0.8a	12.5 ± 0.7a	26.1 ± 1.5b	33.7 ± 2.2c
LDH[2]	1119.1 ± 100.1a	1135.0 ± 39.6a	2320.0 ± 167.3b	1918.1 ± 154.7b

Activities of EROD: 7-ethoxyresorufin O-deethylase, BFCOD: 7-benzyloxy-4-[trifluoromethyl]-coumarin-O-debenzyloxylase, CbE: carboxylesterase, GST: glutathione S-transferase, CAT: Catalase, GPX: total glutathione-peroxidase, GR: glutathione reductase, AChE: acetylcholinesterase, PrChE: propionilcholinesterase, LDH: lactate dehydrogenase. The hepatosomatic (HSI) index is also indicated. Units of enzymatic activity: [1] pmol/min/mg prot, [2] nmol/min/mg prot, [3] μmol/min/mg prot. In order to test statistical differences, a GLM (generalized lineal model) was performed for each variable. For each variable, not significantly different observations are labelled by the same letter. All significant differences are $p \leq 0.05$

higher tubule diameter (mean = 222.99 μm; CI = 184.09–261.88) than males downstream WWTP (mean = 164.80 μm; CI = 117.63–211.97).

Discussion

Former studies carried out in the same area of the Ripoll River revealed that the site downstream from the textile dye plant, even after wastewater treatment, was still highly polluted (Colin et al. 2016, 2017; Maceda-Veiga et al. 2013; Blanco et al. 2019). In this study, several CEC, such as pharmaceuticals, sunscreen compounds, fragrances, antiseptics, fire retardants, surfactants, pesticides and plasticizers, were detected in water. Among them benzotriazole, benzothiazole and their metabolites were found at higher concentrations in the polluted site at both sampling times. Traditional fish

Table 5 Gonadosomatic index (GSI) values for females and males of *S. laietanus* at the two sampling sites (upstream and downstream WWTP). For males, no data from July was analysed. Means and 95% confidence interval (CI) are shown. A GLM (generalized lineal model) was performed for each sex. Not significantly different observations are labelled by the same letter. All significant differences are $p \leq 0.05$

	GSI ♀♀				GSI ♂♂			
	Upstream		Downstream		Upstream		Downstream	
	Mean	CI 95%	Mean	CI 95%	Mean	CI 95%	Mean	CI 95%
March	4.76a	3.46–6.06	3.47a	2.42–4.52	4.40a	2.97–5.82	2.14a	1.23–3.04
April	5.87b	4.27–7.46	4.58a	3.02–6.13	6.35b	4.51–8.18	4.09a	2.49–5.68
June	6.62b	4.97–8.27	5.33a	3.74–6.93	4.35a	2.93–5.77	2.09a	1.20–2.97
July	2.89a	1.87–3.91	1.60a	0.94–2.26	–	–	–	–

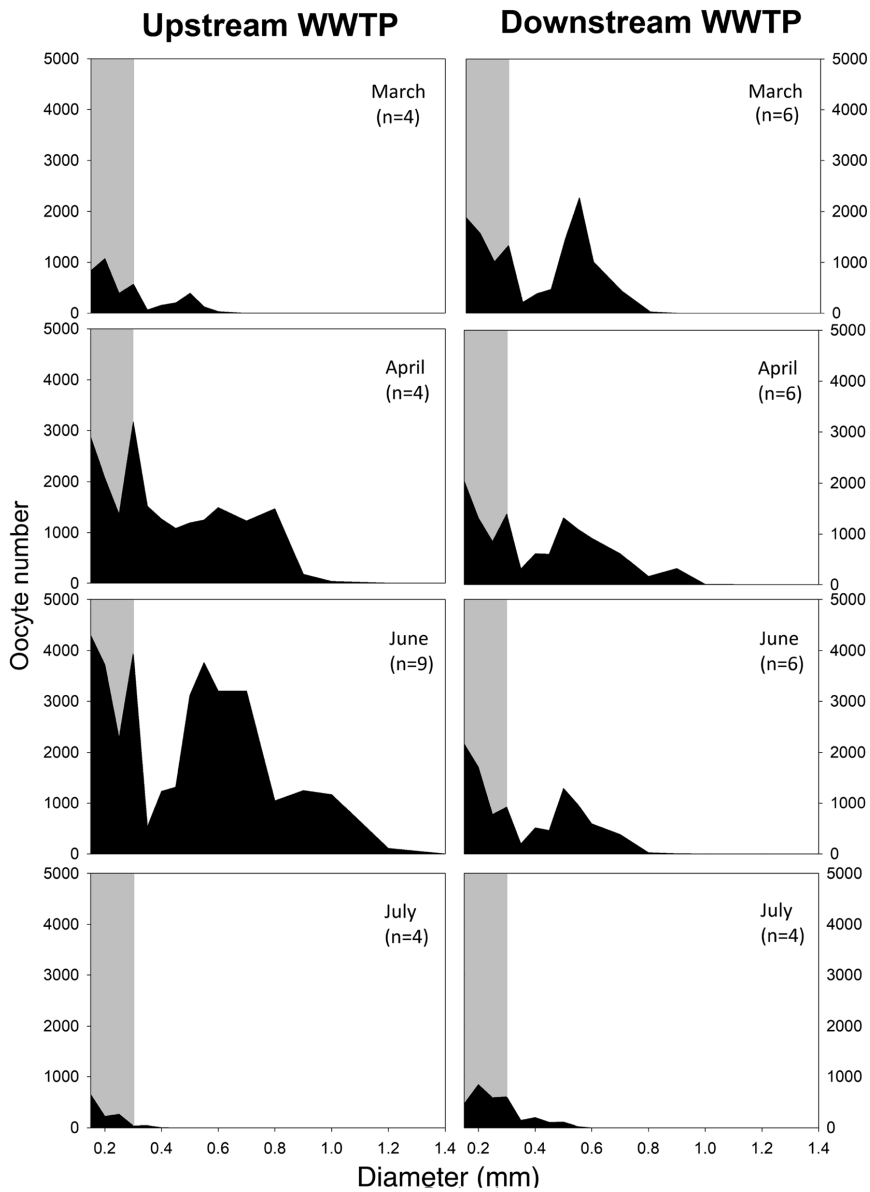

Fig. 2 Size distributions of oocytes over the reproductive period of *S. laietanus* in the two studied sites (upstream and downstream from the WWTP) of the Ripoll River. Previtellogenic oocytes ≤ 0.35 mm in diameter (grey area) and vitellogenic oocytes > 0.35 mm in diameter (white area) are shown. Females upstream WWTP showed a peak of ripe oocytes (> 0.8 mm) in June. Females downstream had lower amounts of vitellogenic oocytes (in maturation process) throughout the entire breeding period with no clear reproductive peak, and a small group of mature oocytes in April

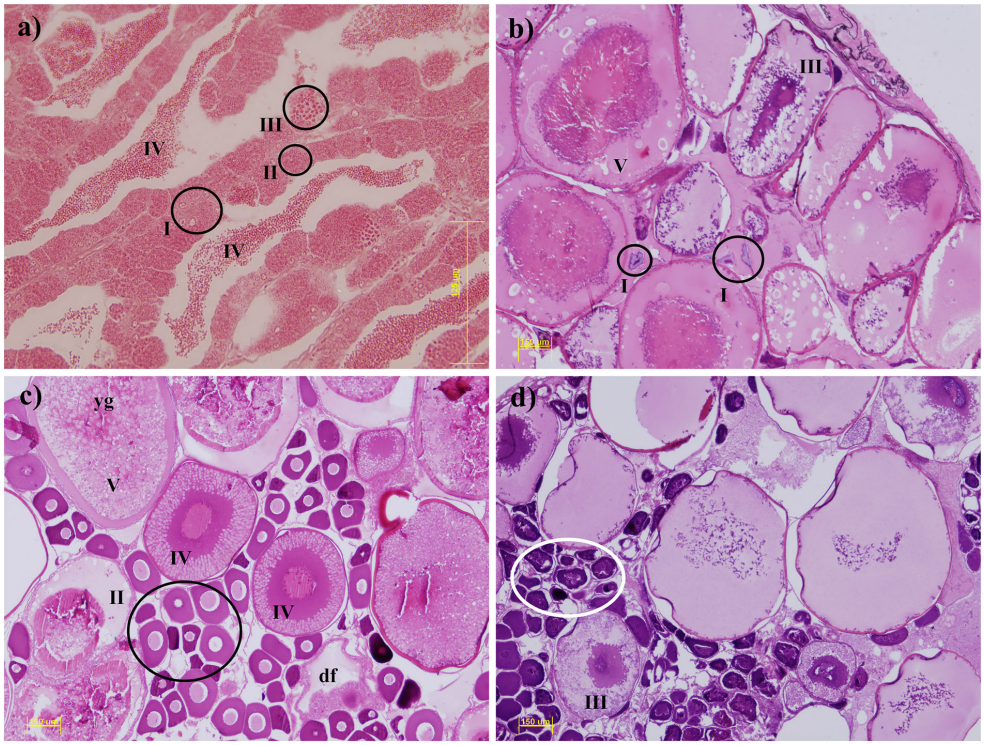

Fig. 3 Photomicrographs of *S. laietanus* gonadal sections stained with haematoxylin and eosin stain, showing ovaries and testes at different developmental phases. **a** Mature testicle, showing spermatozoa (IV) at the centre of the seminiferous tubule and all the stages of development: spermatogonia (I), spermatocytes (II) and spermatids (III) (upstream male). **b** Mature ovary, showing oocytes in phase V (vitellogenic), as well as phase III and phase I (upstream female). **c** Overview of female gonad where phase II, phase IV and phase V (vitellogenic) oocytes are shown (upstream female). **d** Detail of an atretic oocyte (AO) found in some specimens of the study and phase I and phase III oocytes (downstream female). Black and white circles indicate maturation phases with a small size. *yg* yolk granule, *df* follicles after spawn

biomarker responses considered in this study confirmed an elevation of detoxification enzyme activities and oxidative stress parameters in fish sampled downstream from the WWTP. A study carried out on two cyprinid fish species from the same locations in summer 2012, which also included some of the biomarkers considered here (EROD and BFCOD), reached similar conclusions (Blanco et al. 2019). According to these authors, males from two species (*S. laietanus* and *Barbus meridionalis*) displayed delayed sexual maturation on the polluted site, and females of *B. meridionalis* expressed gonadal aromatase (an enzyme that converts testosterone to estradiol) induction. Our study confirms that, even 2 years later, the endocrine disruption on fish from this area still persists and affects *S. laietanus* as well. The inclusion of fish liver biomarkers confirmed chemical exposures to lipophilic organic microcontaminants: from dioxin-like chemicals (reflected as EROD activity increase) to drugs of broader nature (as BFCOD activity enhancement). Both parameters, together with conjugation enzymes (GST), carboxylesterase activities (CbE) and antioxidant defences (GPX and GR), were significantly elevated downstream from the WWTP, confirming the exposure to a broad nature of chemicals. Nonetheless, the observed differences in biomarkers response between seasons (spring and summer) could be attributed to changes in water temperature and the reproductive status of fish (Van der Oost et al. 2003).

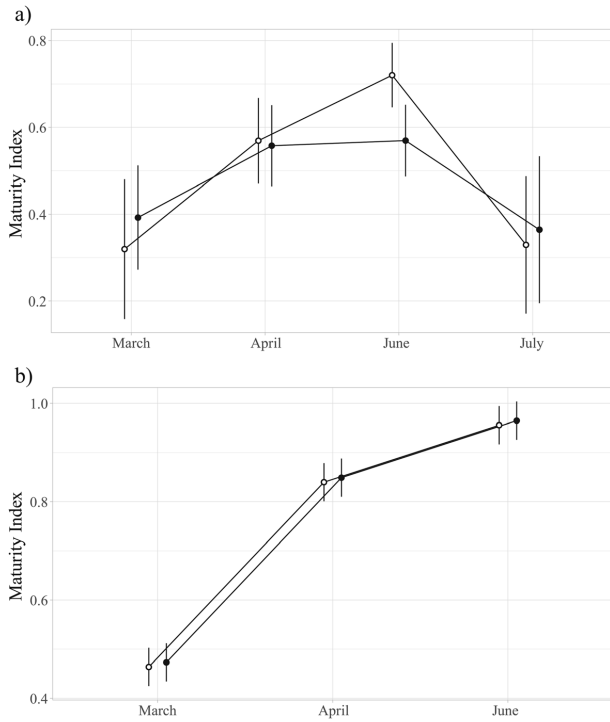

Fig. 4 **a** Maturity Index for *S. laietanus* females over the reproductive period, shown on a 0 to 1 scale. White circles correspond to females upstream WWTP showing a maturation peak in June. Black circles correspond to females downstream WWTP not showing this peak. **b** Maturity Index for *S. laietanus* males over the reproductive period represented on a 0 to 1 scale. White circles correspond to males upstream WWTP. Black circles correspond to males downstream WWTP. Males from upstream and downstream WWTP showed the same maturation peak in June. Average values and 95% confidence intervals (CI) are shown

Water characteristics in the polluted site may account for the reproductive disorder observed in fish of this study. Females upstream the WWTP followed the expected reproductive pattern characteristic of *S. laietanus* with a maximum number of vitellogenic oocytes in June (Casals 2005). By contrast, females downstream presented vitellogenic oocytes during the entire spawning season with no clear reproductive peak and a reduced amount of mature oocytes in April, which indicates an alteration of their gonadal cycle and reproductive output. The lower fecundity rates found for females downstream the WWTP were concomitant to a reduced gonadal development relative to body weight (GSI) and to the absence of a peak for this index throughout the

Table 6 Oocyte size for the four stages of development and atretic oocyte of *S. laietanus* expressed in μm^2. Means and 95% confidence interval (CI) are shown. In order to test statistical differences, a generalized lineal mixed model (GLMM) was performed for each sex

	Upstream		Downstream	
	Mean	CI 95%	Mean	CI 95%
Stage II	111.36*	85.31–137.42	233.23	203.68–262.78
Stage III	2380.28	2042.90–2717.65	2767.76	2423.73–3111.80
Stage IV	791.57*	155.71–1427.43	2079.36	1499.42–2659.31
Stage V	5729.56	4878.79–6580.33	5137.75	3929.48–6346.02
Atretic	81.94	74.05–89.83	85.52	77.02–94.01

* Significant differences by site. All significant differences are $p \leq 0.05$

breeding period. Significant reductions in GSI in sewage effluent exposed fish have been reported for several species (Hecker et al. 2002; Jobling et al. 1998; Tetreault et al. 2011; Vajda et al. 2008). The histological analysis of females also confirmed the absence of a downstream peak of reproduction. Females collected at both sites showed the five oocytes stages of maturation defined in this study. However, the maturity index (calculated from the proportion of oocytes in the different stages of maturation) was higher for females upstream the WWTP in June but this was not observed in the females downstream. No major histological alterations in the morphology of female gonads were recorded in the current study and in other sewage effluent exposed fish despite the occurrence reproductive alterations (Hecker et al. 2002; Jobling et al. 2002; Solé et al. 2003).

Similar results were observed for males. Males downstream the WWTP had lower GSI values as described by Kaptaner et al. (2016) for another cyprinid fish species. Males of both sites showed the four stages of maturation defined in the histological analysis. Although for males the maturity index was not significantly different between the two sampling sites, on the other hand males in the polluted site had narrower seminiferous tubules. Reduction in diameter of seminiferous tubules has been related to a decrease in male fecundity due to less space to stock sperm (Gimeno et al. 1998; Smith 1978). The lower value of GSI in males downstream may confirm this assumption. Gimeno et al. (1998) reported that a reduction in the diameter of the seminiferous tubules is related to fish exposed to 17 β-estradiol (E2) in *Cyprinus carpio*. Although a high frequency of intersex among males of fish species in wild populations exposed to wastewater discharges has been described (Jobling et al. 1998; Tetreault et al. 2011), feminization in males has not been observed in this study.

In teleost female fish, the process of oogenesis is mostly E2-dependent and oocyte maturation is regulated by the progestin 17α,20β-dihydroxypregn-4-en-3one (17,20β-P). According to Scott et al. (2010), the last one can be affected by environmental contaminants such as diazinon and synthetic progestogens. An desynchronised spawning reproductive pattern, such as the one revealed for the ovarian development in females of this study, was also reported in other fish field studies due to chemicals of an oestrogenic nature (Vajda et al. 2008; Woodling et al. 2006). However, for males, the effects of EDCs are unpredictable and variable (Leino et al. 2005). Some of the CEC classified as EDCs affect females but not males of teleost fishes (Dietrich and Krieger 2009). The EDCs detected at higher concentrations in the polluted site of this study were benzotriazole (up to 1.3 μg/L), benzothiazole (up to 0.5 μg/L) and their respective metabolites. Both compounds have been detected in several rivers of the Iberian Peninsula such as the Llobregat, Ter, Ebro and Tordera (Herrero et al. 2013; Matamoros et al. 2010a). Formerly, these EDCs were clearly associated with the activity of the textile dye plant of concern (Matamoros et al. 2010b). The antiestrogenic activity of benzotriazole has not been fully demonstrated in fish under experimental conditions (Harris et al. 2007). A laboratory experiment reported a degeneration of the ovaries and a stimulation of spermatogenesis in fish females at concentrations starting from 5 mg/L, while in males an increase in GSI and alterations in seminiferous tubules was found (Liang et al. 2014). However, this concentration was higher than that found in this study downstream WWTP site. Other laboratory studies have revealed that benzotriazole was weakly oestrogenic to fish (Tangtian et al. 2012) but does not cause serious abnormalities in testes (Dietrich and Krieger 2009). Benzothiazole have effects on the structure of the gills and on the swimming of fish (Evans et al. 2000).

The highly prescribed ibuprofen was detected downstream in the summer sampling (134 ng/L). This nonsteroidal anti-inflammatory drug is responsible for causing endocrine alterations in steroidogenic enzymes in an in vitro study for male fish (Fernandes et al. 2011). Ibuprofen has also been related to the induction of vitellogenin in male fish, a fewer broods per pair and with a delay in hatching of eggs in *Oryzias latipes* (Han et al. 2010). Other compounds classified as EDCs (such as bisphenol A, diazinon, oxybenzone and methylparaben) were found in the polluted site of this study. However, these and other compounds (galaxolide and tonalide) were found at concentrations ten times lower than those that can cause effects on oocyte production (Bhandari et al. 2015; Coronado et al. 2008; Kim et al. 2014; Maxwell and Dutta 2005; Mihaich et al. 2012; Staples et al. 2011) or on testicular properties (Goodbred et al. 2015; Staples et al. 2011). The effects of nitrogen compounds on the reproduction of fish are not clear. In females of *Pimephales promelas*, the ammonium can both reduce the production of eggs (Armstrong et al. 2012) as it may not have any effect (Armstrong et al. 2015). In this same species, although it appears that chronic exposure to nitrates can induce a vitellogenic induction in both sexes, it has not been

shown that it has effects on the GSI or that it favours the occurrence of intersex between males (Kellock et al. 2018). Nitrate exposure also has no effect on GSI in salmonids (Good et al. 2017). Other studies suggest that neither nitrites nor nitrates have clear effects on fish reproduction (Bjerregaard et al. 2018).

In nature, some chemicals could express synergistic or antagonistic interactions with others, and this must be taken into account in field studies as the resulting effects of the mixed exposures could be either under- or overestimated (Ginebreda et al. 2014). Some of these CECs are lipophilic and could be present at higher concentrations in fish than in their environment (Lefebvre 2016). Therefore, the extrapolation of observations from experiments in the laboratory to real polluted environments can fail. Water pollution is one of the main causes of the decline of freshwater fish around the world, but little is known about how mixtures of compounds in water can interact and aggravate their effects on fish. A malfunction in reproduction and a lower fecundity because of EDCs could partly explain the dramatic decline of native fish populations in many freshwater ecosystems. Greater efforts should be directed to enforce the European Water Framework Directive (2000/60/EC) (2000) to avoiding wastewater spillages harmful to fish.

Conclusion

Biomarker responses confirmed that fish sampled downstream from the WWTP were undergoing additional chemical stress. Both females and males had lower GSI values downstream the WWTP. Downstream females showed a desynchronised spawning, a lack of reproductive peak and a lower fecundity value. Males had narrower seminiferous tubules. The chemicals benzotriazole and benzothiazole, in mixture with other compounds, could be responsible for the observed reproductive disorders in this fish species, although further studies are needed in order to clarify their role as ECDs in nature.

Acknowledgements We thank J. Correas and E. Prats for their help at the microscopy laboratories of the University of Barcelona. Thanks also to J. D. Rodríguez-Teijeiro for the assistance and help in the field. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors are grateful to three anonymous reviewers and Víctor Bonet for the English review.

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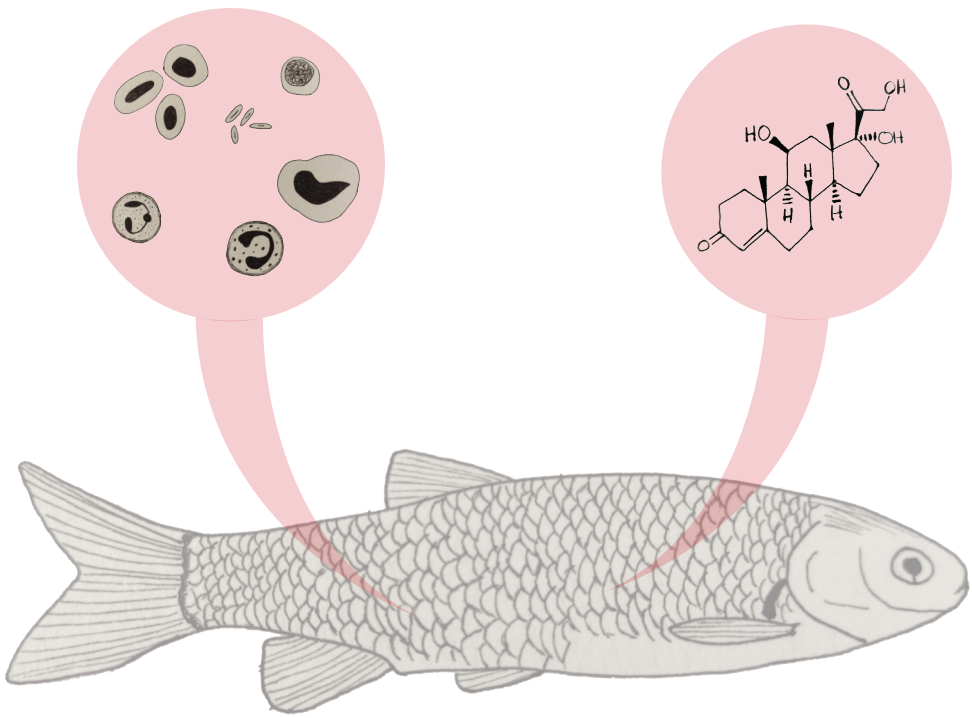
Sección 1.2.

Hacia métodos no invasivos para medir el bienestar de los peces: la medición de la concentración de cortisol en el moco epidérmico de los peces como biomarcador de la calidad del hábitat



Carbajal et al. (2019). Towards non-invasive methods in measuring fish welfare: the measurement of cortisol concentrations in fish skin mucus as a biomarker of habitat quality. Animals, 9, 939. https://doi.org/10.3390/ani9110939

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Towards Non-Invasive Methods in Measuring Fish Welfare: The Measurement of Cortisol Concentrations in Fish Skin Mucus as a Biomarker of Habitat Quality

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Received: 20 September 2019; Accepted: 6 November 2019; Published: 8 November 2019

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Simple Summary: The analysis of circulating cortisol has been by far the most common method used as a means to assess fish stress responses and, thus, animal welfare. To avoid many of the drawbacks inherent to blood sampling, cortisol can be less-invasively detected in fish skin mucus. The measurement of cortisol in skin mucus however, has, to date, only been demonstrated as suitable for farm fish, although its application to free-ranging animals would offer many advantages. The present study was therefore designed to evaluate the applicability of skin mucus cortisol analysis as a potential tool to assess habitat quality. To that end, wild fish residing in environments of different habitat quality were sampled for blood and skin mucus. First, several physiological endpoints typically used as indicators of exposure to pollutants were accurately related to the habitat quality in the Catalan chub (*Squalius laietanus*). Second, cortisol levels in blood were also compared between habitats, and they were successfully correlated to skin mucus cortisol concentrations. Finally, we contrasted the patterns of response of all the endpoints assessed to skin mucus cortisol levels across the sites. The strong linkages detected in this study provide new evidence that the measurement of cortisol in skin mucus could be potentially used as a biomarker of habitat quality in freshwater fish.

Abstract: Cortisol levels in fish skin mucus have shown to be good stress indicators in farm fish exposed to different stressors. Its applicability in free-ranging animals subject to long-term environmental stressors though remains to be explored. The present study was therefore designed to examine whether skin mucus cortisol levels from a wild freshwater fish (Catalan chub, *Squalius laietanus*) are affected by the habitat quality. Several well-established hematological parameters and cortisol concentrations were measured in blood and compared to variations in skin mucus cortisol values across three habitats with different pollution gradient. Fluctuations of cortisol in skin mucus varied across the streams of differing habitat quality, following a similar pattern of response to that detected by the assessment of cortisol levels in blood and the hematological parameters. Furthermore, there was a close relationship between cortisol concentrations in skin mucus and several of the erythrocytic alterations and the relative proportion of neutrophils to lymphocytes. Taken together, results of this study provide the first evidence that skin mucus cortisol levels could be influenced by habitat quality. Although results should be interpreted with caution, because a small sample size was

collected in one studied habitat, the measurement of cortisol in skin mucus could be potentially used as a biomarker in freshwater fish.

Keywords: non-invasive; bioindicator; pollution; stress; welfare; constructed wetland; glucocorticoid; urban river

1. Introduction

Throughout their lifetime, wild fish face many challenges of the aquatic environment that can impose considerable stress and reduce their welfare [1]. These challenges can be either natural or have an anthropogenic origin, and, depending on the magnitude and duration, they can cause acute or chronic stress responses [2,3]. Acute stress responses, such as those triggered by a predator attack or certain unpredictable weather conditions, can facilitate survival [4], whereas long-term stressors, like exposure to environmental pollution, are associated with a wide range of maladaptive effects [5] that may, ultimately, lead to loss of biodiversity [6,7]. Accordingly, understanding the causes and effects of environmental disturbances on fish physiology may help developing conservation strategies to enhance restoration and protect freshwater ecosystems [5,8].

An economical and practical option that can give a substantial amount of information about the overall health status of individuals is the peripheral blood test [9,10]. The analysis of red blood cells (RBCs) allows the detection of DNA damage and alterations by the assessment of erythrocytic nuclear abnormalities (ENA), circulating micronuclei (MN), and senescent (SE) and immature (IE) erythrocytes [11,12]. The detection of RBC abnormalities has actually been widely used as an indicator of exposure to genotoxic and mutagenic contaminants [13–15]. In parallel, relative white blood cell (WBC) count can be obtained, which offers a very common measure of stress and innate immune response [16]. In particular, the relative proportion of neutrophils to lymphocytes has been successfully applied as a measure of prolonged pollutant exposure [9,17,18]. Other uses of blood samples in ecotoxicology include the quantification of glucocorticoid (GC) hormones, such as cortisol, to assess the stress response [19,20]. Cortisol is the main GC in teleost fish secreted after the activation of the hypothalamic–pituitary–interrenal (HPI) axis in response to acute and chronic stress [2,21]. Analyses of cortisol levels in blood and, more recently, in whole-body and the surrounding water have been effectively used to monitor environmental stress responses [22–24]. Blood, whole-body, and the surrounding water sampling, however, present clear limitations when being applied in wild population studies. First, blood collection is an invasive technique that the process by itself may provoke further stress and thus it can potentially compromise the animal's welfare. Similarly, whole-body cortisol analysis involves sacrifice of the specimens [22]. And finally, collection of the holding water requires fish restriction in a bucket, which can cause additional stress. Moreover, this technique is difficult to apply in the wild [24]. Fish scales can also accumulate cortisol [25,26]; however, their potential as biomarkers of habitat quality deserves further investigation [27]. Cortisol analysis in fish skin mucus has recently gained considerable attraction, especially because the sampling method is much less invasive compared to the aforementioned techniques [28–30]. Skin mucus cortisol levels have been shown to reflect circulating concentrations in several species of farm fish [26,30–32], but there is yet no evidence of such a relationship in free-ranging species. In addition, this method has, to date, only been applied in strictly controlled environments [30,33], hence its applicability in uncontrolled, natural environments remains to be explored. Therefore, the present study aimed to examine whether skin mucus cortisol concentrations (MCC) from the freshwater fish Catalan chub, *Squalius laietanus* [34], are affected by the habitat quality to further develop non-invasive biomarkers in free-living fish. Catalan chub was chosen, as this species has demonstrated to be a good candidate for freshwater biomonitoring using blood tests [11]. It is well known that understanding changes in cortisol levels is not a simple process, especially when measuring cortisol in wild animals by using alternative samples

other than blood [6,9]. Given that the measurement of cortisol in skin mucus is a novel method, other physiological endpoints of the effects of pollution in fish were assessed to better interpret cortisol fluctuations in this matrix. Several hematological parameters (RBC anomalies and altered WBC counts) were measured in parallel, since, as previously mentioned, they have successfully been used as indicators of health condition in the Catalan chub [11], as well as in many other species (reviewed above).

This study was carried out in a populated and industrialized urban river, where efforts are being made to minimize the environmental impacts and recover the aquatic fauna throughout the performance of constructed wetlands. Catalan chub were sampled from a non-impacted upstream site and two downstream polluted sites, located within the constructed wetland system, in order to compare fish residing environments of different habitat quality. Initially designed specifically for wastewater treatment, constructed wetlands are nowadays an important component of urban ecosystems since they play a crucial role in environmental pollution control [35–37]. Constructed wetlands are macrophyte-based systems that remove pollutants through a combination of physical, chemical, and biological processes [36,38]. Wetlands' performances, though, need to be periodically monitored [39]. The described methodologies for wetland monitoring include physical and chemical techniques that provide information about the amount of pollutants present in the water. Nevertheless, these tools do not give insight into how living organisms cope with water contaminants [39]. On this basis, the present study was carried out in a constructed wetland system to highlight the need to apply techniques that provide information about how animals perceive and adapt to their environment.

2. Materials and Methods

2.1. Study Area and Field Sampling

In order to study the influence of the habitat quality on skin mucus cortisol concentrations, individuals were sampled from two sites within a wetland system (Besòs River Park, NE Spain), each of which represents a different stage of biodegradation of water pollutants (P1 and P2), and a reference non-impacted upstream site located outside the wetlands (Figure 1). The reference site was set in a small tributary (Riera d'Avencó), 49.6 km distant from the site P1. The sampling site P1 was placed at the beginning of the constructed wetland, 2.9 km distant from P2, which was located at the end of the overall wetland system and 3.6 km to the river mouth. The Besòs River is an urban river adjacent to the City of Barcelona (Catalonia, NE Spain). During the 1970s and 1980s this river was declared the most polluted river in Europe. Fish populations are slowly recovering, making it easy to find an abundance of differences between nearby sites.

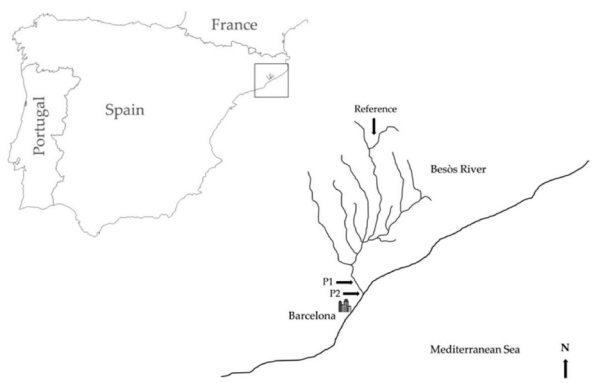

Figure 1. Map showing the location of the three sites sampled in the present study (Reference, P1 and P2) within the Besòs River, in north-east Spain.

Sampling areas were determined following the protocols from the European Committee for Standardization (CEN prEN 14011:2002). Fish were sampled by electrofishing using a portable unit which generated up to 200 V and 3 A pulsed DC in an upstream direction. This capture method was employed as it is considered an easy and safe method, very popular for studying stream fish [2,40,41]. To minimize the potential influence of the sampling technique on the results, fish were all handled identically by the same experienced operator.

At the start of the study (17 May 2017), several physico-chemical parameters and contaminants of emerging concern (CEC) were measured as a basis of the water quality from each sampling site (Table 1). Results of the water analyses provided evidence that the sampling sites classified as polluted presented features typically identified in disturbed environments [11,42,43].

To avoid the circadian rhythm being a source of variability, samplings were performed in the morning (10:00–12:00) from 17 May to 15 June 2017, once the constructed wetland system had been operative for 3 months (23 May, P1; 17 May, P2; 15 June, reference site). Unequal sample size was collected within the polluted sites probably due to sub-optimal habitat conditions in P1 (n = 6) compared to P2 (n = 17) or the reference site (n = 22). All procedures followed the national and institutional regulations of the Spanish Council for Scientific Research (CSIC) and the European Directive 2010/63/EU.

Table 1. Occurrence of contaminants of emerging concern (CEC) and physico-chemical data from river water samples collected within the wetland system (P1 and P2) and the reference non-impacted site located outside the system (Reference) in May 2017, when fish were sampled.

Compound	Sites		
	P1	**P2**	**Reference**
CEC (µg/L)			
Volatile organic compounds			
Tetrachloroethene	<LOD	0.6	<LOD
Pesticides			
Simazine	0.13	0.13	<LOD
Diuron	<LOD	<LOD	<LOD
Isoproturon	0.04	0.04	<LOD
Pharmaceutical products			
Diclofenac	1.61	0.29	<LOD
Alkylphenols			
4-tert-octylphenol	0.025	<LOD	<LOD
Nonylphenol	0.14	<LOD	<LOD
Physico-Chemical Data (mg/L)			
NH_4^+	12.6	10.6	0.07
NO_3^-	3.31	2.83	0.18
PO_4^-	0.8	1	0.4
TOC [1]	9.25	6.39	2.16
COD [2]	29.9	30.5	5.88
SS [3]	7	9.5	0.5
Turbidity (NTU [4])	4.47	3.01	0.66

Concentrations of compounds under the instrumental detection limit (LOD, Limit of detection) are not included. [1] TOC, total organic carbon; [2] COD, chemical oxygen demand; [3] SS, suspended solids; [4] NTU, nephelometer turbidity units.

2.2. Sample Collection

Fish were sampled after applying the combined stressor of capture and a brief period of confinement, given that stress-induced cortisol levels offer a considerable understanding of the overall stress response [44]. In this context, growing evidence suggests that circulating cortisol increases can

be detected from as short as 1–2.5 min following exposure to stressors [1,21]. Accordingly, fish were caught using the portable electrofishing unit, and confined in buckets of 20 L for approximately 15 min. Afterwards, specimens were anesthetized with MS-222 (100 mg/L) and immediately after, blood and skin mucus were sampled. A heparinized insulin syringe was used to collect approximately 0.2 mL of blood by caudal vein puncture. A drop of blood was smeared for hematological analyses, and the remaining fluid kept on ice until transported back to the laboratory. Samples were then centrifuged (1500× *g*, 5 min) and plasma was collected and stored at −20 °C. Skin mucus was collected following the method described by Schultz and colleagues [45] with some modifications. Briefly, a polyurethane sponge was used to absorb the skin mucus by applying light pressure to the left and right flank as this method has been shown to be less stressful than using a spatula [45]. The sponge was then introduced into a cylinder of a syringe and compressed with the barrel to collect, into a centrifuge tube, the skin mucus. Afterwards, samples were centrifuged (2000× *g*, 10 min) and the supernatant was stored at −20 °C until analysis. Morphological variables, including length (mean ± SD: P1 = 208.5 ± 34.2 mm; P2 = 211.6 ± 22.8 mm; reference = 159.9 ± 35.5 mm) and weight (mean ± SD: P1 = 126.7 ± 61.1 g; P2 = 117.0 ± 50.0 g; reference = 58.7 ± 42.2 g) were measured. Fulton's body condition factor (*K*), calculated according to the formula $K = 100{,}000 \text{ body weight (g)} \text{ total length (mm)}^{-3}$ [46], was assessed, since it can reflect the energetic state of individuals [47]. Fish were released into the corresponding capture site once the samples had been collected.

2.3. Hematological Analysis

Immediately after being collected, a drop of blood was placed on glass microscope slides, drawn across the surface and, once air-dried, slides were fixed in absolute methanol for 10 min. This procedure was run in duplicate for each specimen. Upon arrival in the laboratory, one of each duplicated slides was stained with Diff-Quick® to assess the frequency of abnormal RBCs and for the WBC count. RBCs (1000) of each individual slide were scored to calculate the frequency of ENA, SE, and IE. The ENAs analyzed were defined as lobed, kidney-shaped, fragmented, and vacuolated nuclei following the directions of Pacheco and Santos [48]. The relative count of all types of WBCs (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) was performed for 100 WBCs following the directions of Tavares-Dias [49]. The neutrophil and lymphocyte count was used to calculate the relative proportion of neutrophils to lymphocytes (hereafter, N:L ratio). The second duplicated slide of each individual was used to assess the number of micronucleus after performing an acridine orange staining. RBCs (3000) of each slide were scored to calculate the frequency of MN.

2.4. Cortisol Extraction and Biochemical Validation

To analyze cortisol levels from blood and skin mucus, a commercial enzyme immunoassay (EIA) kit (Cortisol ELISA KIT; Neogen® Corporation, Ayr, UK) was used.

Biochemical validation of the EIA was carried out following the methods described by Carbajal and collaborators [50]. Samples of plasma and skin mucus extracts from several individuals were first pooled and used in each validation test. Precision was evaluated with the intra-assay coefficient of variation (CV), calculated from all duplicated samples analyzed. All samples from each matrix evaluated were analyzed in single assays; therefore, the inter-assay CV was not assessed. The dilution test was applied to assess the specificity of the EIA kit by comparing observed and theoretical values from pools diluted with EIA buffer. To test the assay's accuracy, the spike-and-recovery test was used, where known volumes of pools were mixed with different volumes and concentrations of pure standard cortisol solution. Finally, we evaluated the sensitivity of the test, given by the smallest amount of cortisol concentration detected.

2.5. Statistical Analysis

The computer program R software (R-project, Version 3.0.1, R Development Core Team, University of Auckland, New Zealand) was used to analyze the data. A $p < 0.05$ was considered statistically

significant. Normality of the data was assessed using Shapiro–Wilk tests, and parametric or non-parametric tests were applied accordingly. Differences in cortisol levels and hematological data between sites were assessed using one-way ANOVA with Tukey's pairwise post-hoc tests. Non-normally distributed data were assessed by using Kruskal–Wallis test, followed by a multiple comparison test. Length and *K* were run as covariates in the models to account for potential differences across sites due to the age of the fish or the energy accumulated in the body, respectively. These covariates were then removed when results showed no influence on the response variable ($p > 0.05$). Pearson and Spearman correlation tests were applied to test for correlations between skin mucus cortisol levels to levels of the hormone in blood and the hematological variables.

3. Results

In total, 45 Catalan chub from sites P1 (mean *K* ± SD = 1.17 ± 0.14, N = 6), P2 (mean *K* ± SD = 1.30 ± 0.07, N = 17), and reference (mean *K* ± SD = 1.18 ± 0.08, N = 22) were captured, sampled, and returned to the river immediately after sampling.

3.1. Biochemical Validation of the EIA

Plasma and skin mucus intra-assay CV was 8.8% and 7.7%, respectively. The dilution test obtained for plasma showed an R^2 = 98.4% and a mean percentage error of 104.1 ± 4.1%. In the skin mucus dilution test, an R^2 = 99.7% and a mean percentage error of 108.7 ± 8.7% was obtained. Also, in the dilution test, obtained and theoretical concentrations of plasma and skin mucus extracts showed significant correlation coefficients ($r = 0.99$, $p < 0.01$). In the spike-and-recovery test, the average of the recovery percentage was 107.6 ± 10.0% for plasma and 109.6 ± 9.1% for skin mucus. The sensitivity of the assay for plasma and skin mucus assessment was 0.07 ng cortisol/mL and 0.03 ng cortisol/mL, respectively. The biochemical validation of the EIA showed reliable results that demonstrated the assay's precision, specificity, accuracy and sensitivity in measuring plasma and skin mucus cortisol levels of the Catalan chub.

3.2. Hematological Parameters and Cortisol Levels

Concerning the RBC alterations, the frequency of IE (Figure 2A) and SE (Figure 2B) was significantly lower in the reference site compared to P2 ($p < 0.05$). In addition, significantly lower frequencies of ENA (Figure 2C) were detected in the reference site compared to both polluted habitats ($p < 0.05$). Despite no differences detected between polluted and reference sites in the frequency of MN ($p > 0.05$), a higher frequency of MN was detected in P1 compared to P2 ($p < 0.05$).

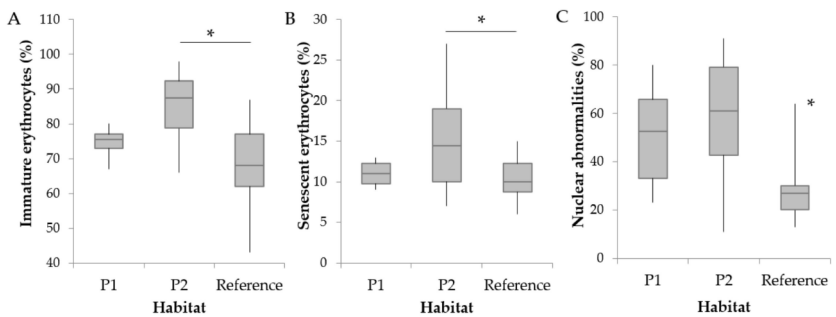

Figure 2. Frequencies of (**A**) immature erythrocytes, (**B**) senescent erythrocytes, and (**C**) erythrocytic nuclear abnormalities determined in the Catalan chub sampled at two polluted sites (P1, n = 6; P2, n = 17) and a reference non-impacted upstream site (n = 22) in the Besòs River. Asterisks (*) indicate significant differences between habitats ($p < 0.05$).

When accounting for differences in WBC counts between habitats (Table 2), we detected a higher N:L ratio in P1 and P2 compared to the reference site ($p < 0.05$), and a significant interaction effect of site with length on the N:L ratio ($p < 0.05$). Although no differences were detected between habitats in the proportion of monocytes and eosinophils ($p > 0.05$), a higher proportion of basophils was detected in P2 compared to the reference site ($p < 0.05$).

Table 2. Mean values and standard deviation of white blood cell parameters (‰) determined in the Catalan chub from polluted (P1, n = 6; P2, n = 17) and reference (n = 22) sites in Besòs River. Different letters indicate significant differences among sites ($p < 0.05$).

White Blood Cell Type	Sites		
	P1	P2	Reference
N:L ratio	10.54 ± 4.49 ^{a}	8.00 ± 3.46 ^{a}	4.79 ± 2.91 ^{b}
Monocytes	4.50 ± 3.67	7.47 ± 3.83	6.05 ± 2.82
Eosinophils	0.67 ± 0.82	1.35 ± 1.17	1.18 ± 1.30
Basophils	0.33 ± 0.52 ^{ab}	1.24 ± 1.20 ^{a}	0.36 ± 0.58 ^{b}

Plasma cortisol concentrations (PCC) (Figure 3A) and mucus cortisol concentrations (MCC) (Figure 3B) were significantly lower in the reference site than in both polluted sites ($p < 0.05$).

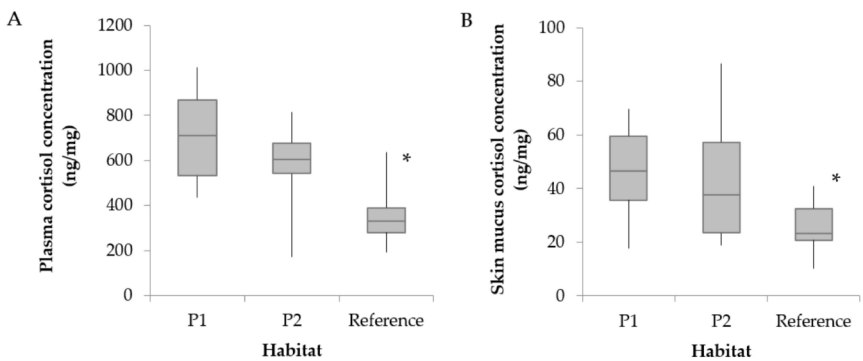

Figure 3. Cortisol concentrations in (**A**) plasma and (**B**) skin mucus of the Catalan chub sampled at two polluted sites (P1, n = 6; P2, n = 17) and a reference non-impacted upstream site (n = 22) in the Besòs River. Asterisks (*) indicate statistically significant differences between habitats ($p < 0.05$).

When studying the relationships between MCC, PCC, and the hematological parameters (Table 3), significant correlations were identified between MCC and PCC ($p < 0.01$), IE ($p < 0.05$), ENA ($p < 0.05$), and the N:L ratio ($p = 0.05$).

Table 3. Correlation (*r*) and *p*-value between hematological variables and skin mucus cortisol concentrations (MCC).

Variable	MCC (*r*)	*p*-Value
Cortisol		
Plasma cortisol concentration	0.55	**<0.01**
Red blood cells		
Immature erythrocytes	0.40	**0.03**
Senescent erythrocytes	0.23	0.24
Erythrocytic nuclear abnormalities	0.41	**0.02**
Micronucleus	−0.001	0.99
White blood cells		
N:L ratio	0.34	**0.05**
Monocytes	−0.01	0.97
Eosinophils	−0.21	0.20
Basophils	0.24	0.17

Bold numbers denote significant correlations between the hematological variables and MCC.

4. Discussion

In this study, we first successfully validated that several physiological endpoints typically used as indicators of exposure to pollutants (abnormal RBCs and altered WBC counts), were accurately related to the habitat quality in the Catalan chub. Cortisol levels in blood were also compared between habitats and they were correlated to skin mucus cortisol concentrations. Finally, we contrasted the patterns of response of all the endpoints assessed to skin mucus cortisol levels across the sites. Lack of an adequate number of samples in one of the polluted sites (P1, n = 6) makes the cross-site comparison difficult. The discussion is therefore focused on differences detected between sites with a larger sample size (P2, n = 17; reference, n = 22). Results highlight the potential of this non-invasive tool to assess habitat quality and the need to combine regular techniques for biomonitoring the wetlands systems' performances with the measurement of cortisol in skin mucus.

4.1. Abnormal RBC Frequencies

There was no consistent pattern in abnormal RBC frequencies when they were compared between the polluted and the reference habitats. As confirmed by the physico-chemical and CEC analysis (Table 1), the site P1, located at the beginning of the wetlands system, presented slightly worse habitat conditions compared to the site P2, placed at the end of the same system. Accordingly, we expected to identify further RBC alterations in P1 than in P2. Nevertheless, relative to the reference site, P1 only exhibited significantly higher frequencies of ENA, while fish from P2 presented greater IE, SE, and ENA frequencies. As mentioned earlier, the sub-optimal conditions of P1 were likely the cause of the small sample size collected in the site, which in turn could have limited the statistical power in detecting potential differences.

Conversely, both polluted P1 and P2 sites showed clearly higher ENA levels than the reference habitat. Particularly in spring, greater frequencies of nuclear abnormalities in the same fish species have already been identified [11]. The ENA test has been demonstrated to be a highly sensitive parameter for pollution assessment [12,51], probably explaining the clear variations detected in these nuclear abnormalities between habitats.

Besides the inconsistencies found between both polluted sites, higher frequencies of RBC disorders detected in the polluted habitat with larger sample size further support the idea that IE, SE, and ENA tests are reliable biomarkers of habitat quality in the Catalan chub [52,53].

Although these commonly noted abnormalities are highly sensitive to pollution, they are not as widely accepted as the use of MN tests [53,54]. The influence of river status on the frequency of MN has been previously studied in the Catalan chub, and, in accordance with our results, no changes

were detected between degraded and reference streams [11]. Nevertheless, in our study, the two polluted areas evaluated differed in MN levels between them, with the highest values detected at the end of the wetland system. Although this result could also be given by the different sample size between study sites, it should be noted that a different contaminant profile can also result in mismatch between habitats [13]. For example, exposure to atrazine and ametrine herbicides resulted in increased MN [55]; in contrast, a different herbicide, pendimethalin, showed increased ENA but not MN [56]. Tetrachloroethene, the only CEC analyzed that presented higher concentrations in P2 than P1, is a dry-cleaning compound widely used in the textile industry, known to have toxic effects in fish [57,58]. Although evidence in humans supports the link between the MN formation and this compound [59], to the authors' knowledge, there are no published studies demonstrating this association in fish. Despite this, other compounds not specifically analyzed in this study could also be the consequence of the differing results between polluted habitats.

Taken together, these findings suggest that the detection of RBC disorders can be potentially used to identify low-quality habitats for the Catalan chub, while being an approach that could contribute to a better understanding of the species' health status than the MN test.

4.2. Variations in WBC Counts

Characteristic changes in blood leukocyte counts have been generally linked to the continuous activation of the HPI axis [2,60–62]. Interestingly, prolonged exposure to environmental contaminants can cause neutrophilia and/or lymphopenia in fish [16–18], likewise in other taxa [9,63]. In line with these published reports, different WBC counts were detected between the study sites. Most notably, the N:L ratio was significantly higher in both polluted sites compared to the reference stream. These between-site differences could be a consequence of the sub-optimal environmental conditions in P1 and P1 habitats. However, the significant interaction detected between length (age) of fish and site suggests caution when interpreting results. Although length did not influence any of the other response variables tested, it remains possible that differences in the N:L ratio were partly exacerbated by the smaller size of fish sampled at the reference site. Age can be an important factor in shaping the stress response [2]; nevertheless, further studies are needed to understand the influence that age/length can have in the leukocyte profile. The number of basophils, a cell type still not assessed in this species, was also higher in P2 compared to the reference site. Although the function of this cell type is poorly understood, probably because its occurrence in teleost fish seems to be very rare [49,64], basophils have been related to acute inflammation processes [16]. Besides this, neither the monocyte nor the eosinophil count appeared to differ between sites, similar to earlier findings on silver carp (*Hypophthalmichthys molitrix*) in response to pesticides [65]. The assessment of these cell types is not common in contemporary research, perhaps due to the controversy concerning the effect of stress on eosinophil and monocyte numbers [2,16]. When only WBC data are available, evaluation of these two leukocyte types can help distinguish stress from infectious responses [16], thus further research on monocyte and eosinophil changes is strongly encouraged.

4.3. Changes in Cortisol Levels

Cortisol levels detected in plasma and skin mucus within the same individuals displayed a close linear relationship, suggesting that cortisol diffuses to the skin mucus in proportion to the amount of circulating hormone. Validation of alternative matrices for HPI axis activity assessment should prove that hormone concentrations in these media are proportional to their abundance in the bloodstream [3,66], as the present study demonstrates for the measurement of cortisol in skin mucus. These results, therefore, increase the applicability of the method as a sensitive-individual measure of the HPI axis activity in wild freshwater fish within their natural environment.

In addition, both plasma and skin mucus cortisol levels differed significantly between habitats of different quality, with the highest hormone values observed in the polluted sites assessed. This association between cortisol concentrations and habitat quality suggests that variation in the HPI axis

activity is likely to be related to the presence of environmental disturbances. Greater stress responses attributed to the effects of pollutants have been reported in several fish species [22,67,68], as well as in other taxa [69–71]. Nevertheless, it is important to note that chronic exposure to certain aquatic contaminants can also have suppressive effects on the stress axis [72–75]. In this context, investigating the toxic mechanisms underlying variation in the HPI axis alteration will be particularly informative.

4.4. Integrated Assessment

Interpreting cortisol fluctuations in free-living vertebrates is certainly a complex practice, particularly when applying alternative matrices for hormone assessment [6,9]. This is why linking cortisol levels to other endpoints of the stress responses can significantly enhance the current understanding on the ecology of stress [76].

In the present study, fluctuations in skin mucus cortisol levels between habitats paralleled those detected in blood, the traditional matrix used for hormone assessments in fish [77]. Relative to habitat quality, changes of the hormone in skin mucus also coincided with variations in the hematological parameters, except for MN levels. Furthermore, the amount of cortisol in skin mucus was directly proportional to frequencies of abnormal erythrocytes (IE and ENA) and to the well-established stress index N:L ratio. Red blood cells are highly sensitive to landscape disturbances [78] and, more specifically, to environmental pollution [79,80]. Accordingly, the measurement of abnormal erythrocytes has been successfully used to assess the health status of the Catalan chub [11,42] and many other fish species [12,13,17]. In the same context, WBC counts, particularly the N:L ratio, increases in individuals exposed to heavy metals and other contaminants proportional, indeed, to the circulating cortisol levels [16]. Given the very clear effect of pollution on leukocyte and erythrocyte profiles, the strong linkages detected in this study provide new evidence that the measurement of cortisol in skin mucus could be potentially used as a biomarker of habitat quality in freshwater fish residing polluted environments.

The use of a robust sample size is recommended in natural settings where individuals are exposed to different environmental conditions [9,81]. However, capturing a relevant number of individuals in wild conditions may not always be possible, especially in highly degraded habitats such as the one included in the present field experiment. An important limitation of our study design may, therefore, be the small sample size in one of the polluted habitats, which requires results of this site be interpreted with caution.

While physical and chemical techniques are commonly applied for wetland monitoring [39], methodologies that provide information about how animals are influenced by their environment are only occasionally used. The incorporation of biomarkers into the constructed wetlands' management would provide complementary data to the conventional analyses. Hence the demonstrated sensitivity of the methods evaluated in the present study to different pollution gradients could be exploited for biomonitoring the wetlands systems' performances. Indeed, the non-invasive measurement of cortisol in skin mucus would largely improve our understanding about the link between the detected chemical concentrations and the biological effects observed.

Author Contributions: Conceptualization, C.E., M.I., D.V., and M.L.-B.; methodology, A.C., P.S., and O.T.-P.; software, A.C.; validation, A.C. and O.T.-P.; formal analysis, A.C.; investigation, A.C. and P.S.; resources, M.L.-B. and D.V.; data curation, A.C.; writing—original draft preparation, A.C.; writing—review and editing, A.C., P.S., O.T.-P., M.L.-B., and D.V.; visualization, O.T.-P. and M.L.-B.; supervision, M.L.-B., C.E., M.I., and D.V.; project administration, D.V.; funding acquisition, C.E., M.I., and D.V.

Funding: This research was financially supported by Aigües de Barcelona and sponsored by Diputació de Barcelona (DiBa) in the context of the Biobesòs Project (Evaluation of the wetlands' efficacy to remove pollutants and improve the biodiversity levels in the Besòs river and its surroundings).

Acknowledgments: The authors thank Pau Fortuño, Narcís Prat, Núria Flor, and Núria Cid from Universitat de Barcelona (Barcelona, Spain) for their excellent help with sampling and technical assistance. We are grateful to Juan Carlos Ruiz and María Monzó from Aigües de Barcelona, and Anna Riera and Antoni Maza from DiBa for the support to make this project possible.

Conflicts of Interest: The authors declare no conflicts of interest.

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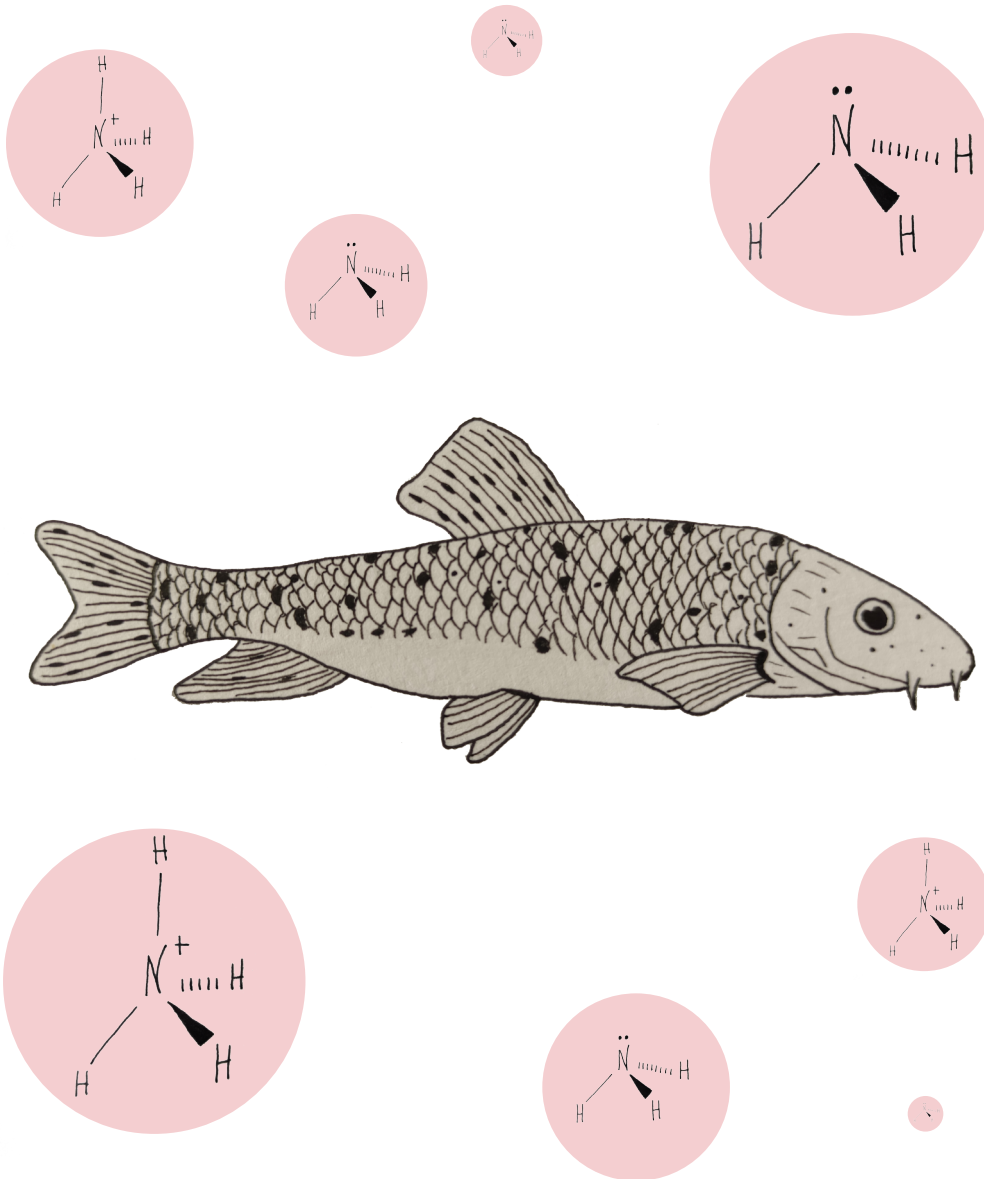
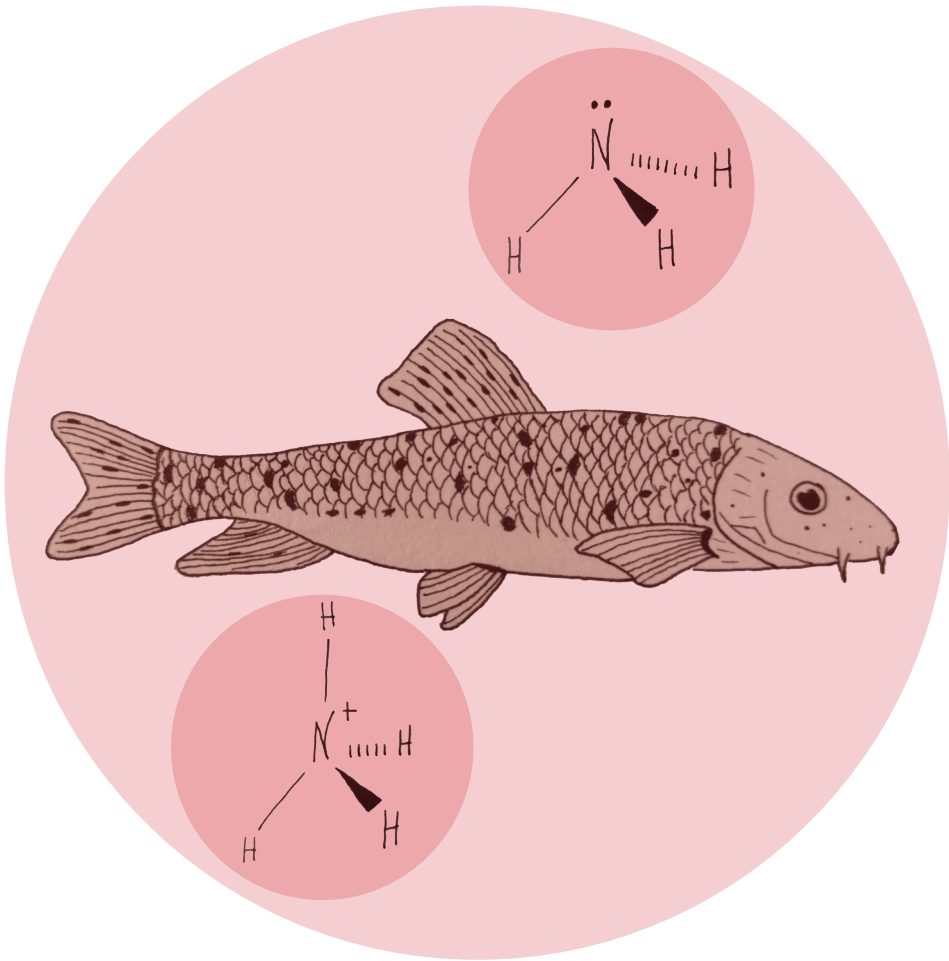
CAPÍTULO 2

EFECTOS DE LA CONTAMINACIÓN SOBRE EL COMPORTAMIENTO DEL BARBO DE MONTAÑA



Sección 2.1.

Mejorar la calidad del agua no garantiza la salud de los peces: efectos de la contaminación por amonio sobre el comportamiento de peces preexpuestos y capturados en la naturaleza



Soler et al. (2020). Improving water quality does not guarantee fish health: effects of ammonia pollution on the behaviour of wild-caught pre-exposed fish PLoS ONE (In revision).

Improving water quality does not guarantee fish health: effects of ammonia pollution on the behaviour of wild-caught pre-exposed fish

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Abstract: Ammonia is a pollutant frequently found in aquatic ecosystems. In fish, ammonia can cause physical damage, alter its behaviour and even cause death. Exposure to ammonia also increases fish physiological stress, which can be measured through biomarkers. In this study, we analysed the effect of sublethal ammonia concentrations on the behaviour and the oxidative stress of *Barbus meridionalis* that had been pre-exposed to this compound in the wild. Wild-caught fish from a polluted site (pre-exposed fish) and from an unpolluted site (non-pre-exposed fish) were exposed, under experimental conditions, to total ammonia concentrations (TAN) of 0, 1, 5 and 8 mg/L. Swimming activity, feeding behaviour and oxidative stress response based on biomarkers were analysed. Pre-exposed fish showed both an altered behaviour and an altered oxidative stress response in the control treatment (0 mg/L). Differences in swimming activity were also found as pre-exposed fish swam less. Lower feeding activity (voracity and satiety) and altered response to oxidative stress were also observed at ≥ 1 mg/L TAN. Biomarker results confirmed pre-exposed fish suffer from a reduction in their antioxidant defences and, hence, showed increased oxidative tissue damage. In summary, pre-exposed fish showed more sensitivity to ammonia exposure than fish from a pristine site.

Keywords: *Barbus meridionalis*, Mediterranean barbel, Swimming behaviour, Feeding behaviour, Biomarkers

INTRODUCTION

Ammonia is a commonly found pollutant in aquatic environments around the world (Camargo & Alonso 2006; CEPA, 2001). This compound can be found naturally, but there is also an additional contribution from sewage effluents, industrial waste and agricultural run-off (CEPA, 2001). The presence of ammonia in freshwater has been associated with the acidification of rivers and lakes, eutrophication and direct toxicity to aquatic organisms (Baker et al., 1991; CEPA, 2001; Camargo & Alonso, 2006). The toxicity of this compound on aquatic organisms will depend on the chemical form of ammonia, pH and temperature (Francis-Floyd et al., 2009). Furthermore, it will depend on the time of exposure (Francis-Floyd et al., 2009). This compound damages the gills, liver, kidney, spleen and other organ's tissues of fish, therefore causing breathing difficulties (Benli et al., 2008; Schram et al., 2010). This may lead to physiological alterations and, eventually, exhaustion or death (Schram et al., 2010). Ammonia can cause cell damage and can also affect the antioxidant defence system thus altering the levels of oxidative stress in fish (EPA, 2013; Sinha et al., 2014). Ammonia can also alter fish behaviour. Fish exposure to sub-lethal concentrations of ammonia can reduce swimming activity (Wicks et al., 2002), foraging behaviour (Tudorache et al., 2008) and the ability to flee from predators (McKenzie et al., 2009; Tudorache et al., 2008).

Behavioural analyses are commonly used in ecotoxicology as indicators of sub-lethal toxicity in aquatic animals, and an increasing body of evidence has demonstrated the effectiveness of this approach in a wide range of exposure scenarios (Bae & Park ,2014; Melvin & Wilson, 2013). Fish exposed to increased ammonia concentrations experience difficulty in eliminating this metabolite from the body (Sinha et al., 2014) and, therefore, prolonged exposures to ammonia promotes its accumulation in fish (McKenzie et al., 2009). Several studies indicated that fish pre-exposed to episodes of pollution by inorganic nitrogen compounds (Boyd, 2013; Shrivastava et al., 2016) and heavy metals (Adeyemi & Klerks, 2013; McGeer et al., 2007; Zheng et al., 2016) could be more tolerant to these pollutants by acclimation. In these studies, it was shown that fish pre-exposed to sub-lethal concentrations of a pollutant exhibited an

increased tolerance to exposure to high concentrations of the same pollutant. Fish pre-exposed to sub-lethal concentrations of ammonia pollution could tolerate high concentrations of this compound by increasing the ammonia excretion rate as well as by favouring the evolution of adaptive mechanisms (Boyd, 2013; Shrivastava et al., 2016). These mechanisms have also been shown to work with other types of stressors such as hypoxia (Rees et al., 2001), salinity (Al-Amoudi, 1987) and temperature changes (Long et al., 2013). All these studies analyse the effect of fish pre-exposure from a biochemical and physiological point of view.

The aim of this study was to analyse, under experimental conditions, the effect of sublethal ammonia concentrations on the swimming activity and feeding behaviour of wild-caught fish that had been pre-exposed for a long time to this compound. The species selected for this study was the Mediterranean barbel, *Barbus meridionalis* (Risso, 1827), a freshwater fish endemic to the NE Spain and SE France. Fish from a long-term polluted river and fish from a pristine stream were exposed to sublethal ammonia concentrations in the laboratory. Fish stress responses were complemented using biomarkers. The analysis of biomarkers may provide valuable information by assessing the activity of enzymes/markers involved in energy metabolism, detoxification, antioxidant defences and oxidative stress. In this study, biomarkers included lactate dehydrogenase (LDH), which is involved in anaerobic metabolism (Diamantino et al., 2001); glutathione S-transferase, a xenobiotic that metabolizes II enzyme response (van der Oost et al., 2003); glutathione (GSH) levels, which aid maintenance of the cell redox equilibrium as well as being a powerful antioxidant (Viarengo et al., 1989); catalase (CAT EC 1.11.1.6—reduces H_2O_2 to water) an antioxidant enzyme involved in detoxifying reactive oxygen species and markers of oxidative tissue damage such as lipid peroxidation (Halliwell & Gutteridge, 1999). It has been suggested that *B. meridionalis* is relatively tolerant to organic pollution (Aparicio et al., 2000) and, globally speaking, more tolerant to pollution than other cyprinid species (Aparicio et al., 2000; Colin et al., 2017; Crivelli, 2006). It was hypothesized that fish previously exposed to ammonia in the wild should have a higher tolerance to this compound than fish coming from unpolluted waters.

MATERIALS AND METHODS

Study area and fish sampling

Two sites (polluted and unpolluted) were sampled in the Besòs River basin (NE Spain) (**Fig. 1**). In both sites there was prior knowledge about the existence of a population of *B. meridionalis* (Sostoa et al., 2002; Vinyoles et al., 2011). The polluted site was located in the Congost River, a 43 Km long tributary in the Besòs basin, 50 m downstream the Granollers WWTP (41º56'97.31"N, 2º27'15.66"E). The unpolluted site was located in the Castelló stream, a pristine 3 Km long tributary inside the San Llorenç del Munt i l'Obac Natural Park (41º65'16.97"N, 2º06'11.18"E). The concentration of total ammonia nitrogen (TAN) in the polluted site (Congost River) ranged from 0.54 mg/L to 24.70 mg/L between 2011 to 2015 (**Table 1**) (data provided for Granollers Town Council). In the unpolluted site (Castelló stream), the concentration of TAN ranged from 0.00 mg/L to 0.02 mg/L during the same period (**Table 1**) (Fortuño et al., 2018). In this stream, there is no urban nucleus or any type of agricultural or industrial activity. Although ammonia is not the only pollutant present at these two sites, it is one of the most frequently found, not only in this river but in all rivers of NE Spain (Camargo & Alonso, 2006). **Table 1** shows the physical-chemical parameters analyzed at the two sites for the sampling month for Granollers Town Council (polluted site) and Fortuño et al. (2018) (unpolluted site). Other contaminants such as contaminants of emerging concern (CEC) (pesticides, metals, industrial solvents, pharmaceuticals and personal care products), could be found in other sites across these basin (Soler et al., 2020).

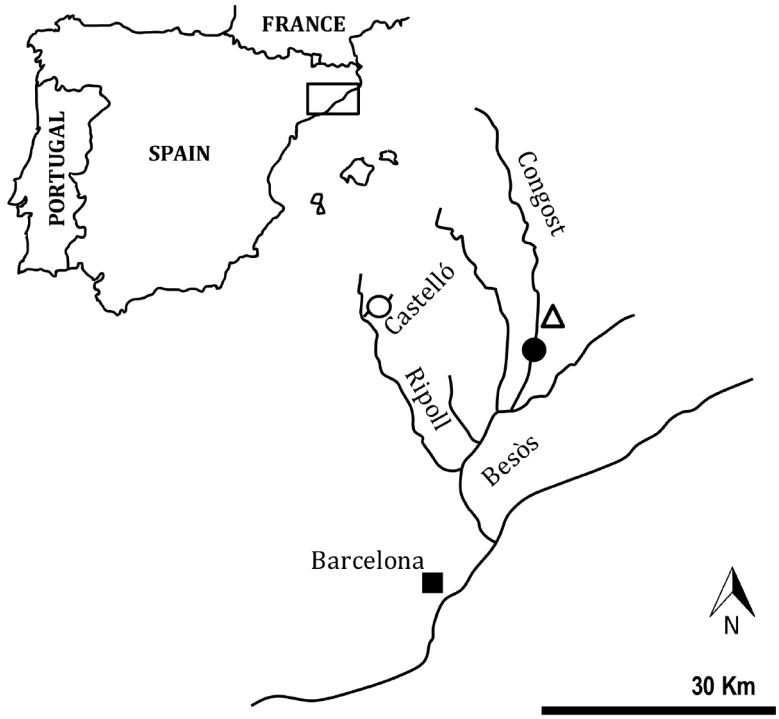

Fig. 1. Map of the sampling sites in the Besòs River basin (NE of the Iberian Peninsula). The black point indicates the location of the polluted site in the Congost River (50 m downstream from a WWTP) where the pre-exposed fish were caught. The white point indicates the location of the unpolluted site in the Castelló stream.

Fish were sampled by electrofishing using a portable unit which generated up to 200 V and 3 A pulsed D. C. A total of 72 individuals (40 in the polluted site and 32 in the unpolluted site) ranging from 5.5 to 10.8 cm were caught in January 2016. No differences in furcal length (FL, mean ± SD = 7.79 ± 1.31 cm) were found between the fish of the two sites. Once in the laboratory, fish of each site were acclimatized separately in 260 L aquaria over 21 days in clean dechlorinated water (there were 10-12 fish per aquaria). Chlorine elimination was achieved by storing water from the drinking supply net in 200 L containers during 48 h. According to Kroupova et al. (2006) fish affected by nitrite poisoning that were placed in clean water for over six days, recovered the normal haematological parameters. Therefore, a period of 21 days seemed sufficient for the fish from the polluted site to recover normal physiological parameters. Aquaria were set in an acclimated room (20ºC) under a 12 h light:

12 h dark photoperiod. All 260 L aquaria had the same equipment (biological filter and air diffusor), substrate (mix of sand, gravel, and coral with a proportion 2:2:1) and enough artificial refugees (PVC tubes and plastic plants) for reducing fish stress. Fish were fed "*ad libitum*" twice a day with frozen red chironomid larvae. A periodical cleaning of aquaria and partial water renovation (one-third of the volume) were carried out every 24 h. Physiochemical water conditions (mean ± SD) were controlled daily in the 260 L aquaria (water temperature = 21.97 ± 0.98 ºC, pH = 8.30 ± 0.27, NO_3^- = 5.63 ± 1.70 mg/L, NO_2^- = 0.00 ± 0.00 mg/L, NH_4^+ = 0.00 ± 0.00 mg/L, and water hardness = 10.50 ± 4.36). These parameters did not show significant differences between aquaria during fish acclimatization.

Table 1. Physical-chemical water parameters from polluted and unpolluted sites.

		January 2016		2011 – 2015	
Physical-chemical parameters		**Polluted**	**Unpolluted**	**Polluted**	**Unpolluted**
General	**pH**	8.0	8.0	8.0 ± 0.4	8.0 ± 0.3
	Oxygen (mg/L)	7.8	5.5	8.1 ± 2.7	8.9 ± 2.4
Salinity	**Conductivity (µS/cm)**	1,112.0	444.7	1,062.5±107.5	613.9 ± 98.0
	Cl (mg/L)	233.6	8.5	158.5 ± 40.8	13.2 ± 1.3
	SO_4 (mg/L)	91.4	13.5	85.0 ± 7.8	16.7 ± 3.9
Nutrients	**NO_2 (mg/L)**	1.3	0.001	0.8 ± 0.7	0.007±0.003
	NO_3 (mg/L)	39.5	0.01	26.0 ± 3.4	0.1 ± 0.1
	TAN (mg/L)	4.8	0.02	2.4 ± 0.1	0.04 ± 0.02
	PO_4 (mg/L)	5.3	0.003	4.1 ± 3.3	0.01 ± 0.01

Physical-chemical water parameters from both sites are shown for January 2016, when fish were sampled as well as the mean values for the period 2011 – 2015 (mean ± SD). All these data were provided by the Granollers Town Council (polluted site) and by Fortuño et al. (2018) (unpolluted site).

Experimental design

After the acclimatization period, fish pre-exposed to ammonia pollution in the wild (hereafter, pre-exposed fish) and fish from the unpolluted site (hereafter, non pre-exposed fish) were exposed to four TAN treatments (0, 1, 5 and 8 mg/L) as follows: each fish was placed in individual 20-L aquaria (40 cm large x 20 cm height x 25 cm deep) and transferred to the room where the experiment was carried out. The aquaria were divided into four groups and a treatment was randomly assigned to each group (**Fig. 2**). For the Congost river (pre-exposed fish) there were ten aquariums per treatment (N = 40), while for the Castelló stream (non pre-exposed fish), there were eight aquariums per treatment (N = 32). Aquaria were positioned in two rows, side by side, within each group (**Fig. 2**). In order to reduce fish stress, the lateral walls between neighboring aquaria were left transparent. To avoid fish interaction with the environment, the external and frontal walls as well as the bottom of aquaria were covered by blue acetate sheets. Before starting the experiment, each fish was acclimatized to its 20 L aquaria for four days and fed daily with red chironomid larvae. During these four days of fish acclimatization, partial water changes were carried out every day, and TAN concentrations were measured with indophenol blue spectrophotometric method (in all aquaria TAN concentration was maintained at 0 mg/L; mean ± SD = 0.00 ± 0.00 mg/L).

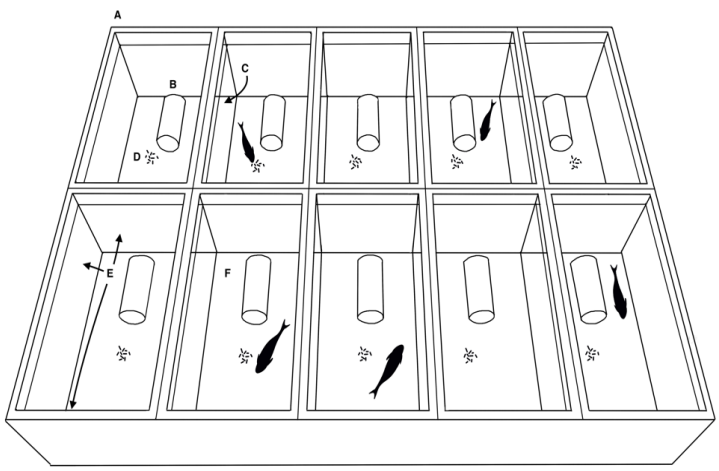

Fig. 2. Individual experimental aquaria used for each TAN treatment. A: Schematic representation of a group of 20 L individual aquaria used for each TAN treatment in the

experiment with *B. meridionalis* (aquaria were placed on a solid deck pallet to facilitate handling). B: PVC tube refuge. C: Transparent lateral walls between neighbouring aquaria. D: Red chironomid larvae used to observe feeding behaviour. E: Exterior and frontal walls of the aquaria covered with blue acetate sheets. F: Bottom of aquaria covered by a blue acetate sheet.

Next, fish were exposed to the assigned TAN treatment for eight days. The experiment was first carried out with the pre-exposed fish. After eight days, the experiment was repeated with the non pre-exposed fish. The TAN concentrations per experimental aquaria were achieved by adding analytical grade ammonium bicarbonate solutions (NH_4HCO_3, Sigma-Aldrich, Barcelona, Spain). These solutions were dispensed with automatic pipettes after water changes. A daily cleaning of aquaria and a two-third of the water volume renovation were carried out with dechlorinated water to guarantee the experimental conditions. TAN concentrations were measured daily by the indophenol blue spectrophotometric method. Once the absorbance values had been recorded for each sample, NH_4^+ concentration was calculated using the equation of the calibration curve and the proportion of the NH3 form was calculated following Thurston et al. (1979) procedures. During the experiment, the aquaria group of each TAN treatment was visually isolated from the researchers with opaque curtains. In order to observe the activity of fish, a PVC tube (4 cm diameter x 13 cm length) was placed in each 20 L aquaria as a fish refuge (**Fig. 2**). In order to observe feeding activity, fish were fed above satiation requirements (20 red chironomid larvae per fish were sufficient to quantify satiety). Fish behaviour was recorded with an overhead shot for each group of aquaria (TAN treatment) using a Sony HD (HDR-SR1E) camera. The experiment lasted for eight days and recordings were made on alternative days (four days) between 9:00 and 12:00 AM. Every day, the recording order of each group of aquaria (TAN treatment) was established at random. Fish were only fed during the recording days. Two behavioural variables per individual were analyzed from video recordings: swimming activity (during 10') and feeding behaviour (until fish stopped eating). The swimming activity was analysed by three variables: (1) "Swimming", amount of time during which fish make displacements of the body using body or fin movement as propulsion (s), (2) "Not visible", amount of time during which the fish was not visible because it was remaining inside the shelter (s), and (3) "Resting", amount of time fish

spent lying motionless on the bottom of the aquaria (s). Total swimming activity was expressed as a percentage of the total observation time (Mas-Muñoz et al., 2011). Feeding behaviour was analysed by measuring: (1) "Latency", defined as the amount of time the fish took to start touching the food (s); (2) "Voracity", defined as the number of chironomid larvae the fish ate in one minute and (3) "Satiety", defined as the amount of time until the fish either stopped eating or they started spitting out the food (s).

The concentration of NH_3 (mean ± SD, mg/L) for each TAN treatment was not significantly different between pre-exposed and non pre-exposed fish (GLM): [0 mg/L] = 0.007 ± 0.010, [1 mg/L] = 0.139 ± 0.077, [5 mg/L] = 0.534 ± 0.218, [8 mg/L] = 0.645 ± 0.237). Physiochemical parameters were controlled daily for each 20 L aquaria during the experiment. No differences in physiochemical parameters (mean ± SD) were found during the experiment between fish from the two sites and between the aquaria of each TAN group (GLM) (water temperature = 21.27 ± 0.45 ºC, pH = 8.33 ± 0.18, NO_3^- = 4.81 ± 0.68 mg/L, NO_2^- = 0.00 ± 0.00 mg/L, and water hardness = 15.05 ± 3.76).

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The scientific procedure of this work was approved by the Animal Ethic Committee of the University of Barcelona (registration Nº 9296), which follows European Directive 2010/63/UE on the protection of animals used for scientific purposes. One of the co-authors holds a category C FELASA certificate that regulates the use of animals for experimental and other scientific purposes.

Biochemical determination

For the biochemical determinations, fish were anesthetized on ice at the end of the experiment and euthanatized by decapitation. Biomarkers were analysed in the liver tissue for each individual fish according to Faria et al. (2009).

The following reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA): potassium phosphate dibasic (K_2HPO_4); potassium phosphate monobasic

(KH_2PO_4); potassium chloride (KCl); ethylenediamine-tetraacetic acid, disodium, salt, dihydrate (EDTA); hydrogen peroxide (H_2O_2); reduced glutathione (GSH); sodium azide; 1-chloro–2,4–dinitrobenzene (CDNB); glutathione S-transferase, from equine liver (GST) (EC 2.5.1.18); monochlorobimane (mCB); sodium pyruvate; β-Nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH), 2,6-di-tert-butyl-4-methylphenol (BHT); 1-methyl-2-phenylindole (MPI); 1,1,3,3-tetramethoxypropane (TMP) and Bradford reagent. All the other chemicals were analytical grade and were obtained from Merck (Darmstadt, Germany).

Except for catalase activity, where a cuvette assay was used (Life Science UV/Vis Spectrophotometer DU® 730, Beckman Coulter – Fullerton, CA, USA), all the bioassays were performed in microplates (Synergy 2 Multi-Mode Microplate Reader, BioTek® Instuments – Vermont, USA).

Liver tissue was homogenized in ice-cold 0.1M phosphate buffer with 150mM KCl and 0.1mM ethylenediamine-tetraacetic acid, disodium, salt, dihydrate (EDTA), then centrifuged at 10 000xg, 4ºC for 30 minutes. The supernatant was collected, aliquoted and stored at -80ºC for biomarker determination.

CAT activity was measured by estimating the decrease in absorbance at 240 nm due to H_2O_2 (50 mM H_2O_2 in 80 mM phosphate buffer, pH 6.5) consumption (extinction coefficient 40 $M^{-1}cm^{-1}$) according to Aebi (1974). Reaction volume and time were 1 mL and 1min, respectively. GST activity towards CDNB was measured as described by Habig et al. (1974). The reaction mixture contained 0.1M phosphate buffer (pH 7.4), 1 mM CDNB and 1 mM GSH. The formation of S – 2,4 dinitro phenyl glutathione conjugate was evaluated by monitoring the increase in absorbance at 340 nm during 5 minutes. Enzyme activity was determined using GST's extinction factor coefficient of 9.6 mM-1 cm-1. Results were normalized by tissue total assay protein content. Reduced glutathione (GSH) quantification was adapted from zebra mussel digestive gland according to Kamencic et al. (2000). It consists on adding 0.1mM of mCB along with 1U/ml of GST to each sample. Then the GSH present in the cells forming a GSH-mCB complex is measured fluorometrically at excitation: emission wave length of

360:460 nm, after an incubation period of 90 minutes at room temperature and protected from light. The total content of GSH was then extrapolated from a GSH standard curve determined under the same physical and chemical conditions as the samples, and the results were normalized by the tissue wet weight (g ww).

Lactate dehydrogenase (LDH) activity was determined according to Diamantino et al. (2001) by monitoring the absorbance decrease at 340 nm due to NADH oxidation. The reaction contained 100 mM phosphate buffer (pH 7.4), 0.15 mM NaOH, 1.18 mM pyruvate and 0.18 mM NADH.

Lipid peroxidation (LPO) was determined by quantifying the levels of malondialdehyde (MDA) according to Esterbauer et al. (1991). The MDA assay was based on the reaction of the chromogenic reagent 1-methyl-2-phenylindole with MDA at 45˚C, giving rise to a chromophore with absorbance at 586nm. Samples were incubated with 5mM 1-methyl- 2-phenylindole in acetonitrile:methanol (3:1 v/v), 5.55% of HCl and 0.01% BHT at 45˚C, for 40 minutes. Absorbance was read at 560nm and MDA content in each sample was extrapolated from the standard curve of 1,1,3,3-tetramethoxypropane (TMP) treated under similar conditions as samples. The final results were normalized by tissue wet weight (g ww).

Total protein concentrations were accessed by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford, 1976).

Statistical analyses

Differences between TAN treatments and sites were analysed by means of a generalized lineal mixed model (GLMM). For swimming activity, “Swimming” and “Not visible” were analysed separately and used as dependent variable. The variable “Resting” was not analysed as it was a complementary variable to the other two. For feeding behaviour, “Latency”, “Voracity” and “Satiety” were analysed separately and used as dependent variables. In all cases “site” (2 levels: pre-exposed and non pre-exposed fish) and “TAN treatments” (4 levels: “0”, “1”, “5” and “8” mg/L TAN) were used as factors together with their interaction.

The gamma distribution was assumed in the analysis of swimming activity and the Poisson distribution was assumed in the analysis of feeding behaviour. The variable "Individual" was added to the model as a random factor.

Biomarker responses across fish from the two sites (pre-exposed and non pre-exposed fish) and TAN treatments were analysed through a lineal model (LM) with the same factors ("site" and "TAN treatments") (Zar, 1996). Differences between TAN treatments against control ones were further compared using Dunnett's post-hoc test (Zar, 1996).

All analyses were conducted with R 3.4.3 (R Core Team, 2017). GLMM assuming a Poisson or a gamma distribution was performed using glmer() (package "lme4": Bates et al., 2015). Non-significant interactions were removed from final models. Homogeneity and normality of residuals were visually checked for all models. All significant differences are $P \leq 0.05$.

RESULTS

Behavioural variables

The "Swimming" and "Not visible" GLMM models showed no significant effect of TAN treatments within and across sites (interaction) ($P > 0.05$). Only a significant effect of site (pre-exposed and non pre-exposed fish) ($P < 0.001$) was shown for these two variables. The swimming activity of fish that had been pre-exposed to ammonia pollution in the field was lower than that of non pre-exposed fish. Non pre-exposed fish swam for a longer time (67% of the time; mean = 643.89 s; 95% confidence interval = 504.10 – 891.34) than pre-exposed fish (57.3% of the time; mean = 548.92 s; 95% confidence interval = 444.92 – 716.43) regardless of the TAN treatments they were in. Similarly, non pre-exposed fish spent significantly less time hidden inside the shelter (mean = 206.76 s; 95% confidence interval = 150.01 – 332.90) than pre-exposed fish (mean = 232.99 s; 95% confidence interval = 165.76 – 392.43).

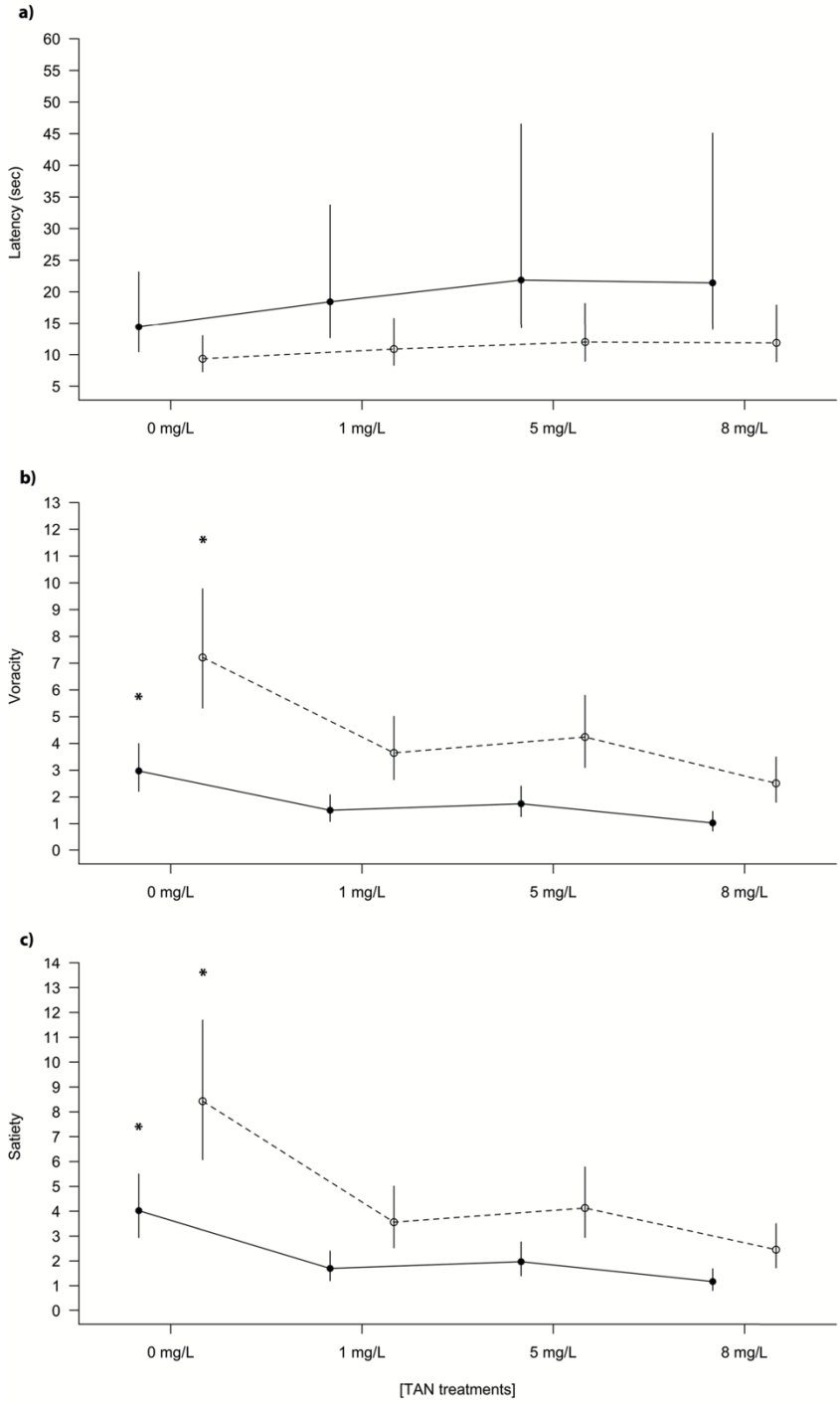

Fig. 3. Feeding behaviour of *B. meridionalis* exposed to the TAN treatments during the experiment. (a) Latency (time taken to start touching the food), (b) Voracity

(number of red chironomid larvae eaten in one minute) and (c) Satiety (total number of red chironomid larvae fish eaten) are shown for the pre-exposed fish from the polluted site (black points and solid lines), and the non pre-exposed fish from the unpolluted site (white points and dashed lines). * indicates significant differences ($P < 0.05$) between the control concentration and the TAN treatments, following a generalized linear mixed model (GLMM).

The analysis of feeding behaviour showed no significant effects of the interaction between TAN treatment and site for none of the variables ($P > 0.05$). For "Latency" GLMM model showed a significant effect between sites ($P < 0.002$) but not for TAN treatments ($P > 0.05$). Non pre-exposed fish had a higher latency than pre-exposed fish (**Fig. 3a**). In contrast, GLMM models for "Voracity" and "Satiety" variables showed a significant effect between sites and TAN treatments within each site ($P < 0.001$). Non pre-exposed fish had a higher voracity (**Fig. 3b**) and were satiated later (**Fig. 3c**) than pre-exposed ones. In both cases (sites), significant differences were found between the control TAN concentration (0 mg/L) and the three TAN treatments (1, 5, 8 mg/L) for "Voracity" and "Satiety" variables.

Biochemical determination

The results of the analysis of biomarkers show that there were significant ($P < 0.05$) differences between sites (pre-exposed and non pre-exposed fish) in three out of the five studied biomarkers (**Table 2, Fig. 4**). TAN treatment within and across sites (interaction) also affected the activities of CAT, GST and levels of LPO. Pre-exposed fish had lower CAT activities and lower levels of GSH, and the activities of CAT and levels of LPO increased across TAN treatments. In fish from both sites the activities of GST were enhanced at 1 mg/L of TAN (**Fig. 4**).

Table 2. Linear model results for testing the effects of site and TAN on the studied biomarkers in *B. meridionalis*.

		df	F	*P*
GSH	**Site**	1.28	46.5	<0.001
	TAN	3.28	2.4	0.091
	Interaction	3.28	0.5	0.713
CAT	**Site**	1.51	50.5	<0.001
	TAN	3.51	1.5	0.215
	Interaction	3.51	2.9	0.043
GST	**Site**	1.52	2.7	0.107
	TAN	3.52	4.7	0.005
	Interaction	3.52	1.1	0.367
LDH	**Site**	1.51	2.8	0.098
	TAN	3.51	0.4	0.782
	Interaction	3.51	2.7	0.054
LPO	**Site**	1.27	12.9	0.001
	TAN	3.27	6.3	0.002
	Interaction	3.27	6.6	0.002

Degrees of freedom (df), Fisher's quotient (F) and probability levels (*P*) are shown.

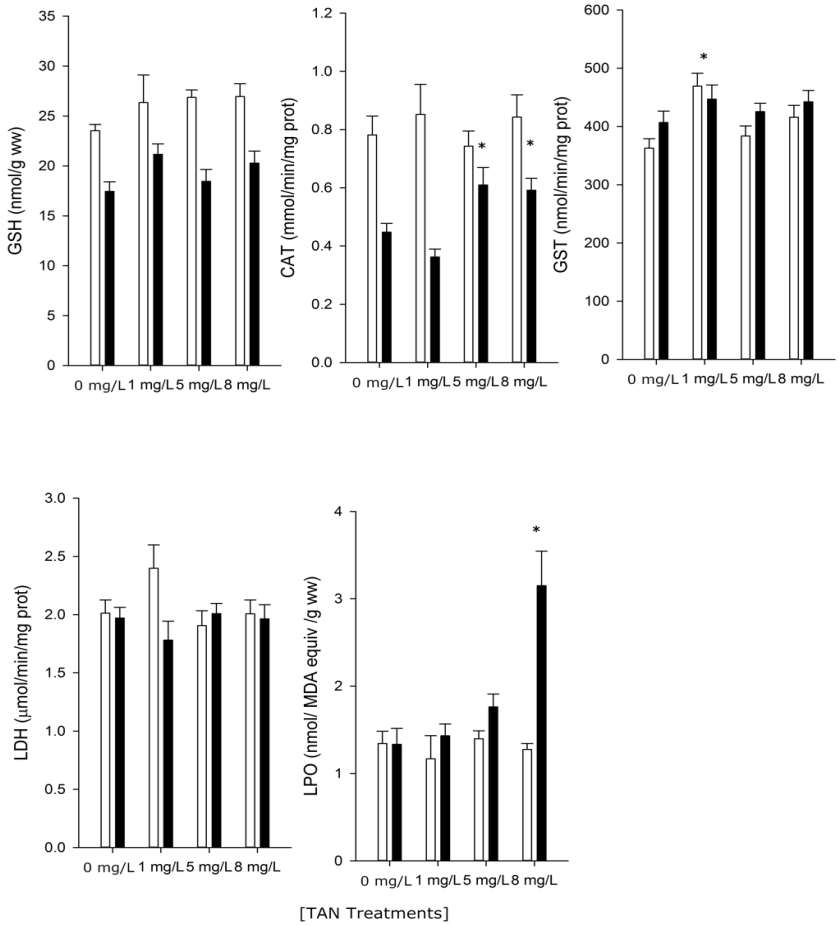

Fig. 4. Antioxidant and oxidative stress responses (Mean ± SE, N=10) of *B. meridionalis* exposed to increasing TAN concentrations during the experiment. Black and white bars indicate the results for the fish from the polluted site (pre-exposed fish) and the unpolluted site (non pre-exposed fish), respectively. * indicates significant differences ($P < 0.05$) within each site from control TAN treatment (0 mg/L TAN) following LM model and Dunnett's post-hoc tests.

DISCUSSION

Chronic exposure to pollution by nitrogen inorganic compounds (NH_4^+, NH_3, NO_2^-, HNO_2 and NO_3^-) has effects on the reproduction, growth and survival of freshwater fish (Camargo & Alonso, 2006). Specifically, exposure to NH_4^+ and NH_3 (TAN) pollution can cause gill damage, anoxia, disruption of blood vessels and osmoregulatory activity (damage to the liver and kidneys), and a decrease in the effectiveness of the immune system (CEPA, 2001). In addition, NH_4^+ ions contribute to an internal reduction of Na^+ which, in turn, increases the toxicity by NH_3 (Camargo & Alonso, 2006). All these effects can result in a reduction in fish feeding activity, fecundity and survival, leading to a reduction of the size of populations (Camargo & Alonso, 2006).

In the present study, wild-caught fish pre-exposed for a long-term period to ammonia pollution in a contaminated river near a WWTP showed an altered behaviour and suffered from an increased physiological stress as compared to non pre-exposed fish from a pristine stream. Analysis of fish swimming activity showed that, regardless of the TAN treatments, pre-exposed fish were less active and spent more time hiding in the refuge than non pre-exposed fish. The only studies on the effects of ammonia on fish swimming activity have been conducted on salmonids, in laboratories or farms. According to Tudorache et al. (2008) and Wicks et al. (2002), the swimming activity of salmonids is reduced at concentrations between 0.2 - 1 mg/L of TAN (that is, at 0.009 – 0.04 mg/L NH_3). Pre-exposed fish spending more time inside the PVC shelters ("not visible" time) might indicate that these fish had their exploratory activity altered. A decrease in the exploratory activity has been reported in several fish species exposed to crude-oil pollution (Jacquin et al., 2017), pesticides (Davy et al., 1973) and pharmaceutical products (Reyhanian et al., 2011).

The feeding behaviour of *B. meridionalis* was also altered. Pre-exposed fish had lower voracity than non pre-exposed fish regardless of the TAN treatments (0, 1, 5 and 8 mg/L TAN). Within each site (pre-exposed and non pre-exposed fish), lower voracity was observed from the lowest TAN concentration (1 mg/L). A reduction in voracity has been reported for salmonids under TAN concentrations from 1 to 3 mg/L (Ortega et al., 2005; Tudorache et al. 2008;

Wicks & Randall, 2002). According to Schram et al. (2010), in a non salmonid fish (*Clarias gariepinus*), food consumption was also drastically reduced at TAN concentrations higher than 1 mg/L. In the present study, latency (the time that the fish took to start touching the food) was lower in pre-exposed fish, regardless of the TAN treatment. A low latency has been related to a low capacity to find food and capture prey (Lima & Dill, 1990). Furthermore, several studies relate lower latency with lower efficiency to flee from predators (Mckenzie et al., 2009; Whitham & Mathis, 2000; Yamamoto & Reinhardt, 2003).

The present study was conducted under a concentration of ammonia within the range of LC_{50} (the tested range was from 0.007 to 0.645 mg/L NH_3). However, the tolerance to NH_3 in cyprinids could be higher. The LC_{50} for cyprinids ranked between 0.685 and 1.720 mg/L NH_3 (CEPA, 2001). For cyprinids, the sublethal concentrations in which negative physiological effects begin to be observed has been described in a range of 0.105 - 0.247 mg/L NH3 (Mayes et al., 1986; Swigert & Spacie, 1983; Thurston et al., 1986). The limits of tolerance to this and other compounds are variable depending on each fish species so that it would be necessary to investigate their effects under natural conditions.

Antioxidant enzyme activities such as those of CAT and reduced glutathione has been reported to be important antioxidant mechanisms against oxidative stress-mediated effects of ammonia in fish (Cheng et al., 2015; Hegazi et al., 2010; Li et al., 2016; Maltez et al., 2017; Pan et al., 2011; Ren et al., 2016; Sinha et al., 2014; Sun et al., 2012; Sun et al., 2014; Yang et al., 2010; Yang et al., 2011; Zhang et al., 2016). Pre-exposed fish (from the polluted site) had lower constitutive levels of the above mentioned antioxidant defences and consequently were unable to detoxify the excess of reactive oxygen species (ROS) generated by ammonia, leading to enhanced tissue levels of oxidative damage measured as LPO. Interestingly, only in fish from the polluted site (pre-exposed fish), the activities of CAT increased in individuals, exposed to ammonia, thus indicating that the exposure to this compound increased ROS and, hence, triggered the antioxidant defences of these fish. In fish from the unpolluted site (non pre-exposed fish), the high constitutive levels of antioxidant defences protected them from ROS generated by ammonia. Sinha et al. (2014) reported that fish

species intolerant to ammonia, such as trout, rely mainly on glutathione-dependent defensive mechanisms, while more tolerant species, such as carps, utilize antioxidant enzymes such as CAT and ascorbate. High tolerant species, such as goldfish, use both of these protective systems, and show more effective anti-oxidative compensatory responses towards oxidative stress induced by ammonia (Sinha et al., 2014). Thus, our results are in line with previous studies, as *B. meridionalis* considered a tolerant species to ammonia.

Results in this study indicated that the exposition of fish to high ammonia concentrations did not guarantee, at least short term, the recovery of a good health status and/or a greater tolerance to a high concentration of this compound. Fish pre-exposed to ammonia pollution in the wild showed an altered behaviour at the control concentration (0 mg/L TAN). This could be a consequence of pre-existing physiological problems due to exposure to ammonia and other pollutants in nature (Smith & Weis, 1997; Weis & Khan, 1991; Weis et al., 2001). The feeding behaviour and the response to the oxidative stress of *B. meridionalis* (both pre-exposed and no pre-exposed fish) follow the same pattern, reacting equally to the first 1 mg/L TAN treatment. However, pre-exposed fish had a more marked response in feeding behaviour and biomarkers under the different treatments of TAN. A reduction in food intake is directly related to both a lower growth and a low rate of protein synthesis (Carter et al., 1992). Reported studies have shown that low protein synthesis rates represent a large proportion of energy costs in fish, and this has a direct impact on the growth efficiency of individuals (McCarthy et al., 1994). Alteration of the behaviour parameters analysed in this study can be extrapolated to other traits such as exploration activity, boldness and ability to avoid predators (Scott & Sloman, 2004). Ammonia can affect social interactions as well, by altering dominance relationships, hierarchical dynamics and predator-prey relationships (Grobler & Wood, 2018; Tudorache et al., 2008).

Ammonia pollution is a common problem in freshwater ecosystems (EPA, 2013). Despite the efforts of implementing the European Water Framework Directive (2000/60/RC) (2000) there are still many WWTPs that do not have tertiary purification systems of urban wastewater, which leads to an increase in nitrogen compounds in aquatic ecosystems (Arenas-Sánchez et al., 2019;

Grizzetti et al., 2011). Improving water quality is an important key to enhance the conservation of river ecosystems. However, our results indicated that fish that previously survived in a polluted environment did not recover their health in more purified waters. In summary, although habitats are improving their environmental quality, the survival of fish populations that have been pre-exposed to contamination could be compromised. In freshwater ecosystems, which have suffered an 83% decline in vertebrate populations from 1970 to 2014 (WWF, 2018), all factors affecting the survival of individuals are of great relevance.

ACKNOWLEDGEMENTS

We thank I. Ramirez and F. López of the Department of Biochemistry and Molecular Biomedicine (University of Barcelona), for giving us access to their laboratory. We thank P. Fortuño (University of Barcelona) and X. Romero (biologist and superior technician of environment and natural environment of the Granollers Town Council) for provided data. We also thank J. Guinea and P. Manning for the assistance in the laboratory tasks. The authors are grateful to V. Bonet for the English review. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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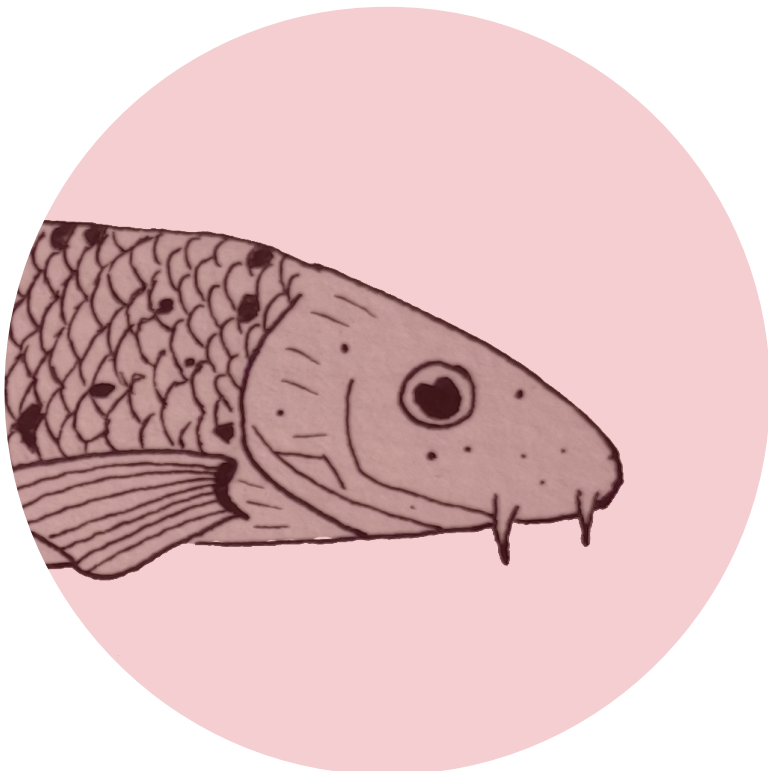
Sección 2.2.

¿Puede el amonio alterar la retención de memoria de los peces de agua dulce silvestres?



Soler et al. (2020). Can ammonia alter memory retention in a wild freshwater fish?

Can ammonia alter memory retention in a wild freshwater fish?

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Abstract: Ammonia is one of the most common aquatic pollutants around the world. Fish behaviour can be altered by a wide range of pollutants but the effects on more complex behaviours such as animal personality and cognition are less studied. Boldness, defined as the propensity of an individual to respond to a novelty or to take risks, is one of the most studied facets on animal personality. The objective of this work was to investigate, under experimental conditions, how exposure to ammonia could alter memory retention in a native fish species (*Barbus meridionalis*). Control (non-exposed) fish (N=10) and experimental (N=14) fish were housed in two 260-L aquaria where total ammonia (TAN) treatments were conducted. Before treatments, fish boldness was evaluated through three variables ("Boldness score", "Hesitancy" and "Feeding") in a specially designed experimental aquaria (at week 1). How these variables varied in subsequent boldness tests (after fish learned through the first experience) was taken as indicative of memory retention and was analysed under exposure to three TAN treatments (1, 8 and 16 mg/L) at weeks 2, 3 and 4, respectively. Exposed fish had similar memory retention than non-exposed ones for "Boldness score" and "Hesitancy" although both groups of fish began to be more cautious at week 4 (between 16 and 24 days of experiment). However, at week 4, exposed fish showed a tendency to completely lose learning (a similar value for the "Boldness score" of week 1 was found for the exposed fish). Fish response under experimental conditions may be merely indicative of what is happening in nature. Additional stressors (such as time of exposure to pollutants and mixture of pollutants) and local adaptation to water pollution can modify, in the wild, results from laboratory studies.

Keywords: Appetitive learning, *Barbus meridionalis*, Boldness, Fish behaviour,

Animal personality, Water pollution

INTRODUCTION

Ammonia is a commonly found pollutant in aquatic environments around the world (Camargo & Alonso 2006; CEPA, 2001). This compound can damage fish tissues and organs, thus leading to physiological alterations and, sometimes, death (Schram et al., 2010). Ammonia can cause cell damage and can also affect the antioxidant defence system thus altering the levels of oxidative stress in fish (EPA, 2013; Sinha et al., 2014). Ammonia can also alter fish behaviour. Fish exposure to sub-lethal concentrations of ammonia can reduce swimming activity (Wicks et al., 2002), foraging behaviour (Tudorache et al., 2008), the ability to flee from predators (McKenzie et al., 2009; Tudorache et al., 2008) and can cause disturbances in social behaviour of species relevant to aquaculture (Grobler & Wood, 2018).

Many pollutants affect a wide array of fish behaviours such as activity, exploration, avoidance, sociability, aggressiveness as well as sexual and feeding behaviours (Jacquin et al., 2020). For instance, guppies (*Poecilia reticulata*) exposed to crude-oil showed decreased exploration (Jacquin et al., 2017). In the crucian carp (*Carassius carassius*), plastic nanoparticles supplied through the food chain entered the brain causing fish to explore less and for longer distances (Mattsson et al., 2017). Feeding behaviour is also altered by exposure to microplastics in planktivorous reef fish (Critchell & Hoogenboom, 2018) but many other pollutants, such as pesticides and pharmaceutical compounds (contraceptives, antidepressants, anxiolytics, etc.) can alter fish behaviours such as swimming (Beauvais et al., 2001), mating (Baatrup & Junge, 2001; Kristensen et al., 2005), response to predators (Eisenreich et al., 2015; Saaristo et al., 2017), and even migratory behaviour as occurs in the salmon (*Salmo salar*) (Hellström et al., 2016; Klaminder et al., 2019). However, effects on more complex behaviours such as animal personality and cognition are less studied, specially in wild species (Saaristo et al., 2018; Zala & Penn, 2004). Chemical pollution can induce abnormal behaviours and may alter cognitive abilities such as learning and memory.

Animal personalities often consist in a suite of interrelated traits, referred to as behavioural syndromes that are consistent over time under different contexts (Sih et al., 2004) and that have important implications for fitness and evolutionary trajectories (Conrad et al., 2011; Dochtermann & Dingemanse, 2013). Personality can be divided into five categories: activity, shyness-boldness, exploration-avoidance, aggressiveness and sociability (Réale et al., 2007). The boldness–shyness continuum is one of the major personality axes in animals, including fishes. It is defined as the propensity (boldness) or aversion (shyness) of individuals to respond to a novel object or environment, expose themselves to risks and respond to predators (Carter et al., 2013; Wilson et al., 1994). Boldness and fast exploration have both been linked to better learning ability (DeRoy et al., 2020; Dugatkin & Alfieri, 2003; Kareklas et al., 2017) although slower individuals may spend more time attending to environmental cues, make choices more accurately, and may be more responsive to environmental changes (Sih & Del Giudice, 2012; White et al., 2017). Learning ability has not always been associated to boldness (Sommer-Trembo & Plath, 2018). Learning is required by animals to respond to environmental changes (Brown, 2012). The efficiency with which organisms learn and forage may have long-term fitness consequences (Dukas, 2004). Learning requires both the ability to comprehend the association between a given stimulus and a particular phenomenon, object or behaviour and the ability to retain this acquired information (i.e., memory).

There is extensive information on fishes' learning ability and memory retention for foraging (Brydges et al., 2008; Ingraham et al., 2016) and predator avoidance (Brown, 2001; Brown et al., 2011; 2013), among others. However, fewer studies have evaluated how pollutants can alter personality in animals. In fish, physiological and personality traits are tightly linked (Jacquin et al., 2020). Pollutants often trigger important stress responses and changes in metabolism, so that they can alter the structure of behavioural syndromes (Killen et al., 2013). In addition, many contaminants affect fish cognitive performances, with potential cascading effects on fitness (De Castro et al., 2009). For example, the perch (*Perca fluviatilis*), when exposed to psychiatric drugs, becomes more active and bolder, and shows a lower latency to feed (Brodin et al., 2013). Similarly, guppies exposed to a neutral household detergent (one of the most common aquatic pollutants) had an altered personality being shier

and more inactive (Lopes et al., 2019). Hormonal growth promoters, veterinary pharmaceuticals that enter aquatic ecosystems, act at low doses and increase exploratory activity in females of eastern mosquitofish (*Gambusia holbrooki*) (Bertram et al., 2018). However, to our knowledge, there is no research on the effect of river pollutants on learning and retention of learning in fish.

The aim of this work was to investigate how the exposition to high concentrations of ammonia (≥ 1 mg / L) affected memory retention in wild fish that experienced a learning event. The species selected for this study was the Mediterranean barbel, *Barbus meridionalis* (Risso, 1827), a freshwater fish endemic to NE Spain and SE France. It has been suggested that *B. meridionalis* is relatively tolerant to organic pollution (Aparicio et al., 2000) and more tolerant to pollution than other cyprinid species (Aparicio et al., 2000; Colin et al., 2017; Crivelli, 2006). However, a recent work shows that fish of this species that had been pre-exposed to ammonia pollution in the field had an altered behaviour even after being detoxified with clean water for a month in the laboratory (unpublished data). I this study, we predicted that fish that had been exposed to ammonia would have worse memory retention than fish that had not been exposed. Applications in conservation are discussed.

MATERIAL AND METHODS

Fish sampling and maintenance

The sampling site was located at the Congost River (41º73'71.84N'', 2º26'68.75E''), a 43 km-long effluent of the Besos River basin (NE Spain). This site is located upstream large industrialized areas, near Santa Eugenia of Congost (**Fig. 1**). Fish were caught by electrofishing using a portable unit which generated up to 200 V and 3 A pulsed D. C. A total of 24 individuals ranging from 76 to 154 mm were collected in September 2016. Once in the lab, fish were randomly distributed into two 260-L treatment aquaria (hereafter, "control" and "experimental" aquaria) and acclimatized for 21 days in dechlorinated water. The control aquaria housed N=10 fish which would not be subsequently exposed to ammonia in the experiment (hereafter, "non-exposed"), and the

experimental aquaria housed N= 14 fish that would be exposed (hereafter, "exposed"). Chlorine elimination was carried out by storing water from the drinking water supply in 200 L drums for 48 h. The two treatment aquaria were in an acclimated room (20ºC) and had the same equipment: a biological filter, an air diffuser, substrate (mix of sand, gravel, and coral with a proportion 2:2:1) and shelters (artificial plants and PVC tubes) for reducing fish stress. These aquaria were regularly cleaned and one third of the water volume was partially renewed every 24 h. Physicochemical conditions remained constant during the acclimatization period (Mean ± SD: Water temperature = 22.01 ± 0.99 ºC, pH = 8.20 ± 0.23, NO3- = 5.53 ± 1.60 mg/L, NO2- = 0.00 ± 0.00 mg/L, NH4+ = 0.00 ± 0.00 mg/L; hardness = 10.40 ± 4.26). A 12:12 (light: dark) photoperiod was established. The two treatment aquaria were isolated from the outside by opaque curtains that prevented any interaction with external elements. During acclimatization, fish were fed "ad libitum" twice a day with frozen red chironomid larvae.

Fish were measured as furcal length (FL, mm) and weighed (W, g). The Fulton index was calculated as $K = 10^5 W FL^{-3}$ in order to evaluate the physical condition of non-exposed and exposed fish at the beginning and at the end of the experiment. Fish were tagged with Visible Implant Elastomer (VIE) following the guidelines of Northwest Marine Technology Inc. (Shaw Island, Washington). A single colour was used (fluorescent red), only visible under ultraviolet light. A small mark was made on the base of the impair fins (dorsal, anal or caudal) and in one of the two body sides (right or left). By making combinations with these two marks, it was possible to differentiate all fish in the two aquaria (control and experimental). Marks apparently did not affect fish behaviour or health.

Measuring boldness

Boldness tests were carried out in an aquaria specifically designed for that purpose (hereafter, "boldness aquaria") following Brown et al. (2007). This aquaria (50 x 70 x 30 cm) was uncovered to be able to introduce the fish and it had a small door (6 cm) for the exit of each individual to the open area (**Fig. 2**). The water level was kept at 27 cm throughout the experiment. Conditions

similar to those of the two treatment aquaria were maintained during the tests (temperature, ammonia concentration and substrata). To avoid any interference during the tests, opaque curtains were installed around the boldness aquaria and the room was kept without light. Fish behaviour was recorded with a video camera (Sony Handycam HDR-SR1E) located 120 cm above the boldness aquaria.

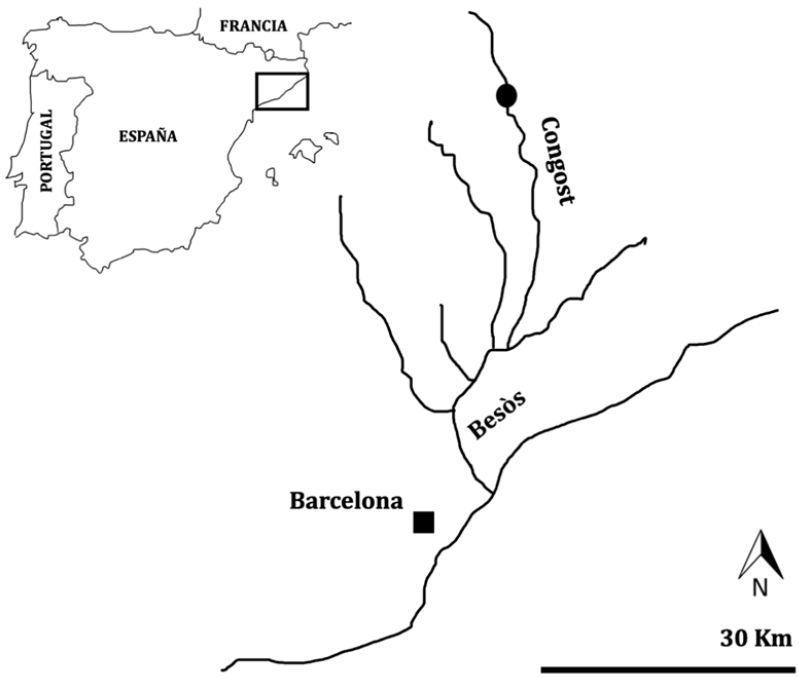

Fig. 1. Map of the sampling site location in the Congost River (Besos River basin, NE Spain). The black point indicates the location where fish were sampled near the town of Santa Eugenia of Congost (Tagamanent, Catalonia) in September 2016.

Boldness tests were performed individually from 8:00 to 17:00 on October 17 (for exposed fish) and October 18 (for non-exposed fish) before starting ammonia treatments. The fish were deprived of food during the 24 h prior to the test. Fish performed the tests in a randomly established order. In order to place them inside the "boldness box" (**Fig. 2**), the fish were carefully caught from their treatment aquaria with a hand net. After a 5 minutes settling time, we opened the boldness box window by raising the trapdoor (**Fig. 2**) and the fish was free to emerge. The maximum duration of the test was 3600 s. After this time, if a fish did not exit the bold box or did not complete the test, the

test was terminated. After the test (and before being returned to their aquaria) the fish were anesthetized with MS-222 (tricaine methanesulfonate), identified with an ultraviolet light, measured and weighed. In order to differentiate the fish that had carried out the tests from those that had not, a separation barrier was placed inside each 260-L treatment aquaria until all the fish had carried out the test. After each test, all the water from the boldness aquaria was replaced by dechlorinated water that had been stored in 200-L drums for the previous 48 h. Once all the fish had performed the test, we removed the divisions from the treatment aquaria.

Fish boldness was measured by using three variables that corresponded to three points within the boldness aquaria (b1, b2 and b3; **Fig. 2**). These variables were defined as follows: "Boldness score" (b1), defined as the time taken for the fish's snout to emerge from the boldness box through the trapdoor (s); "Hesitancy" (b2), defined as the time taken for the fish's snout to emerge from the methacrylate arc (s); and "Feeding" (b3), defined as the time taken for the fish's snout to touch the food reward (red chironomid larvae) (s) (**Fig. 2**). The test finished once the fish had taken the food.

TAN treatments and memory retention tests

In order to investigate whether fish retained learning from the first boldness test when exposed to ammonia, boldness tests were repeated with fish exposed successively to three total ammonia concentrations treatments (TAN treatments; **Fig. 3**). Before the first TAN treatment was added, the biological filter of the two treatment aquaria was disconnected in order to prevent any interference with the ammonia levels. The control aquaria was kept at 0 mg/L of TAN and the experimental one was set at a concentration of 1 mg/L of TAN. Fish were under this treatment for seven days. During the following two days (and starting with fish in the experimental aquaria), boldness tests were carried out following the same procedure as the first test. In the boldness aquaria, the TAN concentration was the same as that of the fish in the treatment aquaria. After this treatment, the experimental aquaria was brought to a TAN concentration of 8 mg/L and the tests were re-run in the same way. Finally, the experimental aquaria was brought to a concentration of 16 mg/L of TAN. The

tests were repeated for both exposed and non-exposed fish (the latter were maintained throughout the experiment at a TAN concentration of 0 mg/L). The experiment began on October 19 and, for exposed fish, ended on November 13 (it ended a day later for non-exposed fish). Therefore, the exposed fish were under the effects of ammonia for 24 days.

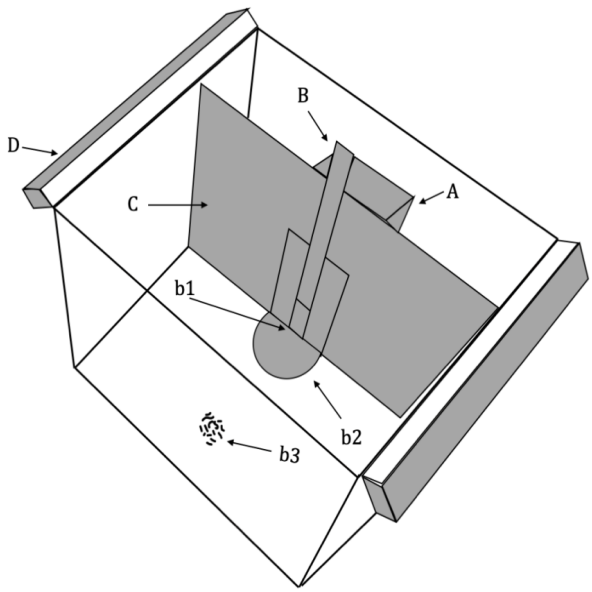

Fig. 2. Schematic representation of the experimental aquaria designed to perform the fish boldness tests. A: boldness box, dark space where each specimen was placed before starting the test (30 x 11 x 11 cm); B: exit trapdoor; b1: emergency line, it was the line that marks the boldness score; b2: methacrylate arc from which the hesitancy time was calculated; b3: food reward (red chironomid larvae); C: methacrylate wall; D: neon lamps.

During the week of exposure to each TAN treatment, the fish were fed "ad libitum" with red chironomid larvae twice a day, with the exception of the day before the tests when they were not fed. Every 24 h, a partial water change (one third of the volume) was carried out at the treatment aquaria in order to keep the physicochemical parameters of the water constant. These parameters were controlled daily in the aquaria (**Table 1**). A total of seven 200-L drums were prepared with the TAN concentrations corresponding to each moment in order to reproduce the conditions of treatments in the boldness

aquaria. The TAN concentrations were measured daily by the indophenol blue spectrophotometric method. Once the absorbance values were recorded for each sample, the $NH4^+$ concentration was calculated using the equation of the calibration curve and the proportion of NH3 was calculated following the procedures described in Thurston et al. (1979).

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The scientific procedure of this work was approved by the Animal Ethic Committee of the University of Barcelona (registration Nº 9296), which follows European Directive 2010/63/UE on the protection of animals used for scientific purposes. One of the co-authors holds a category C FELASA certificate that regulates the use of animals for experimental and other scientific purposes.

Statistical analyses

All study variables were transformed by Tukey's ladder of power to be adjusted to normality. A Lineal Model (LM) was used to analyse possible differences in fish length and body condition between aquaria treatments (control and experimental).

Differences in the boldness variables ("Boldness score", "Hesitancy" and "Feeding") between fish from the two aquaria treatments (control and experimental) were tested before starting the experiment (in week 1, when all fish were at 0 mg/L of TAN) through a General Model. Three Lineal Mixed Models (LMM) were conducted to identify any differences in boldness. The three variables of boldness were considered the response variable in the models. Two factors representing "aquaria treatments" (control and experimental) and "week" with three levels ("week 2", "week 3" and "week 4") together with their interactions, were considered the independent variables. The variable "Individual" was added to the models as a random factor. Differences between the three levels were further compared using Tukey's post-hoc tests.

All analyses were conducted with R 3.4.3 (R Core Team, 2017). LM and LMM, assuming a normal distribution, were performed using lm() (package

"stats") and lmer() (package "lme4": Bates et al., 2015). Non-significant interactions were removed from final models. Homogeneity and normality of residuals were visually checked for all models. All significant differences are $P \leq 0.05$.

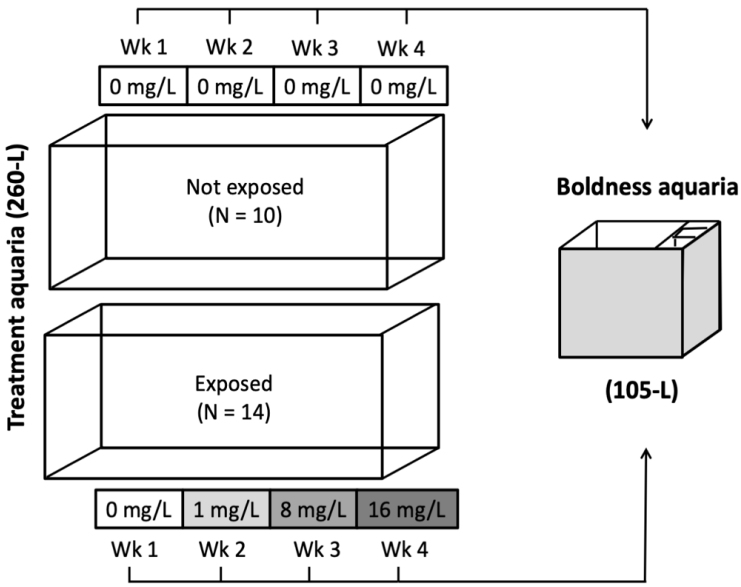

Fig. 3. Schematic representation of the experimental design. Non- exposed and exposed fish were housed in two 260-L aquaria where total ammonia treatments (TAN, from 1 mg/L to 16 mg/L) were performed. Fish boldness was evaluated before the experiment started (when the two aquaria were at 0 mg/L of TAN). Weekly (every eight days), the TAN concentration in the aquaria of the exposed fish was increased. Boldness tests to evaluate the fish memory retention were conducted at each concentration.

Table 1. Physicochemical parameters in the two treatment aquaria (control and experimental) throughout the experiment.

	[TAN mg/L]	Tª (ºC)	pH	NO_3^- (mg/L)	NO_2^- (mg/L)	NH_3 (mg/L)	Hardness
Control	[0]	21.77 ± 0.57	8.03 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.001 ± 0.001	14.05 ± 1.41
Experimental	[0]	21.99 ± 0.56	8.29 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	13.14 ± 0.38
	[1]	21.64 ± 0.86	8.23 ± 0.05	1.43 ± 0.97	0.13 ± 0.05	0.09 ± 0.03	13.25 ± 0.5
	[8]	22.14 ± 0.45	8.14 ± 0.11	2.00 ± 0.50	0.32 ± 0.27	0.55 ± 0.13	15.29 ± 2.56
	[16]	21.86 ± 0.38	8.19 ± 0.12	2.93 ± 1.04	0.43 ± 0.34	1.17 ± 0.21	14 29 ± 0.76

RESULTS

All fish (both exposed and non-exposed to TAN treatments) emerged from the boldness box (b1, "Boldness score") and crossed the methacrylate arch (b2, "Hesitancy") throughout the experiment. Some fish, however, did not reach the food reward (b3, "Feeding"). The number of times that the fish non exposed to ammonia (N= 3, 7.5% of cases) did not risk reaching the food reward in the maximum time established (≤ 3600 s) was not different from that found for the exposed fish (N = 5, a 8.9% of cases; $\chi^2 = 0.06$, 1 d.f., $P > 0.05$).

Fish from the control aquaria were not different in length from those from the experimental one (FL ± SD = 113 ± 15.3 mm; $F_{1,22} = 1.49$, $P > 0.05$). The fish body condition was not significantly different between the two groups either at the beginning (K ± SD = 1.27 ± 0.09; $F_{1,22} = 1.37$, $P > 0.05$) or at the end of the experiment (K ± SD = 1.18 ± 0.06; $F_{1,22} = 1.64$, $P > 0.05$).

Fish boldness

Before starting the ammonia treatments, that is, when all fish were at a 0 mg/l TAN concentration , no differences were found for the three boldness variables analysed in this study between fish from the control aquaria (which would not be exposed later in the experiment) and fish from the experimental aquaria (which would be exposed) (b1, "Boldness score": $F_{1,22} = 2.54$, $P > 0.05$; b2, "Hesitancy": $F_{1,22} = 2.17$, $P > 0.05$; b3, "Feeding": $F_{1,22} = 4.5$, $P > 0.05$; **Fig. 4**).

Memory retention

The "Boldness score" showed no significant effect of the interaction between "aquaria treatments" (non-exposed and exposed) and "week" ($F_{2,44} = 0.94$, $P > 0.05$). The LMM model showed no significant effect between "aquaria treatments", that is, between non-exposed and exposed fish ($F_{1,22} = 3.18$, $P > 0.05$). Fish from the control aquaria took a similar time to stick their snout out the exit door of the boldness box as compared to fish exposed to ammonia (**Fig. 4a**). Only a significant effect for "week" was found ($F_{2,46} = 10.61$, $P < 0.05$). "Boldness score" at week 4 (mean ± SD = 305.4 ± 178.7 secs), when 24 days

had elapsed since the fish had experienced their learning event (that is, the first boldness test) had increased as compared to that of weeks 3 (mean ± SD = 213 ± 177.7 secs; Tukey test: z = 3.14, *P* <0.05) and 2 (mean ± SD = 158.8 ± 138 secs; Tukey test: z = 4.49, *P* <0.05) when 16 and 8 days had elapsed, respectively, since they had experienced learning (**Fig. 4a**). This result indicates that, at week 4, both the non-exposed and the exposed fish began to be more cautious and it took longer for them to get out of the boldness box, becoming, therefore, more similar concerning behaviour to their first day of the boldness test.

Similar results were found for "Hesitancy". No significant effect of the interaction between "aquaria treatments" and "week" was found ($F_{2,44} = 1.57$, $P > 0.05$). The LMM model for this variable did not show a significant effect for "aquaria treatments" ($F_{1,22} = 3.02$, $P > 0.05$) but it did show a significant effect for "week" ($F_{2,46} = 11.36$, $P < 0.05$). As above, for "Hesitancy", at week 4 (mean ± SD = 389.5 ± 240.1 secs) the fish began to delay for longer the crossing of the methacrylate arc than at week 3 (mean ± SD = 271.3 ± 257.3 secs; Tukey test: z = 3.33, *P* <0.05) and week 2 (mean ± SD = 185 ± 142.4 secs; Tukey test: z = 4.62, *P* <0.05) (**Fig. 4b**). For "Feeding," no significant effect of the interaction between "aquaria treatments" and "week" was found ($F_{2,37.7} = 0.09$, $P > 0.05$). No significant effect was found neither for "aquaria treatments" ($F_{1,21.72} = 0.7$, $P > 0.05$) nor for "week" ($F_{2,39.72} = 3.17$, $P > 0.05$).

Although both non-exposed and exposed fish began to be more cautious at week 4, the exposed ones showed a tendency to lose learning earlier. Thus, analyzing the differences between weeks (including the week 1 of the learning day) within each aquaria treatment, it was found that at week 4 the exposed fish had a similar value of "Boldness score" (**Fig. 4a**) and "Hesitancy" (**Fig. 4b**) than at week 1 ("Boldness score": $F_{3,39} = 11$, $P < 0.05$; Tukey test: z = 1.41, $P > 0.05$; "Hesitancy" $F_{3,39} = 9.77$, $P < 0.05$; Tukey test: z = 0.65, $P > 0.05$). On the other hand, non-exposed fish at week 4 had a significantly lower "boldness score" than at week 1 ("Boldness score": $F_{3,27} = 7.2$, $P < 0.05$; Tukey test: z = 2.54, $P < 0.05$) although the same result was not found for "Hesitancy" (": $F_{3,27} = 5.58$, $P < 0.05$; Tukey test: z = 1.92, $P > 0.05$).

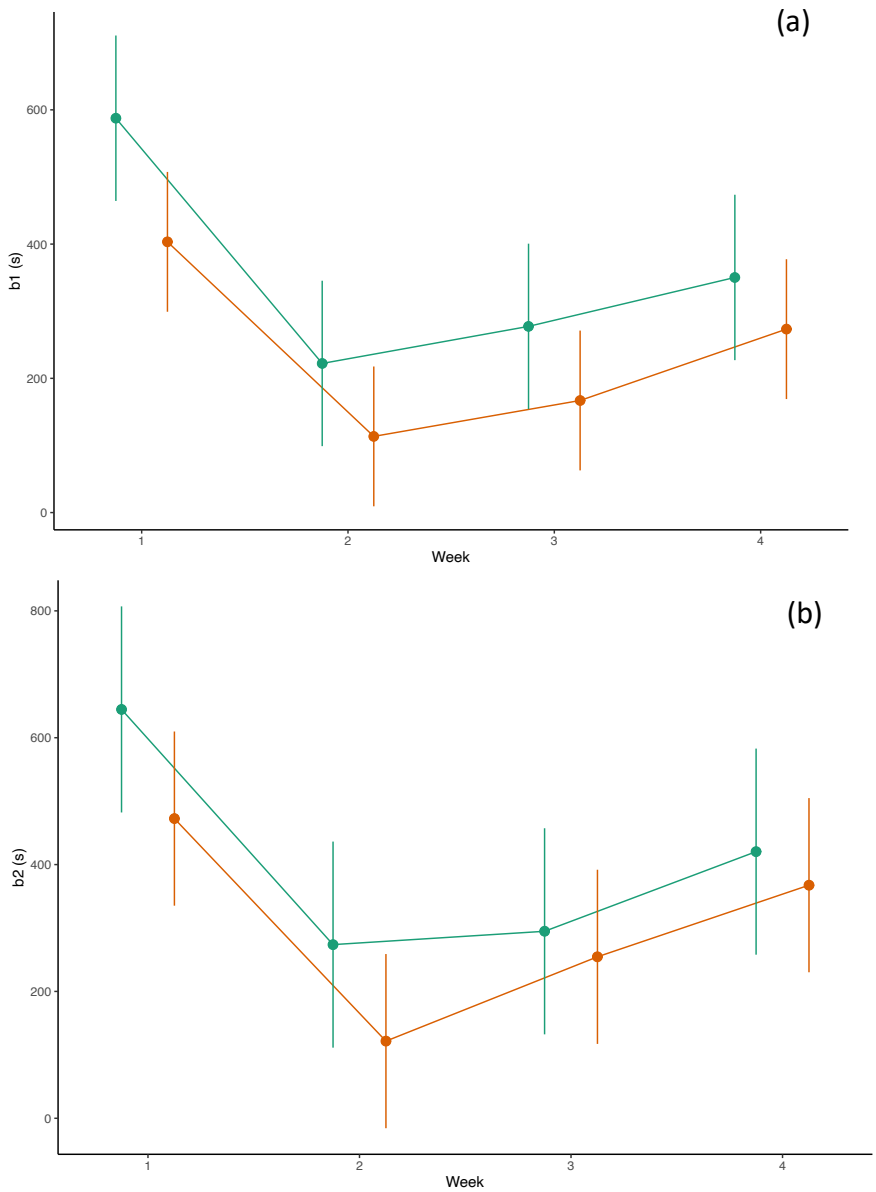

Fig. 4. "Boldness score" (a) and "Hesitancy" (b) for *B. meridionalis* at week 1, when all fish were at 0 mg/L of TAN, and in subsequent weeks, when control fish continued to be ammonia-free (green line) and experimental ones (orange line) were exposed to 1 (week 2), 8 (week 3) and 16 mg/L of TAN (week 4).

DISCUSSION

Many studies have shown that teleost fish store memories that can last a few seconds, days or several months (Madeira & Oliveira, 2017; Miller, 2017). For instance, the zebrafish (*Danio rerio*) remembers and discriminates objects briefly seen 24 h before (Oliveira et al., 2015). The damselfish (*Stegastes fuscus*) stores learned information for more than 15 days (Silveira et al., 2019). The lionfish (*Pterois volitans*) remembers the location of a food reward for up to 6 weeks (DeRoy et al., 2020). The cod (*Gadus morhua*) is able to retain memory for more than 3 months (Nilsson et al., 2008). The cleaner fish *Labroides dimidiatus* was able to remember being caught in a barrier net for up to 11 months (Triki & Bshary, 2019). Among fish, there may also be much interindividual variation in the ability to retain memory. For instance, the juveniles of the red sea bream (*Chrysophrys major*) have an individual variability on memory retention ranging from 3 to 60 days (Kaneko et al., 2018). Fish memory can be classified according to its duration and this may depend on the type of learning task (appetitive or aversive). The duration of memory for appetitive learning is usually longer than for aversive learning (Kaneko et al., 2018). Even the intervals between trials in the training protocols can influence memory retention with longer intervals leading to longer memory retention (Kaneko et al., 2018). The terminology of short-term memory refers to memory for less than 24 h, while long-term memories can last years. In the ecological context, long-lasting memories may confer clear advantages that affect the fitness of fish (Silveira et al., 2019).

In this study, a learning event based on a boldness test with food reward (an appetitive conditioning task) was carried out before exposing the fish to ammonia treatments (week 1). In subsequent tests performed at eight-day intervals (in weeks 2, 3 and 4 of the experiment), fish (non-exposed and exposed) had a significant decrease in "Boldness score" (time to exit the boldness box) and "Hesitancy" (time to cross the methacrylate arc), thus indicating that they possess learning and memory retention. In a similar experiment conducted by Daniel & Bhat (2020) in wild-caught zebrafish (*Danio rerio*), in which boldness tests were also used to test whether learning ability could be altered by the presence of a predator, a decrease in the emergence time of a refuge chamber and in the exploration time in an adjacent area were indicative that there had been learning and they had memory retention. The

results in this study showed that fish exposed to ammonia had a memory retention similar to non-exposed ones, despite total ammonia concentration (TAN) was increasing from 1 mg/L (at week 2) up to 16 mg/L (at week 4). Overall, the fish showed a similar "Boldness score" and "Hesitancy" throughout the experiment. At week 4 (24 days after the start of the experiment), both the non-exposed and the exposed fish began to be more cautious and took longer to exit the boldness box and to cross the methacrylate arc. This was indicating that the fish began to lose learning (between 16 and 24 days of the experiment). In *B. meridionalis* it can be considered that there was long-term memory even under the influence of ammonia. However, at week 4, only the exposed fish reached a "Boldness score" value similar to that of fish in the learning event (week 1), that is, when they were facing the novelty. It is difficult to know if the worst memory retention of these fish could be a consequence of being at the highest ammonia concentrations in the experiment (TAN concentration of 16 mg/L) or if it could be a consequence of having been exposed to ammonia for 24 days. Although the same result was not obtained for "Hesitancy", there was an evidence for exposed fish to have poorer memory retention than non-exposed ones. It is surprising, on the other hand, that the high concentrations of ammonia to which the fish were subjected in the experiment did not severely affect their cognitive capacity.

Memory is essential to enhance future survival and reproduction as it helps in storing and retrieving useful information to solve particular environmental problems (Triki & Bshary, 2019). This is essential in an environment, where resources (such as food, habitat and matings) and risks (such as predation) are changing. Memories are a vital part of the animal's life and may reduce its energy expenditure and improve survival. Owing to their relevance for animals' life, many species exhibit well-developed learning and memory abilities (Brown et al., 2006). Chronic pollution could lead to local adaptation or maladaptation, due to plastic and/or genetic changes caused by pollutants. Behavioural and cognitive responses are central in adaptive processes, because they are shaped by past evolution, and they can in turn facilitate or impede adaptive responses to pollution and other stressors (Sih et al., 2011). However, there is little information on how pollutants alter the cognitive abilities of fish and there is no research on the effect of pollutants as

prevalent in urban wastewater as ammonia or pharmaceuticals. On the effects of ammonia on learning, we are only aware of a study carried out in rats which concluded that hyperammonemia reduces cognitive abilities in this animal (Arias et al., 2014). It has been shown that the neural basis of learning and memory is well conserved across vertebrate species (O'Connell & Hofmann, 2011; Salas et al., 2006) and, similarly, there are also widespread examples of learning and memory abilities in fish that are similar to those of other vertebrate groups (Brown, 2015).

It has been suggested that *B. meridionalis* is more tolerant to contamination than other native cyprinids in the Iberian Peninsula, but their feeding behaviour and swimming activity may be altered by exposure to ammonia under experimental conditions (unpublished data). Poor water quality, water scarcity and competition triggered by the introduction of invasive species has been identified as the most significant threats for fish species in the Iberian Peninsula, including *B. meridionalis* (Maceda-Veiga et al., 2017a; 2017b). In this species, reproductive disorders induced by endocrine disrupting chemicals have also been reported in the wild by Blanco et al. (2019). Fish populations having evolved under chronic pollution have divergent responses, suggesting local adaptations to pollutants (Jacquin et al., 2020). Perhaps the time-periods of exposure to pollutants by fish in laboratory experiments are much shorter than those to which they are exposed in the field. Furthermore, in the field, mixtures of pollutants are often found, but little is known about the effect of these mixtures on organisms. Fish response under experimental conditions may be merely indicative of what is happening in nature, so that it would be necessary to contrast the information obtained in the laboratory with work carried out in the field. Additional stressors (such as time of exposure to pollutants and mixture of pollutants in the wild), as well as local adaptations of fish to pollution may aggravate or mitigate, respectively, the effects found in laboratory studies. In the same way, within the same species it is possible to find divergent information and changes over time concerning its tolerance to pollution.

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DISCUSIÓN GENERAL

En la presente tesis doctoral se ha buscado investigar en cómo la contaminación presente en las aguas de nuestros ríos está afectando, de una manera casi irremediable, tanto a la biología reproductora como al comportamiento y estrés fisiológico de los peces, comprometiendo por lo tanto la viabilidad de las poblaciones. A lo largo de los trabajos que conforman esta tesis se han ido estudiando los principales efectos de ciertos contaminantes sobre los peces. Este grupo de vertebrados es uno de los que corre un mayor riesgo de verse afectado por la contaminación, ya que su hábitat natural está considerado como punto de deposición final de la mayoría de los productos químicos de origen antropogénico (Kime, 1998). Desde un punto de vista global, los ecosistemas de agua dulce han sufrido un descenso de sus poblaciones de vertebrados del 83% entre 1970 y 2014 (WWF, 2018). Comprender los factores que pueden estar provocando este declive puede ser clave para la mejorar supervivencia de las especies.

A continuación, se presenta un pequeño resumen de los principales resultados de los dos capítulos de la tesis a modo de recordatorio antes de proceder a la discusión final. En la primera sección del Capítulo 1, se ha visto como la presencia de compuestos considerados como disruptores del sistema endocrino pueden ocasionar un mal funcionamiento en la reproducción de *S. laietanus*. Individuos de ambos sexos capturados agua abajo de la Estación Depuradora de Aguas Residuales (EDAR) de una zona industrial, presentaban un índice gonadosomático (IGS) más bajo y la respuesta de los biomarcadores indicaba la activación de procesos de detoxificación. A causa de la contaminación, las hembras presentaron un menor número de ovocitos vitelogénicos, por lo tanto, un valor de fecundidad más bajo, junto con una ausencia de pico reproductivo y una desincronización del periodo de puesta.

Los machos presentaron túbulos seminíferos de diámetro más estrecho, lo que también pudo conducir a un descenso de la fecundidad (Gimeno et al., 1998; Smith, 1978). En el segundo de los artículos presente en esta tesis (Sección 1.2.) se realizó un estudio de toxicidad en el campo en el que se compararon muestras extraídas de individuos de *S. laietanus* de dos puntos contaminados en el que se estaba aplicando un sistema de depuración terciaria mediante humedales artificiales. Los principales resultados extraídos de este estudio reflejaron los efectos de la mezcla de contaminantes presentes en el río sobre el estrés fisiológico en *S. laietanus*. Se analizó la presencia de anomalías en extensiones de sangre y se midieron los niveles de cortisol tanto en sangre como en el moco epidérmico (método que hasta ahora no se había utilizado en peces salvajes y en estudios de campo). Los resultados obtenidos confirmaron que los peces sufrieron un mayor nivel de estrés fisiológico por el hecho de vivir en hábitats de menor calidad ambiental. Los peces de las zonas contaminadas presentaron una mayor frecuencia de anomalías nucleares eritrocíticas, de eritrocitos inmaduros y senescentes, una mayor proporción de neutrófilos respecto a linfocitos, y unos niveles de cortisol más elevados (sangre y moco epidérmico). Entre estos resultados se resalta la medición del cortisol en moco epidérmico como un método no invasivo para evaluar los efectos de la calidad del hábitat sobre el estado de salud de los peces. En cuanto al Capítulo 2, se estudiaron los efectos de la contaminación por amonio sobre el comportamiento, la personalidad y la cognición de *B. meridionalis* en condiciones experimentales de laboratorio. El amonio es, uno de los contaminantes que más frecuentemente podemos encontrar en los ríos de todo el mundo. Los resultados de la Sección 2.1. reflejaron cómo los ejemplares de esta especie tenían comportamiento alimentario a partir de una concentración 1 mg/L de amonio total (la más baja de las que fueron testadas en el experimento). Sin embargo, los peces provenientes de una localidad contaminada (y que, por lo tanto, habían estado pre-expuestos a amonio en la naturaleza), presentaban un comportamiento alterado desde la concentración control (0 mg/L) y unos niveles de defensas antioxidantes más bajos. Es decir, que los peces pre-expuestos lejos de responder mejor a las concentraciones subletales de amonio en condiciones de laboratorio demostraron ser más sensibles a la contaminación por este compuesto y, posiblemente, venían con un estado de salud mermado desde su hábitat de origen. En la Sección 2.2. se analizó si este contaminante podía provocar alguna

alteración sobre la capacidad de retención de la memoria en *B. meriodionalis.* Los peces expuestos a amonio no tuvieron alterada la retención de memoria, pero perdieron más rápidamente el aprendizaje que los peces que no habían sido expuestos. Sin embargo, tanto los peces que habían sido expuestos como los que no, empezaron a mostrar una pérdida de memoria entre los 16 y los 24 días posteriores a la experiencia de aprendizaje, lo que es considerado una memoria de largo plazo. Los efectos del amonio sobre la capacidad de retención de memoria en condiciones experimentales podrían ser distintos a los que se pudieran encontrar en condiciones naturales. Además, en futuros estudios, se debería investigar cuál puede ser el efecto del amonio sobre la capacidad de aprendizaje de los peces, lo que también sería más realista respecto a lo que podría estar pasando en la naturaleza.

El principal problema de los denominados ríos urbanos es que los sistemas de depuración no se han desarrollado a la misma rapidez que el crecimiento de las ciudades, lo que tiene una repercusión directa en la calidad del agua (Xu et al., 2019). Este es un problema global, y que no solo afecta a los peces y otros organismos que viven en el agua, sino que tiene efectos sobre la población humana. Es más, una de las metas dentro de la Agenda para un Desarrollo Sostenible 2030 es la de asegurar un acceso equitativo y una gestión sostenible del agua, y proporcionar un saneamiento de ésta para todos (United Nations, 2015). También, según el comité de seguimiento para el desarrollo de los objetivos de la Agenda 2030, en 2019, en un estudio realizado sobre 79 países con ingresos altos y medio-altos, se calculó que ¼ parte de estos países trataban menos de la mitad de sus aguas residuales (United Nations, 2020). Cuando se habla de mezcla de contaminantes en los ríos lo que se quiere reflejar es que los peces en sus hábitats naturales, sobretodo en aquellos ríos sometidos a una fuerte presión antrópica (zonas altamente urbanizadas, zonas industriales y con zonas dedicadas a la agricultura-ganadería), se ven expuestos a una gran cantidad de contaminantes en diferentes concentraciones y durante largos periodos de tiempo (exposiciones crónicas). Disueltos en los ríos se pueden encontrar compuestos orgánicos, compuestos nitrogenados, fosfatos y una gran variedad de compuestos químicos, como productos farmacéuticos, pesticidas, sustancias tensoactivas (contenidas en los detergentes), metales pesados, productos retardantes de llama, etc. También hay que tener en cuenta

que estos compuestos pueden presentar interacciones entre ellos, sinérgicas o antagonistas, cosa puede cambiar la magnitud de los efectos sobre los organismos (subestimándolos o sobrestimándolos) (Ginebreda et al., 2014). Además, los peces son animales complejos y la contaminación a la que se ven expuestos en sus hábitats naturales les afecta a diferentes niveles, es decir, un mismo individuo puede verse afectado por un lado por los disruptores endocrinos que pueden afectar a su reproducción y crecimiento, por otro lado por el amonio que le provoca cambios en el comportamiento y posible pérdida de aprendizaje o de retención de memoria, y para finalizar, a toda una mezcla de contaminantes que aumenta sus niveles de estrés fisiológico con claras consecuencias para su salud y bienestar. Por este motivo, es esencial con vistas a la conservación de las especies tener en cuenta los efectos globales que pueden tener todos estos estresores ambientales sobre la totalidad del individuo y, que acabaran teniendo una repercusión en la población. Por ejemplo, la presencia de disruptores endocrinos en los ríos es una grave amenaza para las poblaciones de peces, ya que pueden tener efectos a bajas concentraciones y pueden disminuir el éxito de supervivencia de una población al completo. Una reducción en la fecundidad y/o en la fertilidad, y un mal funcionamiento de las gónadas de los peces puede llevar a una reducción del número de adultos con capacidad para reproducirse (Dietrich & Krieger, 2009). Estos efectos sobre la fecundidad/fertilidad pueden ser transgeneracionales y pueden alterar tanto el comportamiento de apareamiento como al mantenimiento de las crías, actuales y en las generaciones sucesivas (Dietrich & Krieger, 2009). Por lo tanto, un mal funcionamiento en la reproducción y una reducción de la fecundidad por acción de estos compuestos podría explicar, en parte, la dramática disminución de las poblaciones de peces nativos en muchos ecosistemas de agua dulce. Además, son compuestos cuyos efectos van a variar no solo entre especies, sino entre machos y hembras de una misma especie (Dietrich & Krieger, 2009). Pero los disruptores endocrinos no solo afectan a la reproducción y al desarrollo de los animales. Por ejemplo, se ha visto que compuestos como el benzotiazol tiene efectos sobre la estructura de las branquias y sobre la natación de los peces (Evans et al. 2000). En cuanto a la respuesta de los peces a la exposición a diferentes estresores, se manifiesta como un efecto cascada, induciendo cambios fisiológicos internos que provocarán cambios comportamentales (Schreck, 2000). Si se ven expuestos a

un estrés severo, esto puede significar un aumento de la mortalidad de los individuos, pero con un estrés subletal se verán comprometidas tanto funciones fisiológicas como comportamentales (Iwama et al., 1997). Los peces expuestos a un estrés crónico, ya sea por contaminación, agentes infecciosos o depredadores pueden presentar neutrofilia (elevada concentración de neutrófilos), linfopenia (disminución de los linfocitos circulantes) y en algunos casos, monocitosis (elevada concentración de monocitos) (Davis et al, 2008). Este estrés también tiene sus efectos en la reproducción, al interferir con el eje cerebro-pituitario-gonadal (BPG; von Krogh et al., 2019). Este eje BPG, es el encargado de regular la maduración sexual y la reproducción, por lo tanto, una interferencia en este eje traerá como consecuencia una interferencia en la reproducción (von Krogh et al., 2019). Niveles altos de cortisol pueden afectar a los niveles de gonadotropinas, al desarrollo gonadal, a la calidad de los gametos sexuales, a la producción de vitelogenina, a la producción de esteroides sexuales y al comportamiento sexual (Goos & Consten, 2002; Leatherland et al., 2010; Milla et al., 2009; Schreck, 2010; Schreck et al., 2001). El estrés fisiológico también puede implicar cambios comportamentales y cognitivos, afectando actividades esenciales para la supervivencia como la búsqueda de alimento, la evitación de los depredadores, la orientación y migración, el aprendizaje y la selección de hábitats donde establecerse (Iwama et al., 1997). Es más, analizar las alteraciones provocadas por el estrés es uno de los indicadores mas sensibles donde medir cambios fisiológicos y bioquímicos que tienen lugar en el interior del organismo como respuesta a ese estrés (Olla et al., 1994; 1995). Estos cambios en el comportamiento, en algunos casos, pueden ser adaptativos e incrementar las opciones de supervivencia de los individuos al permitirles evitar la exposición a esta perturbación (Iwama et al., 1997). El estrés afecta directamente al sistema nervioso central de los peces (Iwama et al., 1997) y, por lo tanto, unos niveles de cortisol elevados ocasionados por la exposición crónica a diferentes estresores puede provocar también pérdida de memoria y disminuir las capacidades de aprendizaje de los peces, además de provocar muerte neuronal (Iwama et al., 1997). En cuanto a los compuestos nitrogenados (o contaminación por nutrientes, nitrógeno y fósforo) se trata de un problema persistente desde hace años en los ríos de todo el mundo. Es más, en las últimas décadas se ha producido un incremento en la entrada de nutrientes a los ambientes acuáticos, debido a un aumento en la demanda de alimento y energía

con el crecimiento de la población humana (Díaz-Álvarez et al., 2018). En particular, el uso del nitrógeno se ha extendido tanto que la contaminación por este compuesto se ha convertido en una de las mayores amenazas para la biodiversidad en todo el mundo (Díaz-Álvarez et al., 2018; Payne et al., 2017) causando impactos severos sobre los ecosistemas acuáticos y su biota (Camargo & Alonso, 2006; Galloway et al., 2008). Pese a esto, no se tiene el mismo nivel de conocimiento de los efectos que este tipo de contaminación puede tener sobre los peces. Existen bastantes estudios que analizan la toxicidad de compuestos como el amonio y los nitritos, pero no hay tantos sobre los efectos de los nitratos (Cano-Rocabayera et al., 2019). Durante mucho tiempo se consideró a los nitratos como no tóxicos para la fauna acuática, pero hay varios estudios que demuestran que puede causar daños fisiológicos y alteraciones comportamentales en los organismos acuáticos (Alonso & Camargo, 2013; Cano-Rocabayera et al., 2019; Guillette & Edwards, 2005). Los efectos sobre los peces van desde una reducción de la actividad, del crecimiento y de la supervivencia, a una disrupción de los mecanismos de transporte de oxígeno, una alteración de las concentraciones de hemoglobina, y necrosis de los tejidos de los órganos vitales (Cano-Rocabayera et al., 2019; Grabda et al., 1974; McGurk et al., 2006; Monsees et al., 2017). Estos efectos no solo se producen en peces, por ejemplo, exposiciones crónicas a este compuesto pueden reducir y provocar malformaciones en el crecimiento, reducir la velocidad de movimiento y alterar la reproducción en moluscos, anfibios e invertebrados (Alonso & Camargo, 2013; Guillette & Edwards, 2005; Hamer et al., 2004; Krishnamurthy et al., 2008; Soucek & Dickinson, 2016). El vertido de nutrientes a los ecosistemas acuáticos es una gran amenaza, no solo porque producen eutrofización (Dodds & Smith, 2016), sino porque hay compuestos que son directamente tóxicos para la fauna acuática (Isaza et al., 2020). Esta contaminación puede acabar conduciendo a cambios profundos en la biodiversidad acuática y en los procesos biogeoquímicos (Woodward et al., 2012). Por todo esto, es vital conocer tanto la cantidad de contaminantes a los que se ven expuestos los peces como la duración, la severidad y frecuencia de la exposición, a fin de pronosticar sus posibles efectos sobre la salud y el bienestar.

Otra de las amenazas a las que tienen que hacer frente los organismos, no solo los peces sino todos los que viven en ambientes acuáticos, es a la

presencia de nuevos contaminantes como los microplásticos. Como ya se sabe, la contaminación por plásticos está siendo una de las grandes amenazas para la fauna a nivel mundial. Se trata de material con una alta persistencia, baja densidad y con una amplia distribución de tamaños. El término microplástico fue acuñado en el año 2004 y se refiere a todas aquellas partículas de plástico más pequeñas de 5 mm de diámetro (GESAMP, 2015). Se pueden clasificar en dos categorías: primarios y secundarios (Cole et al., 2011). Los microplásticos primarios son aquellos que han sido diseñados para un uso comercial, como los que se pueden encontrar en los productos cosméticos, las microfibras que se desprenden de ropas y otros productos textiles, así como las redes de pesca (Cole et al., 2011). Por otro lado, los secundarios son aquellos derivados de la rotura de plásticos más grandes por exposición a diferentes factores medio ambientales, como es el caso de las botellas de agua (Cole et al., 2011). El principal problema de la contaminación por microplásticos es que estos no se degradan, no se convierten con el tiempo en moléculas inofensivas para el medio ambiente. Es más, son contaminantes que pueden ser ingeridos por los organismos y que acaban formando parte de la cadena alimentaria (Silva-Cavalcanti, et al., 2017). Se han detectado microplásticos tanto en el interior de plancton como en el interior de ballenas. Según un informe de la Organización de las Naciones Unidas (FAO) realizado en 2017, se detectó presencia de microplásticos en el interior de 800 especies de peces, crustáceos y moluscos (Lusher et al., 2017). Pese a la grave amenaza de esta contaminación sobre los organismos y los ecosistemas, los esfuerzos en investigar sus efectos se han centrado sobre los océanos, quedando los ecosistemas de agua dulce en un segundo plano (Blettler et al., 2018). Muchos de los ríos urbanos del mundo se han convertido como puntos de deposición final de la contaminación por plásticos (McCormick et al., 2014; 2016). Poco se sabe, por lo tanto, de los efectos que la ingesta de estos microplásticos puede tener sobre los peces de río. Dentro de los efectos descritos se encontrarían daños físicos, como obstrucción del sistema digestivo, inflamaciones y laceraciones gastrointestinales, que pueden llevar a evitar una correcta absorción de nutrientes (Lusher et al., 2013; Pedà et al., 2016). También tienen efectos fisiológicos al interferir directamente con el sistema inmune de los peces (Greven et al., 2016), y producen cambios comportamentales reduciendo la habilidad para detectar a los depredadores (Lönnstedt & Eklöv, 2016). A estos efectos hay que sumar la capacidad de los

microplásticos para actuar junto con otros contaminantes químicos (facilitando la entrada en los organismos) (Araújo et al., 2002; Crompton, 2007; Lithner et al., 2011) y orgánicos (ocasionando una bioacumulación) (Browne et al., 2013; Holmes et al., 2012; Ziccardi et al., 2016), por lo tanto, se convierte en otro elemento a añadir a esa mezcla de contaminantes que se pueden encontrar en los ríos y en la que los peces deben vivir.

Tampoco se puede perder de vista que el planeta se encuentra en una situación de cambio global que puede empeorar todos estos efectos descritos, al modificar tanto la temperatura como el régimen de precipitaciones en todo el mundo (O'Briain 2019). Se espera que el cambio climático ocasione veranos más cálidos y secos, e inviernos mas húmedos y suaves con períodos de inundación (O'Briain, 2019). Estos cambios en temperatura y precipitación pueden llevar a modificaciones de los cursos de los ríos, de las características de las llanuras aluviales y, por lo tanto, de las comunidades biológicas (O'Briain, 2019). Por ejemplo, el aumento de las temperaturas, y las sequías en verano y las inundaciones en invierno, pueden afectar seriamente a la tasa de colonización y al crecimiento de diferentes especies de plantas con funciones clave para el mantenimiento de las cuencas aluviales (O'Briain, 2019). En las regiones mediterráneas, donde hay una gran cantidad de ríos intermitentes o efímeros, estos efectos serán más acusados (Bonada et al., 2020; Borg Galea et al., 2019). Algunos de estos ríos, como los intermitentes, pueden secar-se parcialmente durante el periodo estival, quedando solo unas cuantas pozas con gran valor ecológico donde se refugian los organismos acuáticos a la espera de la llegada de las lluvias (Bonada et al., 2020). En un futuro cercano las especies de peces que habitan estas zonas pueden verse amenazadas por esa aceleración de la sequía y por la disminución de la conectividad ecológica, al desaparecer por completo algunos de esos ríos intermitentes o efímeros (Bonada et al., 2020; Yang et al., 2018). A parte de los efectos hidromorfológicos, el cambio climático tendrá efectos sobre la calidad del agua, por ejemplo, los cambios en el ciclo hidrológico pueden tener un efecto directo sobre el transporte de los contaminantes en el ecosistema y, cambios sobre las propiedades físicas y químicas de las masas de agua podrían cambiar los procesos de transformación de los contaminantes (Yang et al., 2018). El cambio del clima, sobretodo el aumento de temperaturas en verano con la consecuente disminución de caudal,

aumentará las concentraciones de contaminantes en el agua al disminuir su factor de dilución (Molina-Navarro et al., 2014). En los últimos años ha aumentado notablemente el número de estudios que evalúan los impactos potenciales del cambio climático en la calidad del agua, especialmente en las cargas y concentraciones de contaminantes en el agua.

Cuando la presencia de todos estos estresores ambientales en los hábitats naturales es persistente, se podría decir que a los peces solo les quedan dos opciones, adaptarse o morir. La capacidad de adaptación de los individuos de manera natural, tanto a nivel celular como de su rendimiento general, permite a las especies adaptarse a los cambios ambientales (Cabej, 2012; Crawford & Davies, 1994). Pero las actividades antrópicas, sobretodo la contaminación de las aguas, se han convertido en detonantes de estos cambios evolutivos (Palumbi, 2001). Estos procesos de adaptación evolutiva se producen de manera rápida y permiten tolerar los ambientes contaminados. Estas adaptaciones incluyen cambios fisiológicos, como un aumento de la detoxificación (Maroni et al., 1987), un incremento de la excreción (Hall et al., 1979, Posthuma et al., 1992) y un incremento de la acumulación (Xie & Klerks, 2004a). Un ejemplo sería la resistencia a la contaminación por cadmio de *Heterandria Formosa*, mediante un cambio en sus mecanismos de acumulación de este contaminante (Xie & Klerks, 2004a). La evolución de la resistencia a los metales pesados que se pueden encontrar disueltos en las aguas está considerado un claro ejemplo de selección natural rápida. Otros ejemplos serian los de *Fundulus heteroclitus* (fúndulo) adaptado a los hidrocarburos aromáticos policíclicos (PAH, por sus siglas en inglés) (Di Giulio & Clark, 2015; Williams & Oleksiak, 2011a; 2011b), *Microgadus tomcod* (tomcod atlántico) adaptado a policlorobifenilos (PCB) y a sustancias similares a las dioxinas (Wirgin et al., 2011; Wirgin & Waldman, 2004), *Perca flavescens* (perca amarilla) adaptada a la exposición por cadmio (Bélanger-Deschênes et al., 2013) y, *Anguilla anguilla* (anguila europea) y *Anguilla rostrata* (anguila americana) adaptadas a la exposición por metales pesados y contaminantes orgánicos (Laporte et al., 2016). En la mayoría de los estudios realizados en este campo solo se han analizado los efectos de un único contaminante, por lo tanto, no se sabe cómo la mezcla de contaminantes puede llegar a afectar a esta adaptación. Tampoco se sabe a ciencia cierta cómo funcionan estos mecanismos de adaptación y las consecuencias que podría

tener este proceso sobre la aptitud o eficacia biológica de las poblaciones. A nivel individual, cada pez, tendrá unos costes metabólicos asociados a la adaptación a ambientes contaminados. Algunos ejemplos serian los procesos para metabolizar o excretar los contaminantes, la producción de moco epidérmico, la reparación de tejidos y moléculas, y el reemplazo de células dañadas (Hamilton et al., 2017). En otro estudio realizado con *H. formosa* adaptada a la contaminación por cadmio, se observó una reducción de la fecundidad de los individuos expuestos (Xie & Klerks, 2004b). Esto se puede deber a que, con el fin de aumentar la tolerancia a la presencia del contaminante en el agua, se redirigió energía destinada a la reproducción (Xie & Klerks, 2004b). Otro ejemplo de estas adaptaciones en ambientes con elevada contaminación química, sería favorecer los mecanismos que permiten completar el ciclo de vida más rápido de lo normal, es decir, un crecimiento más rápido y una reproducción a edades más tempranas (Hamilton et al., 2017). De esta manera los peces podrían intentar adaptarse a través de una reproducción temprana en lugar de a través de mecanismos de tolerancia, que son metabólicamente más costosos y que podrían desviar los recursos de otros procesos vitales (crecimiento y reproducción) (Sibly & Calow, 1989). La eficacia de estas adaptaciones también va a depender del tiempo al que los organismos se vean expuestos a la contaminación (Hamilton et al., 2017). Estos mecanismos adquieren gran importancia al permitir a los peces adaptarse de manera rápida y, si se tratara de una contaminación puntual o de solo una parte del hábitat, los costes serian menores, ya que no serian necesarios los mencionados mecanismos cuando hubiera desaparecido la contaminación (Hamilton et al., 2017). Por el contrario, si se habla de hábitats con un tipo de contaminación permanente, estos mecanismos de tolerancia no se desactivarían nunca, dando como resultado la evolución de unas adaptaciones que serían terriblemente costosas en aguas no contaminadas (Hamilton et al., 2017). Llegar a comprender los mecanismos que presentan los peces para adaptarse a la contaminación química adquiere una gran importancia, al ofrecer información de cómo estos cambios en la dinámica evolutiva pueden afectar a la hora de tomar decisiones de gestión y conservación.

Esta situación, en la que se encuentran la mayoría de los ríos del mundo, podría explicar en parte el descenso global de las poblaciones de peces. No se

puede perder de vista que la gran diversidad de peces de agua dulce se encuentra concentrada en áreas que ocupan solo el 1% de la superficie del planeta. En el mundo se han descrito aproximadamente 15,750 especies de peces de agua dulce, lo que corresponde a un 48% de la diversidad de las especies de peces y un 25% de la diversidad dentro de los vertebrados (Darwall & Freyhof, 2015). Los peces continentales se están enfrentando una crisis sin precedentes, con alrededor del 40% de sus especies amenazadas en todo el mundo (Aparicio et al., 2000). Como se ha descrito, este declive no se puede atribuir a una única causa, sino que más bien responde a una suma de factores que actúan de manera sinérgica y que ha ido empeorando a medida que se incrementaba la población humana y, por lo tanto, la presión de esta sobre los ecosistemas de agua dulce. A parte de la contaminación, las poblaciones de peces se ven amenazadas por la fragmentación y destrucción del hábitat, la sobrepesca y la introducción de especies exóticas (Reid et al., 2013). La fragmentación del hábitat sería una de las más importantes después de la degradación de la calidad del agua, ya que impide el desplazamiento hacia hábitats más favorables y deja a las poblaciones cada vez más aisladas, provocando en algunos casos extinciones de poblaciones a nivel local (Aparicio et al., 2000). En el caso de las aguas continentales de Cataluña, actualmente se pueden encontrar 29 especies de peces autóctonas (Aparicio et al., 2016), de las cuales 14 se encuentran gravemente amenazadas (alrededor del 50%), clasificadas como extintas o en peligro de extinción (IUCN, 2012). Solamente dos de estas *Aphanius iberus* (fartet) y *Valencia hispanica* (samaruc), tienen planes de recuperación establecidos. Por este motivo, es necesario no sólo aumentar el conocimiento del estado de las especies de peces de agua dulce sino implementar planes de recuperación de las poblaciones, incluyendo alternativas para mejorar la calidad de las aguas. Cuando se habla de planes de recuperación de los ecosistemas acuáticos continentales hay que tener en cuenta su complejidad. En estos ecosistemas se pueden diferenciar cuatro dimensiones: longitudinal (de cabecera a desembocadura), lateral (interacciones entre el curso de agua y los márgenes de bosques de ribera o llanuras aluviales), vertical (conexiones entre las aguas superficiales y las subterráneas) y las dinámicas temporales (Ward & Stanford, 1989). Es por este motivo que no sólo es necesaria una correcta depuración del agua, sino que las actuaciones deben alcanzar también los sistemas terrestres. Se debe garantizar la recuperación de la vegetación de

ribera (bosques de ribera), la conectividad a lo largo de todo el curso del río, ofrecer refugios para la fauna que depende de estos ecosistemas (tanto acuática como terrestre), implementar políticas que garanticen una correcta gestión de los usos del suelo en las zonas cercanas a los ríos, evitar el derroche de agua y por supuesto, garantizar una correcta depuración de las aguas vertidas a los ríos. Dentro de los tratamientos alternativos para mejorar la calidad de las aguas, y cuya eficacia se analiza en la Sección 1.2., se encuentran los humedales artificiales. La presencia de estos humedales artificiales, al menos en ríos a partir de determinado caudal, no es suficiente para mitigar los efectos de la contaminación sobre la salud de los peces. Estos tipos de sistemas se llevan utilizando durante varias décadas tratando realizar una depuración terciaria de las aguas provenientes de las EDAR. Estos humedales artificiales, utilizados juntamente con una restauración de la vegetación de ribera para crear lo que se conoce como *riparian buffer zones* (franjas de protección de ribera), pueden ser útiles a la hora de retener nutrientes y otros contaminantes (como compuestos emergentes), para la depuración del agua, la creación de nuevos hábitats y el mantenimiento de la biodiversidad (Brown & Batzer, 2011). Pero son sistemas que, sin una correcta gestión, o si se sobrepasan determinadas concentraciones de nutrientes, pueden no ser funcionales y en lugar de retener estos nutrientes liberarlos en el río (Verhoeven et al., 2006). Estos humedales artificiales serían funcionales en ríos y arroyos con caudales bajos, pero no funcionan correctamente en ríos con caudales más grandes ya que, por un lado, físicamente no puede pasar toda el agua del río a través del humedal y, por otro, se pueden saturan más fácilmente al llevar estos ríos una mayor carga de nutrientes. Otra manera de abordar la mejora de la calidad del agua es la implementación de nuevos métodos de depuración de aguas residuales, incluso intentando ir más allá de los ya conocidos tratamientos terciarios. Hay que remarcar que, pese a los esfuerzos que hacen los países en implementar la Directiva Marco del Agua (2000/60/RC), aún hay muchas EDARs que no cuentan con ningún sistema de tratamiento terciario. Dentro de estos tratamientos los que tienen un uso más extendido son las lagunas para deposición y están pensados para la eliminación de productos orgánicos, metales pesados, nitrógeno, fósforo, patógenos y reducir la turbidez, pero no está clara su eficacia para eliminar los compuestos emergentes (Gerba & Pepper, 2019). Pese a esto actualmente hay una gran cantidad de estudios que tratan de abordar, de la forma más eficaz

posible, cómo eliminar la mayor cantidad de contaminantes del agua. Algunas de las técnicas que se pueden implementar en estos tratamientos terciarios para la eliminación de los compuestos emergentes serían los tratamientos con ozono, la radiación ultravioleta (UV), la cloración, la ósmosis inversa, el carbón activado y las técnicas de membrana de baja presión (Dhodapkar & Gandhi, 2019). Como reflexión final decir que, junto con todas las medidas de gestión, sería necesario revisar los Estándares de Calidad Ambiental establecidos por la legislación, ya que sólo tienen en cuenta los efectos aislados de cada contaminante, pero cada vez hay mas evidencias de que estos contaminantes pueden producir una toxicidad conjunta, incluso a concentraciones que por si solos no presentarían riesgos. Es decir, es necesario revisar la legislación incluyendo los posibles riesgos de la mezcla de contaminantes en el agua.

CONCLUSIONES

1. En el río Ripoll (Besòs), la biología reproductiva de *Squalius laietanus* se vi*ó* afectada por la contaminación originada por una industria textil. Las alteraciones observadas pudieron atribuirse, principalmente, a la presencia de benzotriazol y benzotiazol en el agua (dos disruptores endocrinos). Ambos sexos tuvieron una reducción en el índice gonadosomático y, en las hembras, además de una disminución de un 63% de la fecundidad, se observó una desincronización de la puesta durante el periodo de reproducción.

2. El análisis de la histología gonadal puso de manifiesto, además, que hembras (sin ningún pico de maduración a lo largo del ciclo reproductivo) y machos (con túbulos seminíferos más estrechos) de *S. laietanus* también tuvieron afectaciones a nivel tisular.

3. En el curso bajo del río Besòs, principalmente bajo los efectos de contaminación residual urbana, unos niveles más elevados de cortisol en *S. laietanus* estuvieron indicando un peor grado bienestar respecto a individuos procedentes de un lugar de referencia. Se validaron los niveles de cortisol obtenidos en moco epidérmico y, además, éstos se pudieron relacionar con un aumento tanto en el número de anomalías en los eritrocitos como en la proporción de neutrófilos/linfocitos. El análisis de cortisol en moco epidérmico puede ser utilizado como una herramienta eficaz y no invasiva en la determinación del grado de bienestar de esta especie bajo el efecto de diferentes estresores ambientales. Por tanto, podría ser usado como biomarcador de la calidad del hábitat.

4. En condiciones experimentales de laboratorio, el comportamiento alimentario (voracidad y saciedad) y natatorio de *Barbus meridionalis* se vió alterado por la exposición a diferentes concentraciones subletales de amonio. No obstante, los peces que procedieron de una localidad contaminada (río Congost) y que habían estado pre-expuestos a amonio en la naturaleza, mostraron un comportamiento alterado desde la concentración control (0 mg/L) y, además, tuvieron unas defensas antioxidantes más bajas mostrándose, por lo tanto, más vulnerables a este contaminante que los peces procedentes de un río pristino (riera de Castelló). No se detectaron los efectos de este compuesto sobre el comportamiento de natación y la latencia de los peces, pero sí sobre el comportamiento alimentario (la voracidad y la saciedad) desde la concentración más baja testada en el experimento (1 mg/L).

5. En condiciones experimentales de laboratorio, la puntuación de osadía en individuos de *B. meridionalis* que habían sido expuestos a amonio fue similar a la que tuvieron en el día del aprendizaje, indicando esto que tuvieron una peor retención de memoria que los individuos no expuestos. Deberían estudiarse otras facetas de la cognición (como la capacidad de aprendizaje) para demostrar hasta qué punto el amonio podría ser capaz de alterar comportamientos complejos en los peces.

6. Los individuos de *B. meridionalis* (tanto expuestos como no expuestos) fueron capaces de retener el aprendizaje por al menos 16 días, por lo que puede considerarse que esta especie presenta memoria a largo plazo.

7. Urge implementar nuevas técnicas para mejorar la calidad del agua en los ecosistemas fluviales y establecer unos estándares de calidad ambiental basados tanto en aspectos biológicos, como ecológicos y comportamentales de la fauna acuática.

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