ESTUDIO Y MODULACIÓN DEL METABOLISMO DEL GLUTATIÓN EN LA TOLERANCIA AL ESTRÉS OXIDATIVO GENERADO POR PLAGUICIDAS EN ORGANISMOS ACUÁTICOS DE INTERÉS COMERCIAL

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CERTIFICAN: Que la Tesis Doctoral titulada "Estudio y modulación del metabolismo del glutatión en la tolerancia al estrés oxidativo generado por plaguicidas en organismos acuáticos de interés comercial" presentada por el Licenciado en Bioquímica D. Samuel Peña Llopis para optar al grado de Doctor, se ha desarrollado bajo la dirección conjunta de los abajo firmantes en el Instituto de Acuicultura de Torre de la Sal.

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A mis padres, mi

hermano y Silvia

ÍNDICE DE CONTENIDO

LISTA DE PUBLICACIONES

La presente tesis doctoral está basada en los siguientes artículos, referidos en el texto por sus números romanos e incluidos al final de la misma:

I. Peña, S., J.B. Peña, C. Ríos, E. Sancho, C. Fernández y M.D. Ferrando. (2000). Role of glutathione in thiobencarb resistance in the European eel *Anguilla anguilla*. *Ecotoxicol. Environ. Safety* **46**(1): 51-56.

II. Peña-Llopis, S., J.B Peña, E. Sancho, C. Fernández-Vega y M.D. Ferrando. (2001). Glutathione-dependent resistance of the European eel *Anguilla anguilla* to the herbicide molinate. *Chemosphere* **45**(4-5): 671-681.

III. Peña-Llopis, S., M.D. Ferrando y J.B. Peña. (2002). Impaired glutathione redox status is associated with decreased survival in two organophosphate-poisoned marine bivalves. *Chemosphere* **47**(5): 485-497.

IV. Peña-Llopis, S., M.D. Ferrando y J.B. Peña. Fish tolerance to organophosphate-induced oxidative stress is dependent on the glutathione metabolism and enhanced by *N*-acetylcysteine. *Aquat. Toxicol.* (en consideración).

V. Peña-Llopis, S., M.D. Ferrando y J.B. Peña. Increased recovery of brain acetylcholinesterase activity in dichlorvos-intoxicated European eels (*Anguilla anguilla*) by bath treatment with *N*-acetylcysteine. *Dis. Aquat. Org.* (en consideración).

Los artículos están reproducidos con el permiso de sus respectivas editoriales: Academic Press y Elsevier Science.

El primer autor contribuyó mayoritariamente al diseño experimental, la realización de los análisis bioquímicos y estadísticos, la interpretación de los resultados y la redacción de los manuscritos.

ABREVIATURAS NO COMUNES

AChE	acetilcolinesterasa
CL_{50}	concentración letal al 50%
GCL	glutamato:cisteína ligasa
GPx	glutatión peroxidasa
GR	glutatión reductasa
GSH	glutatión reducido[1]
GSSG	glutatión oxidado o disulfuro
GST	glutatión *S*-transferasa
γGT	γ-glutamil transferasa
NAC	*N*-acetil-L-cisteína
RNS	especies reactivas de nitrógeno
ROS	especies reactivas de oxígeno

[1]En el texto de esta memoria y en los artículos adjuntos se utiliza el término 'glutatión' cuando incluye tanto a la forma reducida (GSH) como oxidada (GSSG) o no se quiere especificar su estado redox.

RESUMEN

La utilización de plaguicidas para el cultivo del arroz en campos cercanos a humedales de gran valor ecológico como los Parques Naturales de la Albufera de Valencia y el Delta del Ebro (Tarragona) suponen un riesgo no sólo para los ecosistemas de estos Parques sino también para la pesca y acuicultura que se desarrolla en la zona. La anguila europea (*Anguilla anguilla*) reside en ambos hábitats y está expuesta a cantidades considerables de herbicidas tiocarbamatos como el molinato y tiobencarb. Estos plaguicidas disminuyen y oxidan los niveles del antioxidante intracelular más importante, el glutatión (GSH). Así, se ha descubierto que el principal mecanismo de toxicidad de los herbicidas tiocarbamatos es la generación de radicales libres, produciendo un estado de estrés oxidativo, que sería responsable de la anemia hemorrágica observada en las anguilas. Además, la tolerancia de las anguilas a los plaguicidas estaría mediada principalmente por la capacidad de mantener e incrementar el estado redox del glutatión. Una excesiva oxidación de éste produciría estrés oxidativo que conduciría, según la intensidad, a una muerte celular programada (apoptosis) o no (necrosis), resultando en una disfunción de los tejidos y finalmente la muerte del organismo. Un patrón similar también se encontró en una población natural de la semilla del pectínido *Flexopecten flexuosus*, al igual que el mejillón (*Mytilus galloprovincialis*) cultivado en el Delta del Ebro, que está expuesto a una variación estacional de plaguicidas, siendo el insecticida organofosforado fenitrotión el más abundante. Por otra parte, el antioxidante y precursor del glutatión, *N*-acetilcisteína (NAC) aumentó la supervivencia de las anguilas expuestas al plaguicida organofosforado diclorvós (que ha sido utilizado extensivamente como antiparasitario de peces) al mejorar el metabolismo del glutatión y disminuir la pérdida y oxidación de éste, además de la inhibición enzimática causada por dicho plaguicida. Mientras que la disminución de glutatión se considera un marcador bioquímico de exposición a la contaminación, el estado redox del glutatión se puede utilizar como biomarcador de efecto y susceptibilidad individual a ciertos plaguicidas u otros contaminantes que inducen estrés oxidativo en vertebrados e invertebrados acuáticos. Asimismo, el tratamiento de anguilas intoxicadas con diclorvós mediante baños de NAC aminoró la disminución y oxidación del glutatión muscular y las actividades acetilcolinesterasa y glutatión reductasa del cerebro, que son biomarcadores de neurotoxicidad y estrés oxidativo, respectivamente. Consecuentemente, la NAC mejora la recuperación de las anguilas envenenadas con plaguicidas, representando un antídoto barato y de fácil administración.

Palabras clave: Plaguicidas organofosforados y tiocarbamatos, metabolismo del glutatión, estrés oxidativo, *N*-acetilcisteína, glutatión reductasa, biomarcadores, pez teleósteo, moluscos bivalvos.

1. INTRODUCCIÓN GENERAL

1.1. Agricultura, acuicultura y desarrollo sostenible

1.1.1. Sostenibilidad

Tras la Cumbre Mundial sobre el Desarrollo Sostenible, celebrada entre agosto y septiembre de 2002 en Johannesburgo, todo son buenas intenciones para tratar de compatibilizar el crecimiento económico con la protección medioambiental y así lograr que la satisfacción de las necesidades de las generaciones actuales no merme la capacidad de cubrir las futuras.

La presente tesis doctoral parte de la problemática que conlleva el desarrollo sostenible en zonas húmedas de gran valor ecológico y con una arraigada tradición agrícola y pesquera. Es el caso de los Parques Naturales de la Albufera de Valencia y del Delta del Ebro (Tarragona), en donde el uso intensivo de plaguicidas para el cultivo del arroz tiene una gran incidencia, no sólo en los ecosistemas de dichos Parques, sino también en la pesca y acuicultura de la zona.

1.1.2. El cultivo del arroz

El arroz está considerado como el cereal más importante a nivel mundial por la extensión de la superficie en que se cultiva y la cantidad de gente que depende de su cosecha, además de representar el alimento básico para más de la mitad de la población mundial (Alexandratos, 1995).

El cultivo del arroz (Fig. 1) sigue un ciclo anual que requiere suelos inundados con aproximadamente 7-8 cm de agua hasta unas semanas antes de la recolección del grano maduro. Se siembra entre finales de abril y principios de mayo. Una vez nacida la planta se procede a quitar las malas hierbas (*Echinocloa* sp., principalmente), tanto por procedimientos de escarda manual como química, mediante el empleo de herbicidas tiocarbamatos como el molinato y tiobencarb. Después de una enjugada a finales de mayo, se realiza entre junio y julio una escarda con sulfonilureas como la bentazona, propanil y quinclorac. En el mes de julio se procede al abonado del cultivo, en agosto se vacían los campos y en septiembre se recolecta el arroz.

Fig. 1. Plantones de arroz.

1.1.3. La Albufera de Valencia

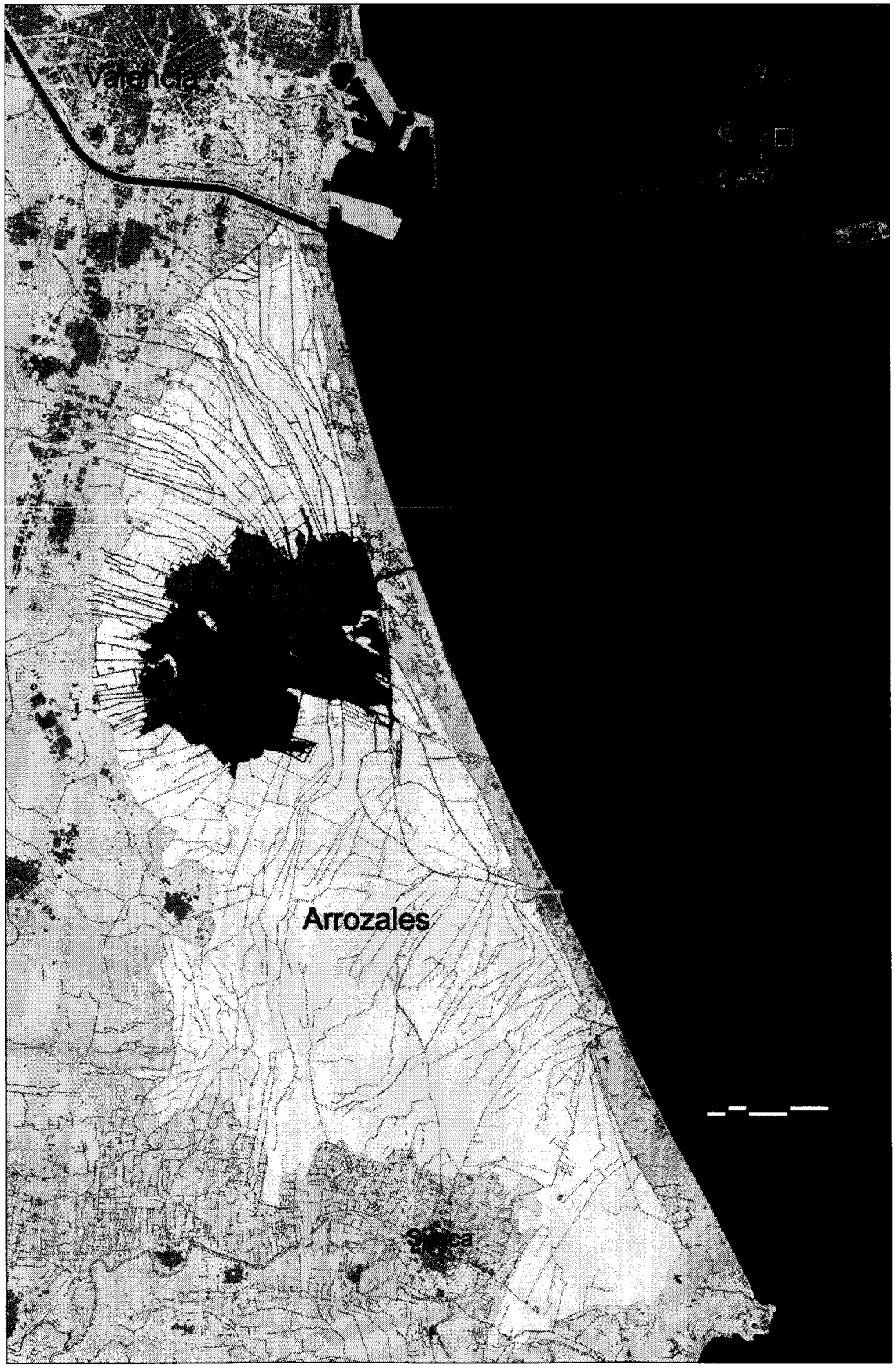
La Albufera de Valencia está ubicada a 10 Km al sur de la ciudad de Valencia, entre las desembocaduras de los ríos Turia y Júcar (Figs. 2 y 3). Tiene una superficie de 21.120 hectáreas (ha), de las cuales 2.800 corresponden a la lámina de agua, 14.000 a arrozales y las restantes 4.000 a restinga litoral y huertas periféricas (Sehumed, 1997).

Este espacio natural es de gran importancia internacional para la conservación de las aves acuáticas y destaca por la abundancia y diversidad de especies. En la Albufera han sido citadas más de 850 especies vegetales, 31 especies de mamíferos, 292 de aves, 18 de reptiles, 8 de anfibios, 33 ícticas y más de 525 especies de fauna invertebrada, de las cuales 475 son insectos, 43 moluscos y 7 crustáceos (Sehumed, 1997). De éstas, algunas son endémicas, como el bivalvo *Unio turtoni valentinus*, los crustáceos *Dugastella valentina* y *Palaemonetes zariquieyi* y los peces ciprinodóntidos samaruc (*Valencia hispanica*) y fartet (*Aphanius iberus*). Por estas razones, la Albufera ha sido incluida en la lista de áreas húmedas de importancia internacional en virtud del convenio Ramsar en mayo de 1990 y declarada como Zona de Especial Protección para las Aves (ZEPA) en abril de 1991.

El uso tradicional más importante de la Albufera ha sido el de la pesca (Fig. 2), la cual está reconocida legalmente desde 1250, que es cuando se sentaron las bases para la Comunidad de Pescadores del Palmar y que después se aplicaría a las de Silla y Catarroja. Ésta se ha basado principalmente en la captura de la anguila europea (*Anguilla anguilla*), la lubina (*Dicentrarchus labrax*), la lisa (*Mugil cephalus*) y la carpa (*Cyprinus carpio*).

Fig. 2. Sistemas de redes para la captura de anguilas, lubinas o lisas en la Albufera.

Fig. 3. Mapa de la Albufera de Valencia (elaborado a partir de material cartográfico de la Consejería de Obras Públicas y Transporte de la Comunidad Valenciana y fotos de satélite de la NASA). →


Valencia
Arrozales

El cultivo de arroz es una actividad que fue introducida por los árabes a finales del siglo XIV, pero desde el cambio a una agricultura intensiva a mediados del siglo pasado, ha ido desplazando a la actividad pesquera como consecuencia de la contaminación de las aguas. En concreto, la captura de anguilas ha disminuido un 90% respecto a la década pasada.

Boluda y cols. (2002) han analizado recientemente las aguas de diferentes canales de irrigación que vierten sobre la Albufera, concluyendo que las fuentes de contaminación y toxicidad sobre el Parque Natural son debidas principalmente a la eutrofización del agua por los múltiples desagües urbanos, a los metales pesados de origen industrial y a los plaguicidas utilizados en la actividad agrícola. De hecho, se detectaron elevadas concentraciones de molinato (3,2-77,1 μg/l) y tiobencarb (90,2 μg/l) en algunos canales de irrigación en mayo, que coincide con el periodo de aplicación de estos herbicidas en los campos de arroz. Por otra parte, la contaminación de la Albufera está acentuada por la escasa profundidad del lago, con una media inferior a los 80 cm, aunque en algunos puntos llega a tener 2 metros.

1.1.4. El Delta del Ebro

El Delta del Ebro (Fig. 4), que está situado al noreste de España y al sur de Cataluña, es consecuencia de la deposición de las tierras aluviales a la desembocadura del río. Tiene una superficie de unas 32.000 ha emergidas, de las cuales 7.736 (un 24% de la superficie total) están protegidas como Parque Natural desde 1986, constituidas por unas 2.000 ha de lagunas, 3.000 ocupadas por las puntas deltaicas del Fangar y els Alfacs, 1.500 de islas, y el resto de tierra yerma. Por otra parte, unas 23.500 ha (un 73% de la superficie total) están dedicadas a actividades agrícolas, destacando el cultivo del arroz con una extensión de unas 21.000 ha (el 66% de la superficie total) (Martínez-Vilalta, 1996).

Al igual que la Albufera de Valencia, el Delta del Ebro es de gran importancia ornitológica y posee una elevada biodiversidad, constituyendo el segundo hábitat acuático más extenso del Mediterráneo Occidental, después de la Camarga (Parque Regional francés), y el segundo de España, detrás del Parque Nacional de Doñana (Huelva-Sevilla, suroeste español).

La explotación de los hábitats naturales y salinas del Delta data del siglo XIII, con la introducción de una economía de mercado basada en la sal y la pesca. A partir del siglo XV se establece una economía de subsistencia fundamentada en la trashumancia, la

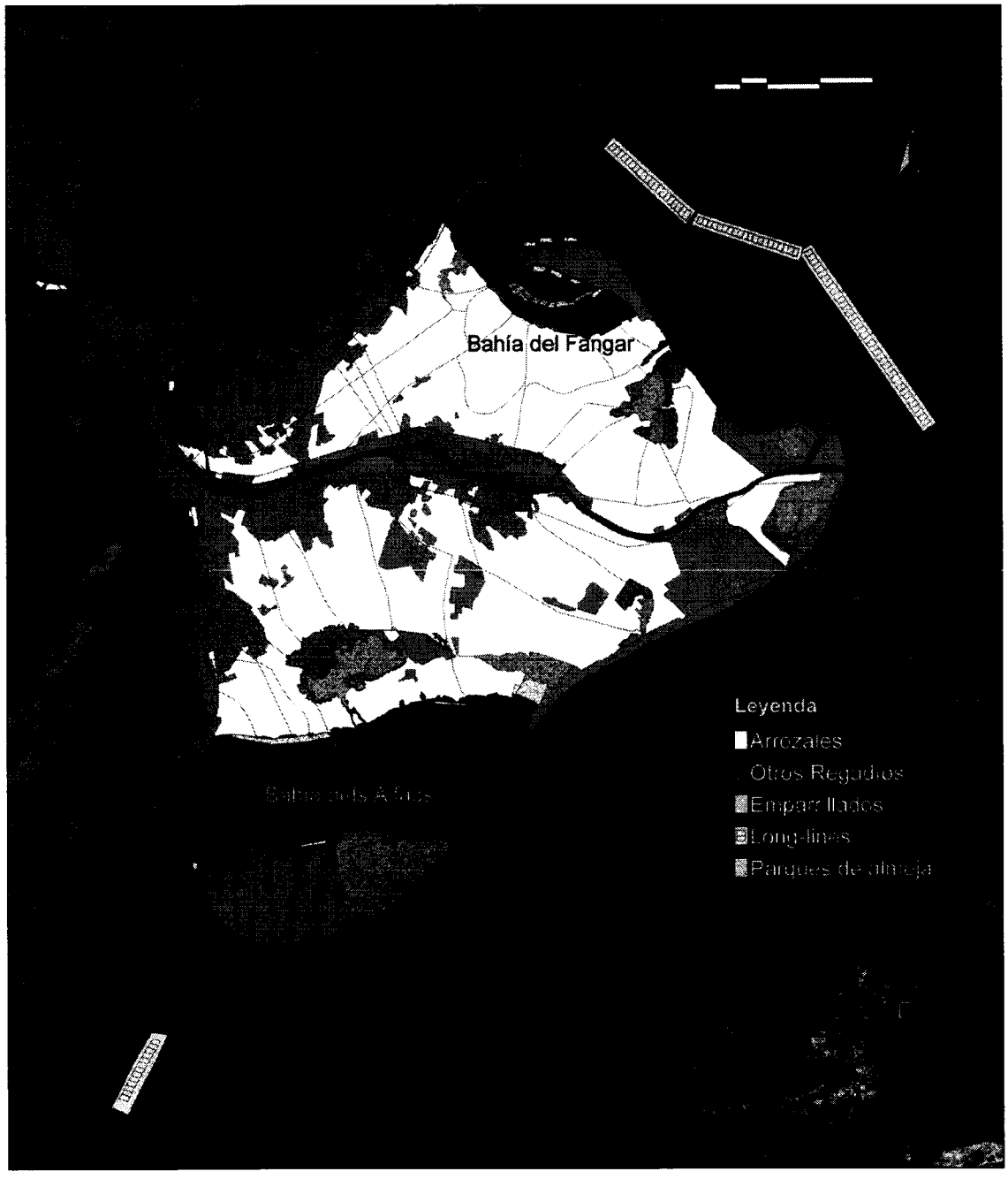


Fig. 4. Mapa del Delta del Ebro (elaborado a partir de material cartográfico del Instituto Cartográfico de Cataluña, el Departamento de Medio Ambiente y la Dirección General de Pesca y Asuntos Marítimos de la Generalitat de Cataluña, y fotos de satélite de la NASA).

agricultura extensiva de secano (trigo, cebada y maíz) y la caza, además de la economía de mercado basada en la pesca continental y la recolección de sosa y regaliz. A partir del siglo XVIII se introduce el cultivo del arroz, pero es a principios del siglo XX cuando se produce la mayor transformación de los hábitats naturales salobres y salinos en hábitats dulces. Así, de 1910 a 1960 los hábitats naturales pasaron de 27.000 ha (80% de la

superficie total) a 11.000 (33%) por desecación de humedales, inundaciones temporales del delta bajo una capa de agua dulce, desaparición de dunas y zonas salobres por la construcción de urbanizaciones, cambios del régimen hidrológico de las lagunas, etc. En 1965 el cambio a una agricultura intensiva lleva a la consolidación de una economía de mercado basada principalmente en el cultivo de arroz, hortalizas y cítricos. Posteriormente, esta economía de mercado se completaría con el turismo, la pesca continental y marítima, y la acuicultura.

El Delta del Ebro recibe anualmente grandes cantidades de plaguicidas provenientes mayoritariamente del cultivo del arroz. Así, el molinato y propanil son los herbicidas más utilizados, con más de 70 y 50 toneladas respectivamente, de ingrediente activo aplicados sobre dichos campos cada año (Santos y cols. 2000). Además, los insecticidas organofosforados son aplicados a lo largo de todo el año, pero masivamente a principios del verano, siendo el fenitrotión el más utilizado, con más de 60 toneladas al año desde 1990.

Mañosa y cols. (2001) inciden en que estos contaminantes – además de los de origen industrial, como los bifenilos policlorados (PCBs) y los metales pesados – tanto pueden acumularse en los suelos y sedimentos del delta, como ser transportados mediante la red extensiva de canales de riego que abastecen continuamente a los arrozales y desembocar directamente al mar en las bahías del Fangar y Alfacs (Fig. 4).

En estas bahías, que son poco profundas y el recambio de agua es poco intenso, se desarrolla el cultivo del mejillón (*Mytilus galloprovincialis*) y ostrón (*Crassostrea gigas*) mediante cuerdas de 3 m de longitud colgadas de estructuras de hormigón y madera denominadas emparrillados (Fig. 5). Además, se cultiva la almeja japonesa (*Ruditapes semidecussatus*) y, en menor medida, la almeja lisa (*Callista chione*) en parques, parcelas acotadas de fondos arenosos en las que se siembra la semilla y se espera a que alcance la talla comercial (Fig. 4).

Fig. 5. Emparrillados del Delta del Ebro donde se cultivan los mejillones suspendidos por cuerdas.

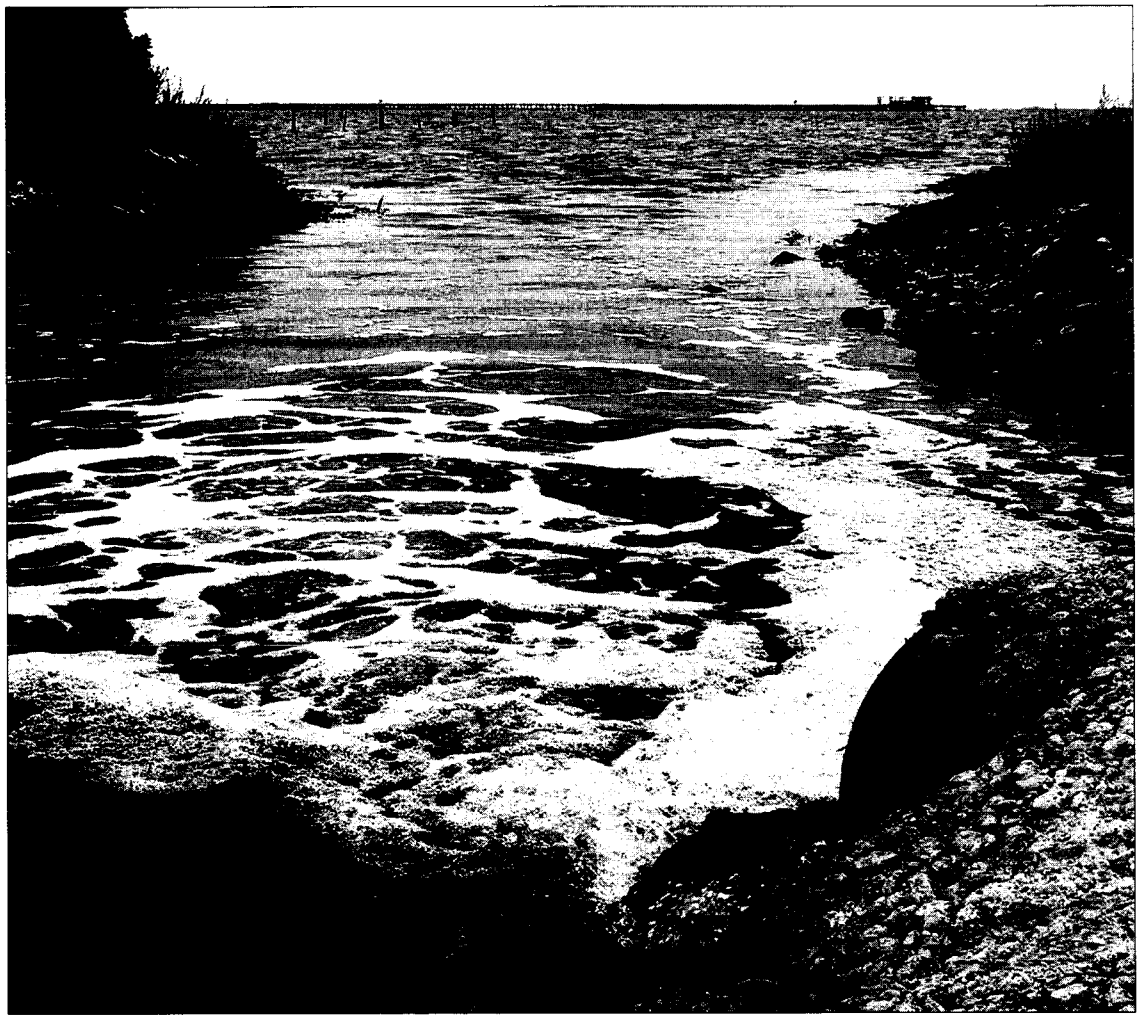


Fig. 6. Vaciado de los campos de arroz frente a los emparrillados de la Bahía dels Alfacs.

De esta manera, el vertido de fitosanitarios y otros contaminantes por la red de desagüe (Fig. 6) puede afectar no sólo a los ecosistemas naturales sino también a las instalaciones de acuicultura próximas. Varios estudios han demostrado la presencia de bajas concentraciones de diversos compuestos tóxicos en los tejidos de los moluscos bivalvos cultivados en estas bahías, siendo el fenitrotión el plaguicida organofosforado mayoritario (Barceló y cols., 1991; Escartín y Porte, 1997; Solé y cols., 2000). El mejillón y ostrón también se cultivan en el Delta en 'long-lines' fuera de las bahías y a mayor profundidad (Fig. 4), por lo que este problema no es tan importante.

1.2. Plaguicidas

1.2.1. Toxicidad de los plaguicidas

Los efectos biológicos de los plaguicidas organofosforados y carbamatos están mediados por una inhibición de la acetilcolinesterasa (AChE), enzima que cataliza la hidrólisis del neurotransmisor acetilcolina y es responsable de acabar la transmisión de los impulsos nerviosos que van desde las fibras nerviosas hasta las células musculares, glandulares y hacia otras células nerviosas en los ganglios del sistema nervioso, tanto autónomo como central (Reigart y Roberts, 1999).

La AChE es una de las enzimas más eficientes ya que es capaz de hidrolizar, dependiendo de la especie, de 2.000 a 15.000 moléculas de acetilcolina por segundo, estando limitada por la difusión celular (Gnagey y cols., 1987). Así, la inhibición de la AChE produce una acumulación de acetilcolina en el espacio presináptico que sobreestimula los receptores de acetilcolina tanto en las uniones neuroefectoras colinérgicas (efectos muscarínicos) como en las uniones neuromusculares del músculo esquelético y en los ganglios autónomos (efectos nicotínicos). Dependiendo del grado de inhibición de la AChE se observan varios efectos tóxicos, tales como hipersecreciones, convulsiones, insuficiencias respiratorias, coma y, en última instancia, la muerte si no se administra un antagonista del receptor muscarínico como la atropina (Fig. 7). Si la sobreestimulación persiste, sin ser lo suficientemente intensa como para provocar la muerte, entonces se produce una liberación de aminoácidos excitatorios como el glutamato y el aspartato, que prolongan las convulsiones y activan al receptor *N*-metil-D-aspartato (NMDA), resultando entonces inefectivo el tratamiento con atropina (Shih y McDonough, 1997). Esto desencadena un influjo excesivo de Ca^{2+} extracelular, que activa las enzimas proteolíticas y la óxido nítrico sintasa, que genera óxido nítrico y otros radicales libres, culminando finalmente en deterioro celular (Beal, 1995).

A causa de la similitud de la estructura química de los organofosforados con la acetilcolina, se tiene una mayor o menor inhibición de la AChE. En el caso de los plaguicidas carbamatos la unión a la AChE tiene lugar mediante una carbamilación en vez de una fosforilación, por lo que la inhibición es menos intensa y los efectos tóxicos de menor duración.

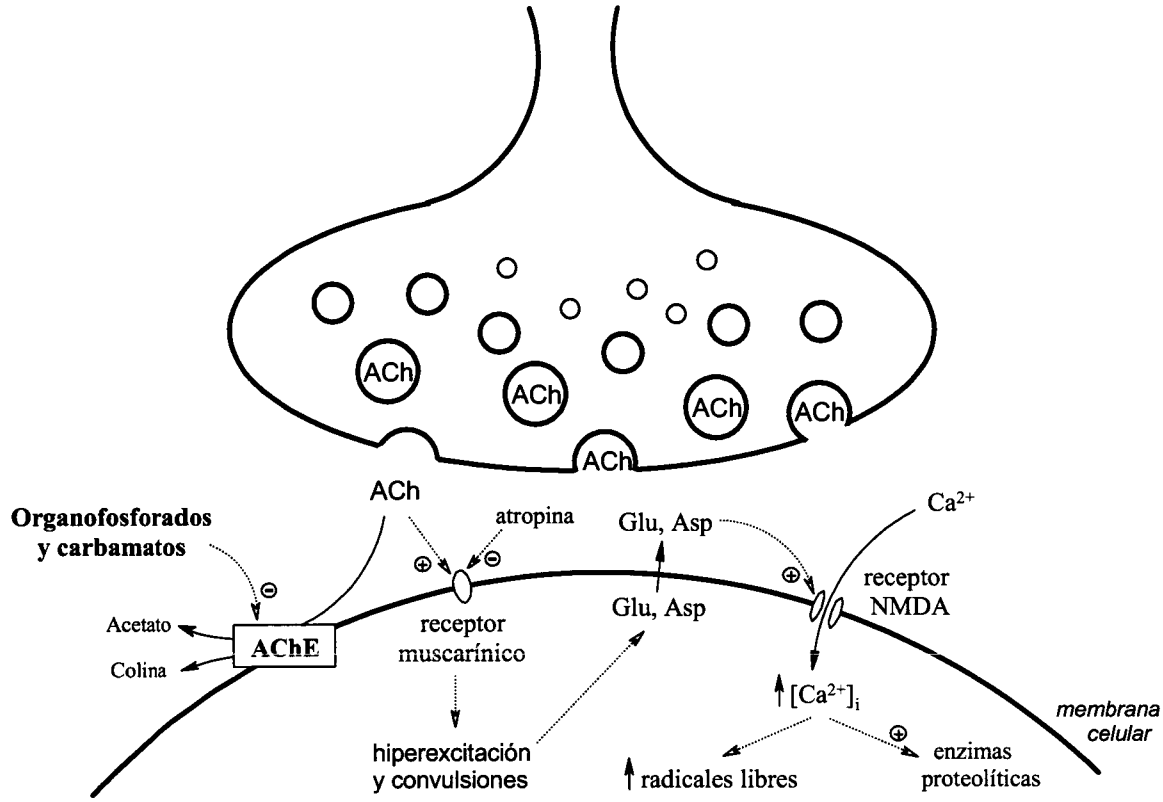


Fig. 7. Mecanismo de toxicidad de los plaguicidas organofosforados y carbamatos en la transmisión sináptica.

1.2.2. Biomarcadores de exposición a plaguicidas

Los cambios a nivel bioquímico, fisiológico e histológico, así como aberraciones en organismos, utilizados para estimar tanto la exposición a contaminantes como sus efectos resultantes se denominan biomarcadores (Hugget y cols., 1992). En general, los biomarcadores se pueden considerar como medidas de las primeras interacciones toxicológicas entre el compuesto químico y su diana o receptor. Esta interacción induce una cascada de procesos bioquímicos que empiezan a nivel subcelular, tales como la perturbación de la expresión génica o la interferencia con rutas metabólicas, que afectan a nivel celular, de órganos, y finalmente se pueden manifestar en una merma de características ecológicas tales como el crecimiento, reproducción y supervivencia. Esto también podría alterar a niveles más altos de organización biológica (población, comunidad y ecosistema).

Según el concepto de deterioro e incapacidad de Depledge (1989), una perturbación inicial causada por una exposición a un tóxico generará respuestas con tal de compensar la homeostasis celular y tisular de un organismo. A mayores dosis de tóxico los ajustes

compensatorios desvían al organismo del estado de salubridad, pudiendo alterar las capacidades reproductivas, hasta que finalmente, cuando la reparación y la compensación ya no fueran posibles, el organismo moriría.

La inhibición de la actividad enzimática de la AChE es un biomarcador específico de la exposición a organofosforados y carbamatos que es más fiable que la determinación analítica del contenido en plaguicidas ya que representa un efecto fisiológico directamente relacionado con el modo de acción del compuesto tóxico y, en general, la inhibición persiste mucho más tiempo en los tejidos (de días a semanas) que los plaguicidas (de horas a días) (Fulton y Key, 2001).

Escartín y Porte (1997) encontraron una menor actividad AChE en las branquias de mejillones obtenidos de los emparrillados del Delta del Ebro en los meses de abril a agosto. En ese periodo es cuando están abiertos los canales de irrigación, permitiendo la circulación del agua hacia las bahías y arrastrando los contaminantes de los campos de arroz hacia las instalaciones de acuicultura. Así, observaron una correlación negativa entre la actividad AChE y la concentración de plaguicidas, pues en los meses que encontraron mayor concentración de fenitrotión en los tejidos de los mejillones, éstos tenían menor actividad AChE.

1.2.3. Utilización de los plaguicidas en acuicultura

El rápido crecimiento y desarrollo de la acuicultura intensiva ha sido paralelo a la utilización de sustancias químicas para aumentar la salud animal. Las infecciones de parásitos causan en los peces estrés y susceptibilidad a desarrollar otras patologías que suponen importantes pérdidas para la industria. Entre las medicinas y productos químicos existentes, los plaguicidas organofosforados, y especialmente el diclorvós, han sido extensivamente empleados como antiparasitarios.

El diclorvós es un plaguicida organofosforado poco persistente que se hidroliza completamente y en poco tiempo en muchos tipos de ambientes y que se degrada rápidamente en el metabolismo de los mamíferos (OMS, 1989). Éste se ha utilizado principalmente para tratar las infecciones del piojo marino (por los copépodos parásitos *Lepeophtheirus salmonis* y *Caligus elongatus*) en el cultivo del salmón atlántico (*Salmo salar*) (Scott, 1993) y de manera esporádica para tratar las monogeneas del género *Pseudodactylogyrus* en el cultivo de la anguila (Imada y Muroga, 1979). El diclorvós es un plaguicida extremadamente soluble en agua, con lo que su bioacumulación en los tejidos

de los peces es prácticamente nula, eliminándose rápidamente, sobre todo por los cortos tratamientos a los que se somete a estos peces (inferiores a 1 hora). Sin embargo, el uso repetido de este plaguicida durante varias décadas ha perdido eficacia por la generación de resistencia en los parásitos (Jones y cols., 1992) y ha sido sustituido por otro organofosforado, el azametifós, para tratar los ectoparásitos del salmón (Roth y cols., 1996) y la anguila (Pretti y cols., 2002).

1.2.4. Efectos de los plaguicidas en especies no diana

Los plaguicidas organofosforados son rápidamente degradados y biotransformados en las condiciones naturales de los cultivos, a diferencia de los plaguicidas organoclorados como el DDT, evitando así su biomagnificación a lo largo de la cadena alimenticia. Estos últimos son altamente persistentes y desde 1977 están prohibidos en España, salvo algunos compuestos como ciclodienos y ciertos isómeros del HCH. Sin embargo, a pesar de que los plaguicidas organofosforados tienen unos tiempos de vida medio en los cultivos que oscilan entre menos de una semana y 10 días (Barceló y cols., 1991), durante el mismo día de aplicación de fenitrotión en el Delta del Ebro, por ejemplo, la concentración del insecticida en el agua puede llegar a ser de 119 a 178 μg/l (Oubiña y cols., 1996), suficiente para matar la fauna de microcrustáceos, y por tanto, alterar el ecosistema.

Por otra parte, el fenitrotión se puede acumular en la grasa de las anguilas de la Albufera de Valencia como han demostrado Sancho y cols. (1998b) pero se elimina rápidamente de los tejidos, recuperándose el metabolismo energético en menos de una semana (Sancho y cols., 1998a).

Las elevadas concentraciones de molinato detectadas por Boluda y cols. (2002) en algunos canales de irrigación de la Albufera de Valencia (3,2-77,1 μg/l) contrastan con los 3 a 5 μg/l detectados por Durand y cols. (1992) en el Delta del Ebro. Estas concentraciones de molinato no son tóxicas para varias especies de fitoplancton representativas de humedales, pues necesitan de 0,65 a 38,0 mg/l para inhibir su crecimiento un 50% (Sabater y Carrasco, 1998). No obstante, según Mañosa y cols. (2001), estas concentraciones serían suficientes para evitar o retardar el desarrollo de la vegetación sumergida, que es necesaria como fuente de alimentación o refugio de muchos invertebrados y vertebrados, destruyéndose así el hábitat necesario para que las comunidades de animales se desarrollen.

1.2.5. Efectos de los plaguicidas en la salud humana

Solé y cols. (2000) inciden en que las bajas concentraciones de contaminantes orgánicos en los moluscos bivalvos cultivados en las bahías del Delta del Ebro no suponen una amenaza para la salud humana, salvo el corto periodo de tratamiento de los campos con organofosforados en verano, que coincide con la extracción y comercialización de los bivalvos. Éstos, antes de ponerse a la venta, se llevan a depuradoras donde se mantienen un mínimo de 48 horas con agua esterilizada con ozono para que eliminen sus contaminantes. No obstante, se ha observado que ciertos plaguicidas necesitan varios días para eliminarse de los tejidos de los bivalvos (Serrano y cols., 1997) y que la exposición crónica a éstos podría ocasionar serios problemas en la salud humana, pues se ha visto asociado a cáncer (Dich y cols., 1997) y a enfermedades neurodegenerativas como el Parkinson (Betarbet y cols., 2000).

1.2.6. Tratamientos convencionales frente a intoxicaciones por plaguicidas

Los tratamientos actuales frente a intoxicaciones por organofosforados consisten en la administración combinada de un antagonista de los receptores muscarínicos (como la atropina), un reactivador de la AChE (una oxima como la pralidoxima) y un anticonvulsionante (como el diacepam) (Reigart y Roberts, 1999). El sulfato de atropina se administra normalmente por vía intravenosa para antagonizar los efectos de las excesivas concentraciones de acetilcolina en los receptores muscarínicos. Sin embargo es inefectiva frente a los receptores nicotínicos, por lo que no evita la debilidad muscular, los temblores, ni la depresión respiratoria. La pralidoxima (2-PAM) cuando se administra en las primeras 48 h después de la intoxicación alivia los efectos muscarínicos y nicotínicos ya que reactiva las colinesterasas y evita el proceso de envejecimiento de las colinesterasas fosforiladas.

Por el contrario, los herbicidas tiocarbamatos carecen de antídotos específicos ya que la inhibición de la AChE es menor que la de los plaguicidas organofosforados, por lo que el tratamiento con atropina y pralidoxima está normalmente contraindicado (Reigart y Roberts, 1999). Únicamente se administra carbón activo para limitar los efectos irritantes y reducir la absorción de los plaguicidas, al igual que antiácidos como el hidróxido de aluminio.

1.3. Radicales libres, estrés oxidativo y antioxidantes

1.3.1. Especies reactivas de oxígeno (ROS) y nitrógeno (RNS)

Un radical libre es una molécula (o fragmento de la misma) que ha ganado o perdido un electrón. Así, al poseer un número impar de electrones en su orbital externo, tiene gran avidez por captar electrones de su entorno o ceder el electrón desapareado. En los organismos vivos existen moléculas que, sin ser radicales libres, reaccionan con otras moléculas para dar lugar a éstos. Un ejemplo sería el peróxido de hidrógeno (H_2O_2), que por la reacción de Fenton con metales de transición da lugar al radical hidroxilo ($HO^{\cdot}$), que es altamente reactivo. De esta manera, se engloban todas estas moléculas en especies reactivas de oxígeno (ROS) o de nitrógeno (RNS), dependiendo de su naturaleza (Tabla 1).

Tabla 1. Principales especies reactivas de oxígeno y nitrógeno.

Especies Reactivas de Oxígeno (ROS)		Especies Reactivas de Nitrógeno (RNS)	
$HO^{\cdot}$	radical hidroxilo	$NO^{\cdot}$	óxido nítrico
HOCl	ácido hipocloroso	NO_2	dióxido de nitrógeno
H_2O_2	peróxido de hidrógeno	N_2O_3	trióxido de dinitrógeno
$LO^{\cdot}$	radical alcoxil lipídico	$ONOO^{-}$	peroxinitrito
$LOO^{\cdot}$	radical peroxil lipídico	ONOOH	ácido peroxinitroso
$^1O_2$	oxígeno singlete		
$O_2^{\cdot-}$	anión superóxido		

1.3.2. Fuentes de ROS y RNS

Las ROS y RNS se generan por el metabolismo aeróbico de las células en una variedad de reacciones. Está bien establecido que la **mitocondria** es la principal fuente de radicales libres dentro de las células. La respiración celular implica la reducción de oxígeno molecular (O_2) a agua por la cadena de transporte de electrones a través de la formación de los intermediarios $O_2^{\cdot-}$, H_2O_2 y $HO^{\cdot}$. Entre un 1 y un 5% de estas ROS se pueden escapar de la cadena de transporte de electrones y dañar los componentes celulares (Kelly y cols., 1998).

Una segunda fuente de ROS y RNS estaría formada por **enzimas oxidantes**, tales como la diamina oxidasa, triptófano dioxigenasa, xantina oxidasa, citocromo P-450 reductasa, que generan $O_2^{\cdot-}$, y la guanil ciclasa y glucosa oxidasa, que generan H_2O_2. Sin embargo, los radicales libres pueden ser beneficiosos tanto en la inmunidad innata como adquirida, pues la fagocitosis de neutrófilos y macrófagos estimula varios procesos como el "estallido respiratorio", donde las NAD(P)H oxidasas de membrana generan enormes cantidades de $O_2^{\cdot-}$, que junto al $NO^{\cdot}$ producido por los macrófagos se forma $ONOO^{-}$, que es un potente agente citotóxico contra organismos patogénicos, al igual que el $HO^{\cdot}$ generado por la reacción de Fenton y el HOCl producido por la mieloperoxidasa a partir de la oxidación del cloruro por el H_2O_2 (Knight, 2000). No obstante, una infección parasitaria puede producir estrés oxidativo en el músculo de los peces (Belló y cols., 2000).

Determinados **xenobióticos** comprenderían una tercera fuente de ROS y RNS, pues pueden inhibir la cadena de transporte de electrones, permitiendo la acumulación de los intermediarios radicalarios (Nohl y cols., 1981), inactivar las enzimas antioxidantes (Kono y Fridovich, 1983; Hasspieler y cols., 1994) o deplecionar las moléculas antioxidantes (Thomas y Wofford, 1993; Hasspieler y cols., 1994). Otros xenobióticos como quinonas, algunos tintes, herbicidas bipiridilos, algunos metales de transición y compuestos nitro aromáticos son capaces de realizar ciclos redox con enzimas como la xantina oxidasa y la citocromo P-450 dependiente de NADPH (Kelly y cols., 1998). Estos compuestos orgánicos son reducidos univalentemente a un intermediario reactivo que transfiere rápidamente su electrón al O_2, produciendo $O_2^{\cdot-}$ y regenerando al xenobiótico. Así, una única molécula de éstas es capaz de generar gran cantidad de ROS antes de ser eliminada, además de consumir equivalentes reductores celulares como el NADPH, lo cual compromete a otros procesos metabólicos.

Finalmente, otras fuentes de ROS serían las producidas por las **radiaciones ultravioleta**, como se ha observado en peces cultivados en jaulas flotantes, donde los daños en el ADN resultan en mortalidad de embriones y larvas, quemaduras de adultos y juveniles, así como, de manera indirecta, estrés oxidativo, fototoxicidad y fotosensibilidad (Zagarese y Williamson, 2001).

1.3.3. Efectos celulares de las ROS y RNS

Las ROS y RNS a bajas concentraciones son indispensables para muchos procesos bioquímicos ya que actúan como mensajeros secundarios en varias vías de señalización

durante un metabolismo celular normal para indicar la diferenciación y progresión celular o el arresto del crecimiento, además de intervenir en procesos inflamatorios y en la defensa frente a microorganismos (Finkel y Holbrook, 2000). Asimismo, el NO· puede actuar también como un neurotransmisor y relajante muscular (Bruhwyler y cols., 1993).

Por el contrario, a elevadas dosis las ROS y RNS son capaces de reaccionar con macromoléculas biológicas y producir inactivación de proteínas, y por tanto de enzimas, peroxidación de los lípidos de membrana y daños en el ADN (tales como hidroxilación, apertura de los anillos y fragmentación) si no son eliminadas apropiadamente por las defensas de antioxidantes en la célula (Kelly y cols., 1998; Fig. 8). Éstas están formadas por sistemas enzimáticos como la catalasa, superóxido dismutasa, glutatión peroxidasa y tioredoxina peroxidasa así como por moléculas de bajo peso molecular como la vitamina C (ascorbato), vitamina E (tocoferoles y tocotrienoles) y glutatión. Cuando hay un desequilibrio entre la generación de radicales libres y su eliminación por parte de los antioxidantes, entonces se produce una situación de estrés oxidativo (Winston, 1991).

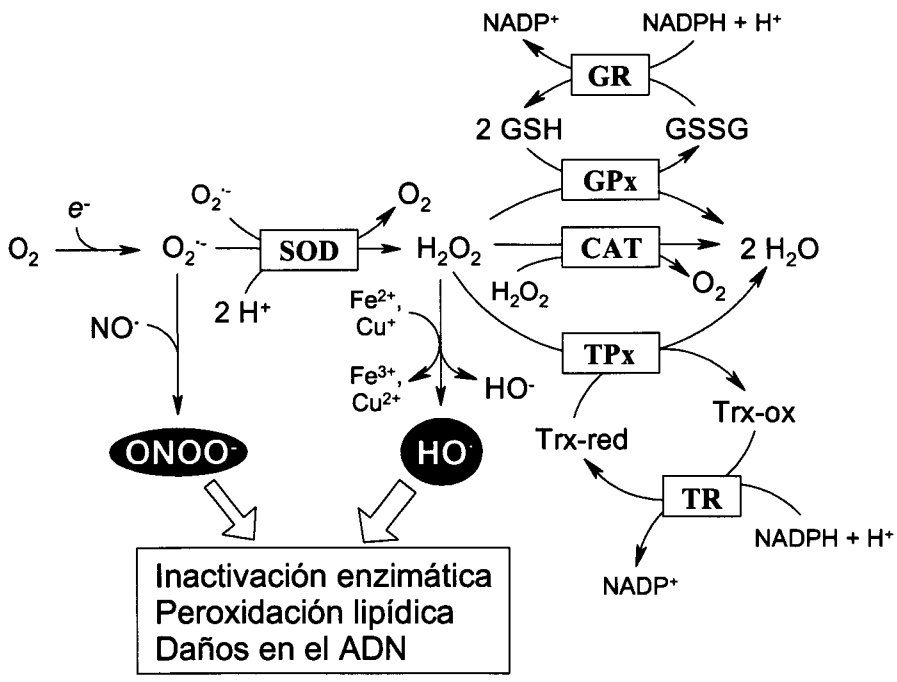


Fig. 8. Esquema básico de formación y eliminación de radicales libres en las células por las enzimas superóxido dismutasa (SOD), catalasa (CAT), glutatión peroxidasa (GPx) junto a la glutatión reductasa (GR) y tioredoxina peroxidasa (TPx) junto a la tioredoxina reductasa (TR). GSH es el glutatión reducido, GSSG el glutatión oxidado y Trx la tioredoxina.

1.3.4. Enzimas antioxidantes

Las **superóxido dismutasas** (SODs) son metaloproteínas que facilitan la dismutación de dos radicales superóxido para formar peróxido de hidrógeno (Fig. 8). En eucariotas se han caracterizado tres isoformas de SODs: En el citosol se localiza una forma que contiene cobre y zinc (CuZn-SOD), en las mitocondrias contiene manganeso (Mn-SOD) y en la matriz extracelular cobre y zinc (EC-SOD).

La **catalasa** (CAT) es un tetramero que se localiza principalmente en los peroxisomas y cataliza la descomposición de dos moléculas de peróxido de hidrógeno en agua y oxígeno (Fridovich, 1978; Fig. 8). Esta enzima no metaboliza alquil hidroperóxidos (Chance y cols., 1979). El hecho de que tenga un alto valor de K_m para el H_2O_2 y esté compartimentado en los peroxisomas indica que su función protectora frente al peróxido de hidrógeno se limite al generado en estos organelos (DeLeve y Kaplowitz, 1991).

Las **glutatión peroxidasas** (GPx) son proteínas diméricas compuestas de dos subunidades que contienen un residuo de selenocisteína en cada una de estas cuatro subunidades que es imprescindible para la actividad enzimática (Tappel, 1978). Las GPx catalizan la reducción dependiente de GSH, de H_2O_2 a H_2O (Fig. 8), así como de otros peróxidos, principalmente hidroperóxidos orgánicos, protegiendo a los lípidos de las membranas celulares frente a la oxidación (Hayes y McLellan, 1999). Esta enzima está acoplada a la **glutatión reductasa** (GR), que es una flavoproteína que reduce el GSSG a GSH a costa de oxidar el NADPH a $NADP^+$.

La **tioredoxina peroxidasa** (TPx) utiliza los electrones de la tioredoxina para reducir al peróxido de hidrógeno (Chae y cols., 1994; Jin y cols., 1997). La tioredoxina (Trx) es una proteína de pequeño tamaño (12 kDa) que contiene un centro activo conservado con la secuencia -Cys-Gly-Pro-Cys-, donde las dos cisteínas forman un centro redox ditiol/disulfuro. La reducción de esta proteína está catalizada por la **tioredoxina reductasa** (TR), que oxida el NADPH a $NADP^+$ como la GR (Arner y Holmgren, 2000).

Las **peroxiredoxinas** (Prx) son una familia de proteínas homólogas a la TPx que han sido caracterizadas recientemente con actividad peroxidasa pero que se desconoce el compuesto que dona los electrones (Kang y cols., 1998), o actúa la tioredoxina y/o el glutatión (Fujii e Ikeda, 2002).

1.3.5. Moléculas antioxidantes

Existen numerosas moléculas que son capaces de inhibir las reacciones de oxidación de las ROS y RNS tanto por la eliminación directa como por la prevención de su formación. La mayoría de antioxidantes son compuestos de bajo peso molecular que son sintetizados endógenamente como el glutatión, el ácido úrico y la bilirrubina u obtenidos por la dieta como la vitamina C (ascorbato), la vitamina E (tocoferoles y tocotrienoles), los flavonoides y los carotenoides. La vitamina E es un importante antioxidante liposoluble ya que bloquea las reacciones radicalarias de peroxidación lipídica y es regenerado por el antioxidante hidrosoluble vitamina C. Por otra parte, los agentes quelantes de metales como la desferrioxamina o proteínas como la transferrina, ferritina, metalotioneína y ceruloplasmina secuestran los iones metálicos y previenen la formación de oxidantes nocivos generados por la reacción de Fenton (Halliwell y Gutteridge, 1989).

1.3.6. La N*-acetilcisteína*

La *N*-acetilcisteína (NAC) (Fig. 9) destaca entre los antioxidantes actuales por su capacidad de eliminar directamente a los radicales libres (Aruoma y cols., 1989) y la posibilidad de desacetilarse para dar lugar al aminoácido cisteína, que es el limitante en la síntesis de glutatión (DeLeve y Kaplowitz, 1991). Debido a su alto umbral de toxicidad y amplia ventana terapéutica, la NAC se ha utilizado con muy pocos efectos secundarios en varias aplicaciones clínicas relacionadas con el estrés oxidativo y/o la deficiencia en glutatión, tales como la sobredosis de paracetamol (acetaminofen) (Prescott y cols., 1977), la infección por el virus de la inmunodeficiencia humana (VIH) (De Rosa y cols., 2000) y en enfermedades pulmonares (Gillissen y Nowak, 1998) y cardíacas (Sochman, 2002). Además, se ha demostrado su efectividad frente a intoxicaciones por metales pesados tales como el metilmercurio (Ballatori y cols., 1998) y el plomo (Gurer y Ercal, 2000), y el plaguicida paraquat (Hoffer y cols., 1996).

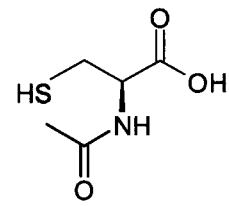
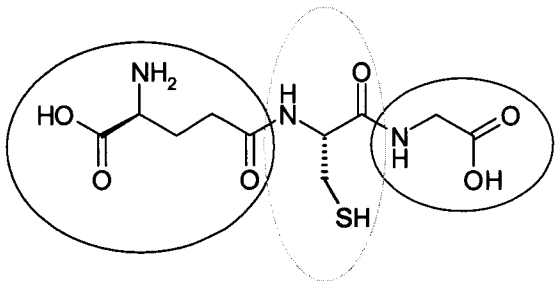


Fig. 9. Estructura química de la *N*-acetilcisteína.

1.4. Metabolismo del glutatión

1.4.1. Estructura del glutatión

El glutatión (L-γ-glutamil-L-cisteinilglicina) es un tripéptido compuesto por glutamato, cisteína y glicina, que está presente en grandes cantidades en prácticamente todos los organismos aeróbicos (Fig. 10). El peculiar enlace peptídico del grupo γ-carboxilo (en vez del α-carboxilo) del glutamato con la cisteína en el extremo N-terminal evita que las peptidasas y proteasas lo degraden, a excepción de la γ-glutamiltransferasa, que se encuentra en la superficie externa de las membranas celulares. Además, la presencia de glicina en el extremo C-terminal protege al tripéptido de la γ-glutamilciclotransferasa, lo que hace al glutatión muy resistente a la degradación intracelular (DeLeve y Kaplowitz, 1991).



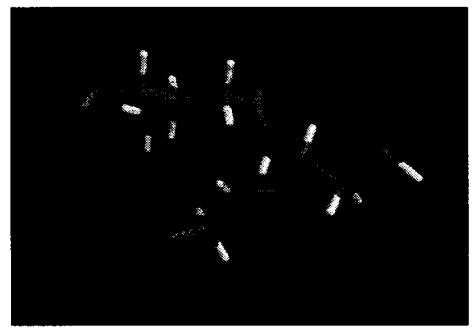


Fig. 10. Estructura química y espacial del glutatión.

Mientras que la cisteína se autooxida rápidamente a cistina, y puede ser tóxica en grandes cantidades por la generación de radicales libres, la cisteína del glutatión estaría protegida por los extremos C- y N-terminal, en el que el grupo tiol (-SH) actuaría como un reductor y nucleófilo (compuesto rico en electrones) intracelular de bajo peso molecular. Esto le permite al glutatión (a parte de ser una forma no tóxica de almacenamiento de cisteína) intervenir en varios fenómenos celulares de gran importancia, tales como la destoxificación de xenobióticos con centros electrófilos, la eliminación de radicales libres, el mantenimiento del estado reducido en los grupos tioles de las proteínas (al actuar como un tampón redox) y la modulación de la función inmune, la transducción de señales, la expresión génica y la síntesis de ADN (Meister y Anderson, 1983; Kretzschmar, 1996; Sen y Packer, 1996; Cooper y Kristal, 1997; Sies, 1999).

El glutatión está presente mayoritariamente en las células en su forma activa y reducida (GSH). Sin embargo, como consecuencia de las condiciones oxidantes, dos moléculas de GSH que hayan captado un radical libre se pueden unir por un puente disulfuro para generar una molécula de glutatión oxidado o disulfuro (GSSG) y eliminar dichos radicales.

1.4.2. Síntesis y transporte del glutatión

El GSH se sintetiza tanto en procariotas como eucariotas exclusivamente en el citosol en dos reacciones consecutivas dependientes de ATP (Fig. 11). La primera está catalizada por la enzima **glutamato:cisteína ligasa** (GCL), anteriormente conocida como γ-glutamilcisteína sintetasa, donde se unen los aminoácidos cisteína y glutamato para dar lugar a γ–glutamilcisteína, a la que se une glicina por la **GSH sintetasa** (GS). La GS parece no tener regulación, en cambio la GCL controla la síntesis de GSH ya que presenta inhibición por retroalimentación negativa del propio GSH (Richman y Meister, 1975) y está regulada por la disponibilidad de L-cisteína (Deneke y Fanburg, 1989). Esto es así porque mientras la concentración de glutamato intracelular es muy superior al valor de K_m de la GCL para el glutamato, la concentración intracelular de cisteína se aproxima a su valor de K_m (0,1-0,3 mM) (Fernández-Checa y cols., 1992).

El hígado es el principal órgano de síntesis del GSH ya que no sólo obtiene la cisteína directamente de la dieta o del catabolismo de proteínas, sino que también es el único órgano donde se sintetiza cisteína a partir de metionina mediante la ruta de la transsulfuración (Reed y Orrenius, 1977). Ésta consiste en la activación dependiente de ATP de la metionina a *S*-adenosilmetionina (SAM) catalizada por la metionina adenosiltransferasa, que es posteriormente desmetilada y retirada la mitad adenosil para dar lugar a homocisteína. Ésta es conjugada con serina para dar lugar a cistationina y por acción de la cistationina γ-liasa resulta en L-cisteína. A pesar de que se ha observado que en condiciones oxidantes la vía de la transsulfuración puede estar activada respecto a la remetilación (Taoka y cols., 1998), el estrés oxidativo y nitrosativo inhiben a la metionina adenosiltransferasa (Ávila y cols., 1997; Sánchez-Góngora y cols., 1997) lo que disminuye la producción de SAM y posteriormente de GSH hepático (Lu, 1998). Así, los niveles de GSH hepáticos están íntimamente relacionados con la dieta y especialmente con el contenido en cisteína (Tateishi y cols., 1974).

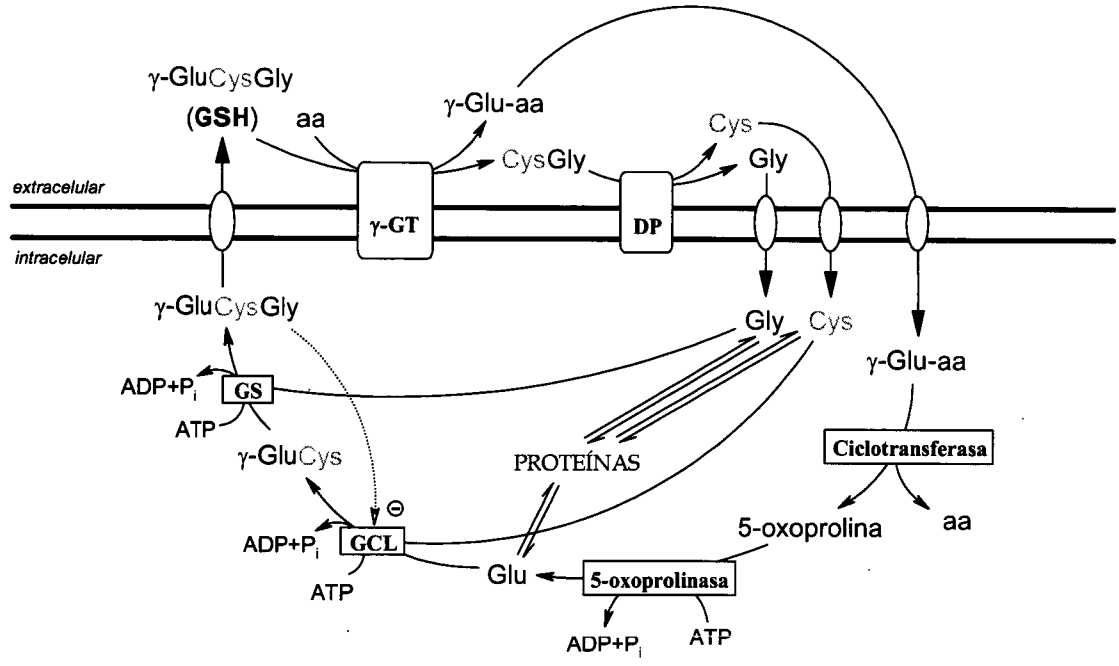


Fig. 11. Ciclo del γ-glutamil para el transporte y síntesis de glutatión (adaptado de Meister y Anderson, 1983). aa, aminoácido; DP, dipeptidasa.

El GSH tiene transportadores específicos para exportarse fuera de las células, pero en cambio no lo tiene para transportarse dentro de éstas (Ookhtens y Kaplowitz, 1998). Esto se debe a que el hígado exporta el GSH hacia el torrente sanguíneo y se distribuye entre los otros tejidos. En la parte externa de la membrana plasmática se encuentra anclada la enzima **γ–glutamil transferasa** (γGT), también conocida como γ–glutamil transpeptidasa, que es la única proteasa que degrada al glutatión y a éste conjugado a otras moléculas. La γGT transfiere la mitad γ–glutamil del GSH a un aminoácido (siendo la cistina la más adecuada) formando γ–glutamil aminoácido y cisteinilglicina (Meister y Anderson, 1983; Fig. 11). El γ–glutamil aminoácido se puede transportar al interior celular y por la ciclotransferasa liberar el aminoácido y formar 5-oxoprolina, que se puede convertir a glutamato. La cisteinilglicina se puede escindir por la dipeptidasa (DP) y generar cisteína y glicina, las cuales se pueden transportar al interior de la célula para sintetizar GSH.

Así la γGT permite la reabsorción de los catabolitos del GSH del plasma y mantiene unas altas concentraciones de GSH en tejidos extrahepáticos (Curthoys y Hughey, 1979; Fig. 11). Se ha observado que ciertas hormonas del estrés, como la adrenalina, fenilefrina y vasopresina, aumentan el transporte del GSH hepático al plasma (Sies y Graf, 1985).

1.4.3. Función destoxificante del glutatión

La destoxificación de xenobióticos o sus metabolitos es una de las más importantes del glutatión. El grupo tiol del GSH puede realizar un ataque nucleófilo sobre el centro electrófilo de un amplio espectro de compuestos (como los metabolitos reactivos formados por el sistema del citocromo P-450 y la mayoría de los plaguicidas) tanto de manera espontánea como catalizado por las **glutatión *S*-transferasas** (GSTs), resultando en la formación de productos menos tóxicos y facilitando su eliminación (DeLeve y Kaplowitz, 1991; Fig. 12). Las GSTs son una familia multigénica de isoenzimas que contienen una región hidrofóbica muy polimórfica para unirse a una gran variedad de sustratos electrófilos. En el hígado las GSTs representan del 5 al 10% de las proteínas solubles (Whalen y Boyer, 1998), por lo que la destoxificación de contaminantes se produce principalmente en este órgano.

El metabolismo de xenobióticos comprende varias fases: En la **fase I** tendría lugar una modificación de la molécula original (oxidación, reducción o hidrólisis), donde actuarían principalmente las oxigenasas de función mixta o monooxigenasas dependientes del

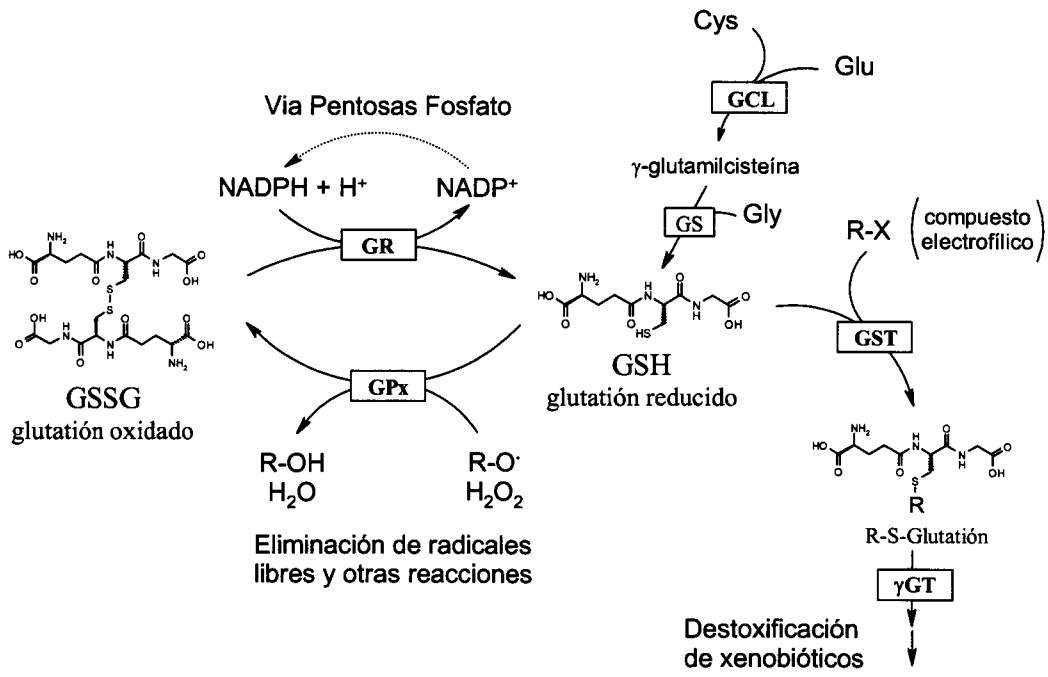


Fig. 12. El metabolismo del glutatión está regulado principalmente por las siguientes enzimas: GCL, glutamato:cisteína ligasa; GPx, glutatión peroxidasa; GR, glutatión reductasa; GS, glutatión sintetasa; GST, glutatión *S*-transferasa; γGT, γ–glutamil transferasa.

citocromo P-450, que oxidarían al compuesto exógeno. En la **fase II** estos compuestos resultantes se conjugarían a grupos más polares como el glutatión, ácido glucurónico o sulfato para solubilizarlos y facilitar su excreción (Commandeur y cols., 1995). Finalmente, en la **fase III** estos compuestos conjugados serían eliminados por proteínas transportadoras de aniones orgánicos multivalentes denominadas MRP (*multidrug resistance associated protein*) (Cnubben y cols., 2001).

La conjugación con glutatión consume GSH irreversiblemente. Estos conjugados se degradarían mediante la γGT al conjugado de cisteinilglicina, que por la dipeptidasa daría lugar al conjugado de cisteína y por la *N*-acetilasa se formaría el ácido mercaptúrico correspondiente (DeLeve y Kaplowitz, 1991). Como los riñones contienen elevadas concentraciones de γGT, la eliminación de xenobióticos por la vía del ácido mercaptúrico tendría lugar preferentemente por la orina.

Además de sustancias exógenas, muchos compuestos formados endógenamente, como las prostaglandinas y leucotrienos se conjugan con el GSH por un mecanismo similar al de los xenobióticos (Wang y Ballatori, 1998).

1.4.4. Función antioxidante del glutatión

El GSH es capaz de reaccionar espontáneamente con $HO^{\cdot}$, N_2O_3 y $ONOO^-$ (Wink y Mitchell, 1998) y regenerar a la vitamina C y ésta a la E. Sin embargo, la eliminación de H_2O_2 y de otros peróxidos está catalizado por la **GPx**, como ya se ha comentado previamente en la sección 1.3.4. Como consecuencia de estas reacciones el GSH se oxida a GSSG, el cual es reducido otra vez a GSH por la **GR** a expensas de NADPH, formándose un ciclo redox (Fig. 12). La principal ruta metabólica de regeneración del NADPH es por la vía de las pentosas fosfato, donde participan las enzimas glucosa-6-fosfato deshidrogenasa, 6-fosfogluconato deshidrogenasa y transaldolasa, pero hay otras enzimas que también pueden generar NADPH citosólico como la isocitrato deshidrogenasa y el enzima málico.

Las **GSTs** también participan en la defensa frente al estrés oxidativo ya que estas enzimas son capaces de eliminar los productos de la peroxidación lipídica como hidroxialquenales. Además, determinadas GSTs poseen actividad peroxidasa y son capaces de desactivar los productos de la peroxidación lipídica como bases de ADN oxidadas e hidroperóxidos lipídicos (Ketterer y Christodoulides, 1994).

1.4.5. Mantenimiento del estado tiol-disulfuro intracelular

El GSH es el tiol no proteínico más abundante de las células, resultando imprescindible para mantener el equilibrio redox intracelular y el estado tiólico de las proteínas (DeLeve y Kaplowitz, 1991). Para conseguir esto, el GSH realiza un intercambio tiol-disulfuro con las proteínas catalizado por la tiol-transferasa:

$$\text{proteína-SSG} + \text{GSH} \leftrightarrow \text{proteína-SH} + \text{GSSG}$$

Como esta reacción es reversible, el equilibrio estará determinado por el estado redox celular, el cual depende de las concentraciones de GSH y GSSG.

La reducción de disulfuros de las proteínas también está catalizado por sistemas como la glutaredoxina, tioredoxina y la proteína-disulfuro isomerasa. La glutaredoxina es una proteína semejante a la tioredoxina que para reducirse utiliza el ciclo redox del glutatión (Björnstedt y cols., 1997).

En condiciones fisiológicas normales, el estado redox está mantenido por la GR a expensas del NADPH. Cuando la capacidad reductora de la célula es insuficiente y se disminuye la relación GSH/GSSG, se produce una oxidación del estado redox celular que activa elementos transcripcionales sensibles al estado redox (Sen y Packer, 1996), ya que muchas vías de transducción de señal para la proliferación, diferenciación celular y apoptosis están reguladas por el estado redox celular (Kamata y Hirata, 1999).

Además, la unión del glutatión a proteínas (glutationilación) está implicada en la regulación de la actividad enzimática y la transcripción de enzimas como la fosforilasa, creatina quinasa, anhidrasa carbónica, ras, GST y GADPH (Sies, 1999).

1.4.6. Regulación de la apoptosis

La muerte celular programada o apoptosis es un proceso complejo caracterizado por una reducción del tamaño celular, condensación de la cromatina y fragmentación internucleosomal del ADN que permite la eliminación por fagocitosis de células no deseadas o no funcionales, previniendo la respuesta inflamatoria a sus componentes intracelulares (Hengartner, 2000). No obstante, a mayores condiciones estresantes el deterioro celular puede ser tan alto que la apoptosis se reprima dando lugar a una muerte celular por necrosis, que está caracterizada morfológicamente por un aumento del tamaño celular y finalmente una ruptura de la membrana celular. Esto libera los constituyentes

intracelulares al espacio extracelular causando un posterior deterioro del tejido y una intensa respuesta inflamatoria.

Las caspasas son una familia de proteasas que contienen residuos de cisteína que están presentes en el citosol como proenzimas inactivas pero que se activan cuando se inicia la apoptosis, jugando un papel esencial en varias etapas de ésta (Cohen, 1997). La caspasa-3 es una enzima clave en el desarrollo de la apoptosis, siendo responsable de la degradación parcial o total de muchas proteínas estructurales y reguladoras como la poli(ADP-ribosa) polimerasa (PARP), láminas, citoqueratinas, etc.

La apoptosis está modulada por el estrés oxidativo (Chandra y cols., 2000), donde la actividad de componentes de la misma, como las caspasas y la permeabilidad de las membranas de las mitocondrias, son dependientes del estado redox intracelular (Hall, 1999).

2. OBJETIVOS

En este estudio se pretende determinar los factores bioquímicos que condicionan la tolerancia a plaguicidas en organismos acuáticos y, a partir de ahí, se propone una nueva estrategia terapéutica consistente en incrementar la capacidad antioxidante de las células con el fin de restablecer el equilibrio entre prooxidantes y antioxidantes, deteriorado por una exposición a plaguicidas. Según esto, los objetivos que se han planteado son:

- Establecer la relevancia del glutatión y el metabolismo de éste en la resistencia a plaguicidas organofosforados y carbamatos en vertebrados acuáticos (la anguila europea) e invertebrados marinos (el mejillón y un pectínido).

- Demostrar si la inyección intraperitoneal y baños del antioxidante y precursor del glutatión *N*-acetilcisteína (NAC) producen un aumento de los niveles de glutatión en las anguilas.

- Evaluar la efectividad de la administración de NAC sobre la supervivencia de las anguilas expuestas a una dosis letal del plaguicida organofosforado diclorvós y determinar si está mediada por el metabolismo del glutatión.

- Evaluar la recuperación de las anguilas tras una intoxicación con una dosis subletal de diclorvós mediante la administración de NAC en forma de baños.

3. MATERIAL Y MÉTODOS GENERALES

3.1. Diseño experimental y metodología

3.1.1. Animales experimentales

Los animales de estudio fueron la anguila europea (*Anguilla anguilla*, L.), el mejillón (*Mytilus galloprovincialis* Lam.) y el pectínido *Flexopecten flexuosus* Poli (Fig. 13). La anguila europea pertenece a la Clase Osteictio, Orden Anguilliforme, Suborden Enquelicéfalo y Familia Anguillidae (Lozano, 1947). El mejillón pertenece a la Clase Bivalvia, Orden Mytiloida, Suborden Mytilina y Familia Mytilidae. *Flexopecten flexuosus* pertenece a la Clase Bivalvia, Orden Ostreoida, Suborden Pectinina y Familia Pectinidae (Boss, 1982).

Las anguilas, además de representar una fuente de alimento para el hombre, se utilizan para monitorizar el estado de la contaminación acuática en determinados lugares (Van der Oost, 1996). Las anguilas se obtuvieron de dos fuentes: una población natural de la Albufera de Valencia capturada por la Cofradía de Pescadores del Palmar y anguilas engordadas en la piscifactoría Valenciana de Acuicultura S.A. En ambos casos se trataba de la anguila amarilla, con pesos inferiores a los 30 g, por lo que se trataba de animales indiferenciados sexualmente, evitándose los efectos de la variación sexual y minimizando la interacción hormonal en los ensayos toxicológicos.

Los moluscos bivalvos, y especialmente el mejillón, son organismos ampliamente utilizados en programas de vigilancia y control de la contaminación en el medio marino ya que están distribuidos alrededor de todo el mundo, son especies sedentarias, relativamente tolerantes a un amplio rango de condiciones ambientales y filtradores, capaces de bioacumular en sus tejidos elevadas concentraciones (hasta 1000 veces superior al agua exterior) de productos químicos que no se detectarían en muestras de agua (Widdows y

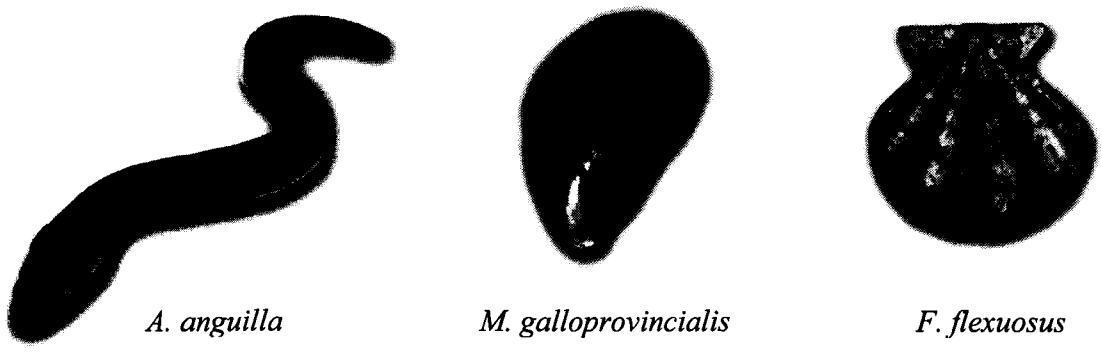


Fig. 13. Fotografías de las especies acuáticas estudiadas.

Donkin, 1992). Además, los bivalvos tienen una gran importancia económica, ya que su cultivo representó el 84% de la producción total de la acuicultura en España en el año 2000 (Ministerio de Agricultura, Pesca y Alimentación), debido principalmente al cultivo extensivo del mejillón en las rías gallegas (79%).

Los mejillones se obtuvieron del cultivo en suspensión de un emparrillado de la Bahía del Fangar en el Delta del Ebro, donde están expuestos a una variación estacional de plaguicidas como se ha comentado anteriormente. Por el contrario, la especie de pectínido *F. flexuosus* se distribuye al oeste y centro del Mediterráneo, y el área adyacente del Océano Atlántico en fondos arenosos y de algas (Wagner, 1991). La semilla de este pectínido se obtuvo mediante la recolección de colectores filamentosos fondeados durante 3 meses (de junio a septiembre de 1998) cerca del arrecife artificial de Cabanes (40° 07' N, 0° 13' E) a una profundidad de 20 m, según Peña y cols. (1998).

3.1.2. Plaguicidas utilizados

Los plaguicidas que se han estudiado y sus correspondientes aplicaciones se describen a continuación (Fig. 14):

El **tiobencarb** (*S*-4-clorobencil dietiltiocarbamato) es un herbicida selectivo que inhibe el crecimiento de los brotes de plantas emergentes por inhibición de la síntesis de proteínas. Este plaguicida tiocarbamato se utiliza para controlar *Echinochloa*, *Leptochloa* y *Cyperus* spp., además de otras monocotiledoneas, en el cultivo del arroz (Tomlin, 1997).

El **molinato** (*S*-etil azepan-1-carbotioato) es un herbicida sistémico selectivo que se absorbe rápidamente por las raíces, se transporta hasta las hojas e inhibe la germinación por inhibición del metabolismo lipídico (Tomlin, 1997). Este plaguicida tiocarbamato se emplea para controlar la germinación de malas hierbas, particularmente *Echinochloa* spp., en el cultivo del arroz.

El **fenitrotión** (*O,O*-dimetil *O*-4-nitro-*m*-tolil fosforotioato) es un insecticida organofosforado no sistémico de acción por contacto o ingestión que inhibe a las colinesterasas. Se utiliza para el control de los insectos en los cultivos de cereales, frutas, hortalizas, arroz, caña de azúcar, vid, césped y en silvicultura. También se utiliza como un insecticida de uso doméstico, sobre productos almacenados y en el control de las larvas de mosquito, que son vectores de la malaria en determinados países (Tomlin, 1997).

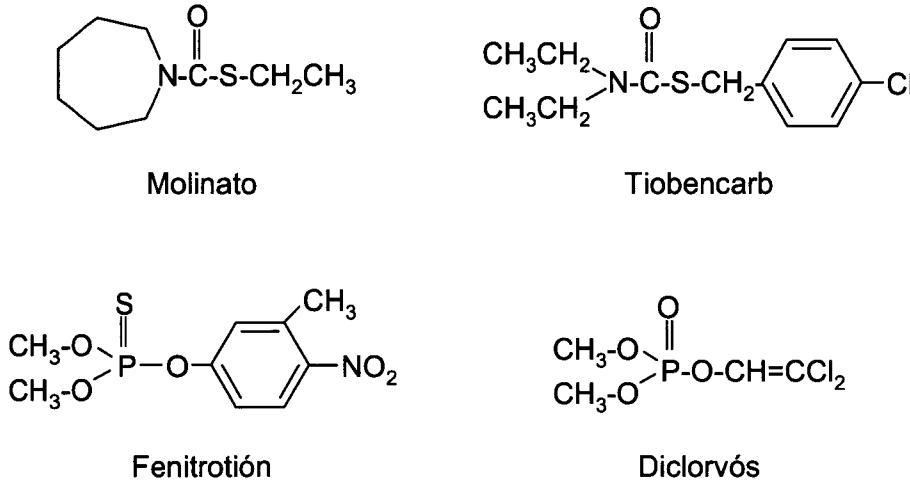


Fig. 14. Estructura química de los plaguicidas estudiados.

El **diclorvós** (2,2-diclorovinil dimetil fosfato) es un insecticida y acaricida de acción por contacto, inhalación e ingestión que inhibe a las colinesterasas. Este plaguicida organofosforado, además de aplicarse en la agricultura y en el hogar como el fenitrotión, se utiliza en veterinaria para tratar parásitos externos, como pulgas y garrapatas, y como antihelmíntico del ganado, animales domésticos y peces de piscifactoría (OMS, 1989).

3.1.3. Ensayos de toxicidad aguda

La evaluación más común de la toxicidad es la medida de la letalidad a corto plazo. Para una sustancia dada esta medida implica la determinación de la concentración media que es letal para una proporción fija, normalmente el 50% de la población de organismos ensayados, después de una exposición continua durante un periodo de tiempo concreto, generalmente 96 h en peces (Johnson y Finley, 1980).

La concentración letal (CL) para el 50% de la población después de una exposición de 96 h se designa como CL_{50}-96 h. Esta abreviatura puede modificarse adecuadamente para hacer referencia a distintos tiempos de exposición y a distintas proporciones de la población.

Los ensayos de toxicidad aguda con anguilas se realizaron de acuerdo a las directrices de la OECD (1992). Para determinar la CL_{50} se utilizaron de 4 a 6 concentraciones diferentes de tóxico, 10 animales por concentración y se efectuaron 3 réplicas del ensayo para obtener una representación de la curva dosis-respuesta estadísticamente más precisa

(Ferrando, 1990). Los ensayos con los herbicidas tiocarbamatos molinato y tiobencarb se realizaron mediante ensayos estáticos con anguilas procedentes de la Albufera de Valencia, en los que los animales estuvieron durante todo el periodo de experimentación en la misma solución de plaguicida, ya que el tiobencarb tiene una vida media de 247,66 h en condiciones de laboratorio, permaneciendo el 85% del plaguicida después de 96 h (Ferrando, 1992). Como el diclorvós se hidroliza rápidamente por los álcalis en el agua, con un tiempo de vida medio de 31,9 días a pH 4, 2,9 días a pH 7 y 2,0 días a pH 9 (Tomlin, 1997), los ensayos con diclorvós en anguilas procedentes de la empresa Valenciana de Acuicultura S.A. se realizaron mediante ensayos de renovación, en los que el agua y el plaguicida eran renovados cada 24 h.

Los ensayos de toxicidad aguda en los mejillones obtenidos de un emparrillado de la Bahía del Fangar y los pectínidos capturados a 20 m de profundidad frente a la costa de Castellón se realizaron según los criterios de la U.S. Environmental Protection Agency (USEPA, 1996a y b) mediante ensayos estáticos con el insecticida organofosforado fenitrotión, ya que este plaguicida es relativamente estable a la hidrólisis en condiciones normales (Tomlin, 1997).

3.2. Análisis bioquímico

En la presente tesis se han puesto a punto varias técnicas de determinación de metabolitos y actividades enzimáticas mediante el lector espectrofotométrico de placas de 96 pocillos (Bio-Rad 3550, Bio-Rad Laboratories, Richmond, CA, EE.UU. y Multiskan Ascent, Labsystems, Helsinki, Finlandia). Además se han desarrollado varias plantillas de Microsoft® Excel que permiten resumir rápidamente en una sola página toda la información disponible, por lo que se consigue una mayor automatización y control en el procesamiento de elevadas muestras.

3.2.1. Determinación del glutatión

El contenido de glutatión total (GSH + GSSG) y únicamente el oxidado (GSSG) se determinó mediante un ensayo sensible y específico del GSH con el ácido 5,5'-ditiobis-2-nitrobenzoico (DTNB, reactivo de Ellman) en presencia de GR y NADPH en exceso según el método de Tietze (1969) (Fig. 15) y adaptado a microplacas por Baker y cols. (1990).

Los tejidos se homogenizaron con 5 volúmenes del ácido 5-sulfosalicílico al 5% por gramo de tejido para precipitar las proteínas, se sonicaron y centrifugaron a 4 °C y 20.000*g* durante 10 minutos. En los sobrenadantes se determinó el glutatión total directamente o se utilizó 2-vinilpiridina para conjugar al GSH en la determinación del GSSG (Griffith, 1980). Todas las medidas se realizaron a 415 nm con longitud de onda de referencia de 595 nm cada 30 segundos durante 3 minutos, como recomiendan Cribb y cols. (1989). Las muestras para la determinación de GSSG fueron cuidadosamente verificadas con papel indicador para evitar sobrepasar el pH 7.5 y producir la autoxidación del GSH. Las concentraciones de glutatión se expresaron como nmoles de equivalentes de GSH (GSx) por gramo de peso de tejido fresco o mg de proteína (GSx = [GSH] + 2·[GSSG]). El contenido en GSH se calculó al restar el valor de GSSG del de glutatión total. La razón GSH/GSSG se expresó como número de moléculas y no moles: [GSH]/[GSSG] = (GSx – GSSG) / (GSSG / 2). Los estándares y blancos se prepararon simultáneamente con las muestras en las mismas condiciones de tamponamiento y acidez.

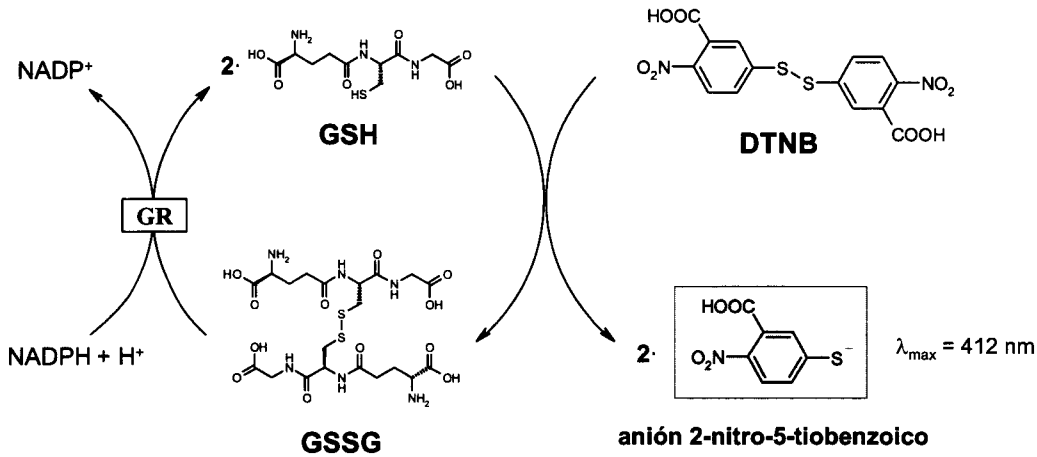


Fig. 15. Esquema de la reacción del GSH con el DTNB para dar un producto de color amarillo con un máximo de absorción a 412 nm.

3.2.2. Determinación cinética de las actividades enzimáticas

La actividad enzimática se puede medir mediante ensayos continuos o por punto final. En estos últimos la reacción enzimática se detiene después de un tiempo de incubación como consecuencia de la desnaturalización de la enzima por inmersión en un baño de agua hirviendo o por adición de un ácido o una base fuerte. Esto es ideal para ensayos enzimáticos no espectroscópicos en los que el tiempo de medida es mayor que el ritmo de

la reacción enzimática, como sería la determinación mediante un método de cromatografía líquida de alta resolución (HPLC). Sin embargo, a pesar de que resulta mucho más fácil realizar una lectura inicial y otra final que realizar múltiples medidas durante la reacción, el método de punto final asume peligrosamente que la señal evolucionará linealmente con el tiempo durante el periodo de incubación elegido. Así, cambios en la temperatura, pH, la concentración de sustrato, de enzima, o la presencia de ciertos inhibidores pueden alterar dramáticamente la linealidad de la señal. Por esta razón la determinación de la actividad enzimática por cinética es la manera más segura y exacta de calcular la velocidad de reacción a partir de la pendiente de la absorbancia respecto al tiempo, y además se obtiene, en general, una menor variabilidad (Copeland, 1996).

La actividad **AChE** (EC 3.1.1.7) se determinó a 415 nm con acetiltiocolina como sustrato según una adaptación a microplaca del método de Ellman (Ellman y cols., 1961) por Doctor y cols. (1987), pero con tampón fosfato 0,1 M, pH 7,27 y EDTA 1 mM como recomiendan Riddles y cols. (1979). La actividad colinesterasa detectada en el músculo y cerebro de las anguilas se trata realmente de AChE, como previamente caracterizaron Lundin (1962) y Ferenczy y cols. (1997), por lo que no fue necesario realizar el ensayo con inhibidores específicos.

La actividad **GCL** (EC 6.3.2.2) se adaptó a microplacas a partir del método indirecto de Seelig y Meister (1985), que utilizan la reacción acoplada de la piruvato kinasa (PK) con la lactato deshidrogenasa (LDH) para determinar la formación de ADP por la GCL a través de la oxidación del NADH. Cada pocillo contenía tampón Tris-HCl 0,1 M, pH 8, KCl 150 mM, EDTA 2 mM, $MgCl_2$ 20 mM, ATP 5 mM, fosfoenolpiruvato 2 mM, L-glutamato 10 mM, L-α-aminobutirato 10 mM, NADH 0,2 mM, 7 U/ml de PK y 10 U/ml de LDH. La actividad enzimática fue evaluada siguiendo el descenso en absorbancia del NADH a 340 nm y 25 °C.

La actividad **GR** (EC 1.6.4.2) se analizó por el método Cribb y cols. (1989) a través del aumento de absorbancia a 415 nm con 595 nm de longitud de onda de referencia y con las siguientes concentraciones finales: [DTNB] = 0.075 mM; [NADPH] = 0.1 mM; [GSSG] = 1 mM según Smith y cols. (1988).

La actividad **GST** (EC 2.5.1.18) se ensayó por la conjugación del GSH con 1-cloro-2,4-dinitrobenceno (CDNB) según Habig y cols. (1974). La mezcla de reacción contenía tampón fosfato potásico 100 mM, pH 6,5, CDNB 1 mM en etanol y GSH 1 mM. La formación del aducto del CDNB, *S*-2,4-dinitrofenil glutatión, se siguió por el incremento de absorbancia a 340 nm.

La actividad **γGT** (EC 2.3.2.2) se midió por el índice de escisión de la γ-glutamil-*p*-nitroanilida para formar *p*-nitroanilina (pNA) a 405 nm durante al menos 10 minutos, por transferencia del grupo glutamil a la glicilglicina, siguiendo el método de Silber y cols. (1986).

3.2.3. Determinación de la actividad caspasa-3

La actividad caspasa-3 (EC 3.4.22.-) se midió en el lector de placas a partir del ensayo colorimétrico manufacturado por Sigma. La hidrólisis del péptido acetil-Asp-Glu-Val *p*-nitroanilida (Ac-DEVD-pNA) para liberar pNA se siguió a 405 nm y se calculó en cada microplaca a partir de una recta de calibrado de pNA, cuyas concentraciones se determinaron en un espectrofotómetro.

Para determinar actividades enzimáticas extremadamente bajas (del orden de pmoles de Ac-DEVD-pNA hidrolizado por min y mg de proteína), las microplacas se incubaron varios días a 25 °C selladas y exentas de luz. Una cinética de pseudo orden cero se comprobó al representar gráficamente la absorbancia respecto al tiempo en cada pocillo. Para validar los resultados se utilizó caspasa-3 recombinante humana. La actividad DEVDasa medida se consideró pseudo-caspasa-3 ya que la caspasa-7 es otro ejecutor de la apoptosis que tiene una función y especificidad de sustrato similar a la caspasa-3 (Fernandes-Alnemri y cols., 1995).

3.2.4. Determinación de proteínas

Las actividades enzimáticas y el contenido en glutatión (Artículos IV y V) se expresaron en relación al contenido en proteínas de cada muestra para estandarizar los valores. El contenido de proteínas se ha determinado por el kit de Bio-Rad basado en el procedimiento colorimétrico de Bradford (1976), usando albúmina sérica bovina como estándar.

3.3. Análisis estadístico

El tratamiento estadístico de los experimentos realizados se detalla en cada uno de los artículos. La relación de las variables con la supervivencia se analizó inicialmente

mediante el análisis de la varianza (Artículo I y II), pero posteriormente (Artículos III y IV) se utilizaron estadísticos más robustos como el modelo de regresión de riesgos proporcionales de Cox (Cox, 1972), que merece ser comentado con más detalle, al igual que el método probit para tratar los datos obtenidos de los ensayos de toxicidad aguda.

3.3.1. Análisis probit

Este método se basa en la hipótesis de la existencia de una relación dosis-efecto de manera que los efectos observados se distribuyen de forma proporcional a los logaritmos de las concentraciones utilizadas en el ensayo (Gaddum, 1953).

En el análisis probit en lugar de hacer la regresión de la proporción de los organismos muertos respecto a las concentraciones de plaguicida, se transforma cada una de las proporciones observadas por el valor de la curva normal estándar bajo la cual se encuentra el área de la proporción observada y, generalmente, se utilizan los logaritmos de las concentraciones. El modelo de regresión se puede entonces representar como:

$$\text{Probit}\,(P_i) = A + B\cdot[\log(\text{concentración}_i)]$$

donde P_i es la transformación probit de la proporción de organismos muertos a una concentración$_i$ de plaguicida, y A y B son la ordenada en el origen y la pendiente, respectivamente.

En principio, la representación gráfica del probit respecto al logaritmo de la concentración debe ser lineal para poder determinar la concentración que produce un 50% de mortalidad en los organismos ensayados, o cualquier otro porcentaje. Sin embargo, si se tienen datos muy heterogéneos o dispersos, se cometería un error al ajustar los datos a una recta. Por esa razón se utiliza el método de la bondad del ajuste de χ^2 basado en los residuos para comprobar que el modelo probit se ajusta a los datos obtenidos para un nivel de confianza del 95%.

La CL_{50} del molinato en las anguilas se calculó mediante un programa informático de análisis probit (Artículo II). La CL_{50} del fenitrotión para los bivalvos marinos se calculó mediante el USEPA Probit Análisis Program 1.5 (Artículo III). La CL_{50} del diclorvós para las anguilas se determinó mediante el paquete estadístico SPSS 10.0 (Artículo IV).

3.3.2. Regresión de Cox

Este es un método de regresión utilizado para evaluar el efecto de múltiples variables independientes predictoras (covariables) en la curva de supervivencia. Aunque este modelo no asume una distribución matemática en particular, sí que asume que los efectos de las diferentes covariables sobre la supervivencia no cambian respecto al tiempo. La razón de riesgo estima el porcentaje de cambio en el riesgo que suceda el evento estudiado (en este caso la muerte en presencia del plaguicida) por cada cambio de unidad en la covariable. Para variables dicotómicas la razón de riesgo representa el riesgo relativo (RR) de morir entre las dos clases de la variable: 0 y 1. Cuando el riesgo relativo es mayor que 1, el riesgo de morir aumenta cuando la covariable aumenta, mientras que cuando el riesgo relativo es menor que 1, el riesgo de morir disminuye cuando aumenta la covariable.

Las razones de riesgo individuales se obtuvieron al incluir únicamente cada variable en el modelo de Cox. Posteriormente se obtuvieron las variables predictoras (covaribles) más relacionadas con la supervivencia al plaguicida mediante un procedimiento de selección por pasos sucesivos hacia delante de todas las variables estudiadas basado en la estimación condicional de los parámetros. Los límites para la inclusión y exclusión de cada variable en el modelo de Cox fueron, respectivamente, de $P \leq 0.05$ y $P > 0.10$. Las covariables se trataron como variables continuas, pero en el Artículo III también fueron dicotomizadas a la mediana. Esto quiere decir que se asignó el valor cero a los bivalvos que para cada variable presentaron un valor inferior a la mediana, mientras que a los que tuvieron un valor mayor que la mediana se les asignó un uno.

La suposición de riesgos proporcionales se aseveró por la inspección visual de las gráficas de supervivencia para el log-menos-log de cada una de las covariables tratadas como variables categóricas, las gráficas de los residuos parciales de Schoenfeld (Schoenfeld, 1982) respecto al tiempo de supervivencia, según Hess (1995), y las gráficas de los residuos martingala respecto las covariables.

4. RESULTADOS Y DISCUSIÓN GENERAL

4.1. Caracterización del metabolismo del glutatión en la tolerancia a plaguicidas en la anguila europea

En varios trabajos (Artículos I, II y IV) se ha puesto de manifiesto la importancia del metabolismo del glutatión en la tolerancia/resistencia a plaguicidas organofosforados y carbamatos en la anguila europea (*A. anguilla*). Inicialmente se observó que la exposición de las anguilas provenientes de la Albufera de Valencia al herbicida tiocarbamato tiobencarb (Artículo I) disminuía los niveles de glutatión en músculo e hígado, y que aquellos individuos que sobrevivían más tiempo en presencia del plaguicida presentaban niveles hepáticos de GSH tres veces mayores que los controles, por lo que habrían inducido su síntesis. Además, se producía una oxidación del glutatión conforme aumentaba el tiempo de exposición.

Cuando las anguilas se expusieron al herbicida tiocarbamato molinato (Artículo II), también presentaron una disminución del contenido hepático y muscular de glutatión, tal y como describen otros autores en peces intoxicados con amonio (Chatterjee y Bhattacharya, 1983), mercurio (Allen y cols., 1988), cadmio (Vaglio y Landriscina, 1999), dietil maleato (Gallagher y cols., 1992), menadiona (Hasspieler y cols., 1994) y contaminación en general (Almar y cols., 1998). La disminución de los niveles de GSH y la interacción de ciertos contaminantes que generan estrés oxidativo con la GCL conduce a una inducción de la síntesis hepática de este tripéptido. Si esta activación se mantiene varios días, el contenido de GSH en el hígado puede aumentar por encima de los niveles normales. Así, se observó que, al igual que las anguilas expuestas al tiobencarb (Artículo I), aquellas que sobrevivieron más de 72 h a una concentración letal del molinato contenían mayor cantidad de GSH hepático que las anguilas no expuestas. Este aumento en el contenido de GSH como consecuencia de la exposición a contaminantes también se observó en peces expuestos a sedimentos contaminados con hidrocarburos aromáticos policíclicos y poliaromáticos (Stein y cols., 1992) y a menadiona (Hasspieler y cols., 1994).

La exposición a molinato produjo una oxidación en el estado redox del glutatión muscular, y únicamente en el hígado de las anguilas que murieron durante las primeras 48 h. Como las anguilas que sobrevivieron más tiempo tenían mayor actividad GR, especialmente a nivel hepático, podían reducir el GSSG a GSH más eficientemente y mantener los niveles de GSH/GSSG. De esta manera, aquellas anguilas que fueron capaces de incrementar el contenido en glutatión, inducir la actividad GR y mantener la relación entre los niveles de glutatión reducido y oxidado en el hígado, mostraron una mayor

supervivencia a estos plaguicidas que aquellas anguilas que perdieron la homeostasis del glutatión.

La toxicidad de los herbicidas tiocarbamatos molinato y tiobencarb, al igual que otros carbamatos, es debida a la inhibición de la enzima AChE (Sancho y cols., 2000; Fernández-Vega y cols., 2002). Sin embargo, este modo de acción no es suficiente para explicar la severa anemia hemorrágica observada en las carpas (Kawatsu, 1977) y anguilas (Sancho y cols., 2000) por el molinato. Esto podría ser debido al estrés oxidativo generado por estos herbicidas ya que una depleción de glutatión resulta en una mayor permeabilidad y menor funcionalidad de la membrana de los eritrocitos hasta producir una hemorragia hemolítica (Uhlig y Wendel, 1992).

Como ya se ha comentado anteriormente, los niveles de glutatión están regulados por varias enzimas bien caracterizadas (Meister y Anderson, 1983). Por lo tanto, el contenido de GSH en las células no es un valor constante ya que depende del equilibrio entre el grado de síntesis (por la GCL), el grado de conjugación (por las GSTs), el grado de oxidación (espontáneamente o catalizado por la GPx) y la capacidad de reducción del GSSG a GSH (por la GR).

La conjugación del GSH sobre muchos plaguicidas, como los tiocarbamatos estudiados, catalizada de las GSTs, conduce a la destoxificación y excreción del xenobiótico. No obstante, si este proceso no está asociado a una síntesis de GSH *de novo* por la GCL, el GSH se puede agotar. Además, como el GSH es capaz de eliminar radicales libres a costa de oxidarse a GSSG tanto de manera espontánea como catalizado por la GPx, si la generación de GSSG es mayor que la reducción a GSH por la GR, el GSSG se puede acumular en el citosol. A partir de determinadas concentraciones, el GSSG es transportado fuera de las células por transportadores específicos (Sies y cols., 1972; Kaplowitz y cols., 1996) para proteger las células de un cambio en el estado redox. Consecuentemente esto implica una disminución en el 'pool' de glutatión.

En principio, una merma de los niveles de GSH únicamente del citosol no implica un daño sobre las células ya que las mitocondrias retienen al GSH por transportadores de alta afinidad (Mårtensson y cols., 1990). No obstante, si se agota el GSH a nivel citosólico y, además, existe un estrés oxidativo, el 'pool' de GSH de la mitocondria se oxida y disminuye. Como la mitocondria es incapaz de exportar GSSG (Olafsdottir y Reed, 1988) y sintetizar GSH (Griffith y Meister, 1985), este orgánulo es más susceptible que el resto de la célula a las consecuencias de una oxidación y diminución del glutatión (DeLeve y Kaplowitz, 1991).

No obstante, los procesos moleculares desde el principio del estrés oxidativo hasta la muerte del organismo aún son inciertos. Carlson y cols. (2000) sugirieron que la citotoxicidad de los compuestos organofosforados podría deberse a la inducción de la muerte celular programada (apoptosis) por vías multifuncionales. Domenicotti y cols. (2000) encontraron una vía de apoptosis que era dependiente de la depleción de glutatión mediante una oxidación en el estado redox celular. Como muchas moléculas de señalización celular (tales como proteína tirosinas quinasas, serina/treonina quinasas y fosfatasas, canales y transportadores de Ca^{2+} y factores de transcripción como NF-κB, *p*53 y AP-1) están reguladas por el estado redox de la célula, cualquier cambio en el potencial redox es esperable que altere las respuestas transcripcionales modificando el comportamiento celular y viceversa (Kamata y Hirata, 1999). Además, según Schafer y Buettner (2001), el estado redox del glutatión es indicativo del estado biológico celular, pasando por la proliferación, diferenciación, apoptosis y, a relaciones de GSH/GSSG muy bajas, necrosis.

Por otra parte, Voehringer y cols. (2000) mediante chips de ADN encontraron que las células resistentes a la apoptosis activaban vías metabólicas para establecer y mantener un alto estado redox intracelular del glutatión. Entonces, la capacidad individual de aumentar las actividades enzimáticas de la gluconeogenesis y de la vía de las pentosas fosfato también determinaría la tolerancia al plaguicida. De hecho, los niveles de GSH y la resistencia al estrés oxidativo se ha encontrado que están modulados por varios de estos enzimas, tales como la glucosa-6-fosfato deshidrogenasa (Salvemini y cols., 1999), la transaldolasa (Banki y cols., 1996) y la isocitrato deshidrogenasa dependiente de $NADP^+$ (Lee y cols., 2002). Además, Godon y cols. (1998), mediante proteómica establecieron que de las más de 100 proteínas cuyos niveles cambiaron después de someter a la levadura (*Saccharomyces cerevisiae*) a estrés oxidativo, una cuarta parte estaban involucradas en la represión de la glucólisis y el ciclo del ácido tricarboxílico para redirigir el metabolismo de los carbohidratos hacia la regeneración de NADPH en vez de ATP.

Así, en los Artículos I y II se demuestra que el principal modo de toxicidad de los herbicidas tiocarbamatos es la generación de radicales libres (posiblemente por desacoplamiento de la cadena de transporte de electrones), que en exceso conducirían a una situación de estrés oxidativo, seguida probablemente de una inducción de la muerte celular, tanto por apoptosis como por necrosis, y finalmente la muerte del organismo.

Posteriormente se profundizó en los mecanismos moleculares que condicionaban la tolerancia a los plaguicidas (Artículo IV). Asimismo, además de poner a punto la

determinación de las enzimas glutatión *S*-transferasa (GST) y glutamato:cisteína ligasa (GCL), se puso a punto la determinación de la actividad caspasa-3, que es una enzima que una vez activada desencadena la muerte celular programada (apoptosis). Se observó entonces que la exposición a una dosis letal del plaguicida organofosforado diclorvós generaba tal cantidad de estrés oxidativo que inhibía la actividad caspasa-3, inactivando la apoptosis a costa de producir necrosis (Fig. 16). Las caspasas contienen residuos de cisteína en su dominio catalítico (Wilson y cols., 1994), lo que las hace susceptibles a la oxidación o la alquilación de tioles, y por lo tanto, están reguladas por el estrés oxidativo y el estado redox celular (Ueda y cols., 1998). La inhibición de la actividad caspasa-3 por un excesivo estrés oxidativo puede causar un cambio de apoptosis a necrosis en el hígado tal y como está descrito en líneas celulares (Lemaire y cols., 1998; Samali y cols., 1999).

La supervivencia de las anguilas al diclorvós dependía principalmente del GSH muscular, el estado redox del glutatión hepático y la actividad AChE del músculo y GR hepática y muscular.

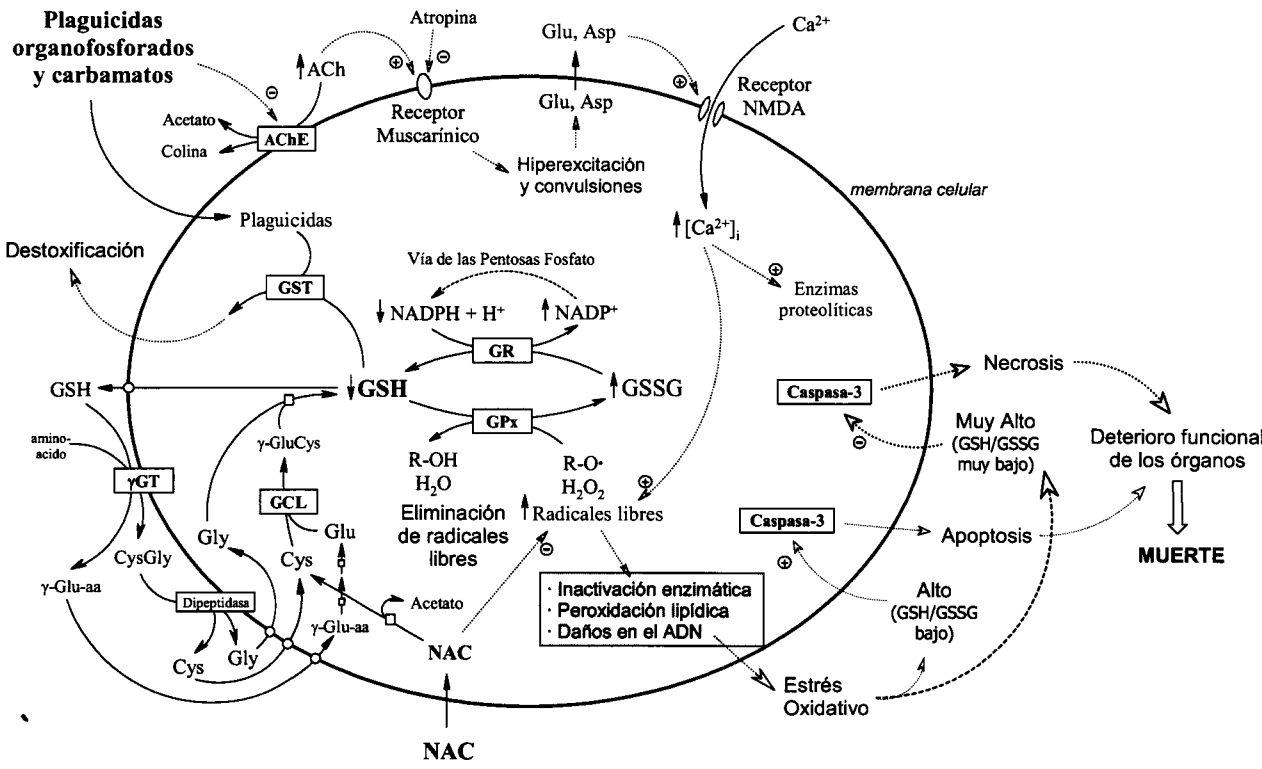


Fig. 16. Esquema propuesto de la toxicidad de los plaguicidas organofosforados y carbamatos en relación con el metabolismo del glutatión.

La tolerancia a contaminantes generalmente sigue una distribución log-normal (como asume el análisis probit para las relaciones dosis-respuesta). Dos hipótesis se propusieron para explicarlo. Gaddum (1953) introdujo el concepto de la tolerancia individual o dosis efectiva individual, donde cada individuo tiene un 'background' innato a tolerar compuestos tóxicos hasta que recibe una dosis igual o mayor que su dosis efectiva individual, y entonces muere. Por el contrario, la mortalidad puede ser el resultado de los procesos estocásticos que ocurren en todos los individuos con la misma probabilidad de morir al recibir una dosis. La primera hipótesis ha permanecido universalmente aceptada hasta que hace poco Newman y McCloskey (2000) encontraron que la tolerancia a concentraciones letales de tóxicos no cumplía ninguna de las dos hipótesis. Por tanto, según la información aportada en este estudio, se propondría una hipótesis intermedia, en la que la tolerancia a los plaguicidas estaría mediada por la capacidad de mantener al glutatión en estado reducido. Cuando esta homeostasis se pierde, se produciría una oxidación celular que desencadenaría una cascada de transducciones de señal, que según la cantidad de plaguicida esto resultaría en una muerte celular por apoptosis o por necrosis, que de no restablecerse las condiciones iniciales podría derivar a la muerte (Fig. 16).

4.2. Papel del glutatión en la tolerancia al plaguicida organofosforado fenitrotión en bivalvos marinos

En primer lugar se determinaron la CL_{50} y CL_{85} de los mejillones cultivados en las bahías del Fangar del Delta del Ebro (Tarragona) y se compararon con las de semillas naturales del pectínido *F. flexuosus* capturadas a 20 m de profundidad frente a la costa de Torre la Sal (Castellón) (Artículo III). Así, los mejillones no sólo tuvieron más del doble de la CL_{50} respecto a los pectínidos (8,4 frente a 3,9 mg/l de fenitrotión) sino que también una mayor supervivencia frente a una concentración letal (la respectiva CL_{85}) de este insecticida. Esto demuestra que los mejillones son más resistentes al fenitrotión que *F. flexuosus*.

En general, los mejillones expuestos al organofosforado mostraron una disminución del contenido de GSH y GSSG en la glándula digestiva, músculo aductor y branquias, mientras que las semillas de pectínidos presentaron una disminución de GSH en la glándula digestiva y manto, además de una oxidación del estado redox del glutatión en las branquias y el manto. Esta depleción del glutatión también la han encontrado otros autores

en bivalvos expuestos a hidrocarburos aromáticos policíclicos (Suteau y cols., 1988), metales pesados (Viarengo y cols., 1990; Regoli y Principato, 1995; Regoli y cols., 1997; Canesi y cols., 1999) y contaminación en general (Doyotte y cols., 1997; Cossu y cols., 2000).

El efecto de la depleción del glutatión en bivalvos marinos es muy parecido al de los vertebrados, pues una disminución de GSH a nivel únicamente citosólico no presenta efectos tóxicos en las ostras (*Crassostrea virginica*), mientras que si se acompaña de una exposición a cobre (que es un generador de radicales libres) se produce estrés oxidativo (Conners y Ringwood, 2000). Sin embargo, mientras que en *F. flexuosus* la supervivencia al fenitrotión es debida principalmente al estado redox del glutatión en la glándula digestiva y músculo y al GSH del hepatopáncreas, en el mejillón las variables que más condicionaron la supervivencia al plaguicida fueron el GSH y el estado redox del glutatión en el músculo. Esto contrasta con lo obtenido con peces (Artículos I, II y IV), donde el hígado es el órgano donde el glutatión juega un papel más relevante.

4.3. Biomarcadores de exposición, efecto y susceptibilidad individual en vertebrados e invertebrados

La disminución del GSH está considerado como un biomarcador de exposición al estrés ambiental, tanto debido a contaminantes naturales como químicos. Sin embargo, la depleción del GSH hepático de las anguilas como consecuencia de la exposición a los plaguicidas estudiados (Artículos I, II y IV) puede inducir su síntesis hasta alcanzar valores incluso por encima de los peces no expuestos, como se ha observado en los Artículos I y II, y se ha comentado previamente. Por otra parte, la disminución del GSH muscular parece ser un factor intrínseco de la exposición a plaguicidas, ya que cuanto mayor es la exposición, menores son sus valores. Como los niveles de glutatión en el músculo dependen ampliamente de la captura del GSH plasmático por la γGT (Griffith y Meister, 1979), si hay una depleción de GSH hepático, menos glutatión será exportado al plasma (Kaplowitz y cols., 1985) y estará disponible para los tejidos extrahepáticos, permitiendo el consumo del GSH muscular. Así, la regeneración de los niveles de GSH en el músculo es menos probable que en el hígado, por lo que la disminución del GSH muscular puede ser

mejor biomarcador de exposición a plaguicidas (y contaminantes en general) que el GSH hepático.

Por otra parte, la supervivencia de las anguilas estaba relacionada con los niveles de GSH, el estado redox del glutatión, la actividad AChE, GR, GCL y caspasa-3, por lo que se podrían utilizar como biomarcadores de efecto y susceptibilidad individual a los plaguicidas estudiados y otros contaminantes que induzcan el estrés oxidativo, ya que las anguilas que no fueron capaces de mantener el estado reducido del glutatión presentaron una mayor mortalidad. Además, de manera específica, la relación GSH/GSSG y la actividad GR se confirman como biomarcadores de estrés oxidativo.

De la misma manera, la depleción de glutatión en los bivalvos marinos se puede utilizar como un biomarcador de exposición a la contaminación. Sin embargo, Sheehan y Power (1999) advierten que los biomarcadores pueden sufrir variaciones estacionales debido a factores naturales como la temperatura, la disponibilidad de comida y el estado reproductivo. De hecho, el GSH sigue una variación estacional en la glándula digestiva de los mejillones, al igual que otros antioxidantes, presentando unos valores más bajos en invierno cuando hay menos comida disponible y las gónadas descansan (Viarengo y cols., 1991, Porte y cols., 2000). Sin embargo, la relación GSH/GSSG es menos probable que presente estas variaciones, ya que sus niveles están regulados por el metabolismo del glutatión. Además, el estado redox del glutatión presentaba una fuerte asociación con la supervivencia al fenitrotión que parecía ser independiente de la especie, posible adaptación a los plaguicidas y el estado de desarrollo de los mejillones y *F. flexousus*. Por consiguiente, el estado redox del glutatión muscular se puede considerar como un biomarcador de efecto y susceptibilidad individual al fenitrotión y a otros contaminantes que produzcan un estrés oxidativo.

4.4. Efecto de la administración intraperitoneal de NAC en la tolerancia al diclorvós en las anguilas

El plaguicida organofosforado diclorvós se ha utilizado considerablemente en acuicultura para tratar infecciones de parásitos externos en peces. La inyección intraperitoneal de 1 mmol de *N*-acetilcisteína (NAC) por kg de peso corporal produjo un aumento en el contenido de GSH hepático y muscular, la relación GSH/GSSG hepática y

las actividades GCL y GR del hígado y la GST hepática y muscular de las anguilas (Artículo IV).

El diclorvós se metaboliza principalmente en el hígado mediante dos vías enzimáticas: una dependiente de glutatión produce desmetildiclorvós, mientras que otra independiente de glutatión da lugar a dimetilfosfato y dicloroacetaldehído (Dicowsky y Morello, 1971). Por lo tanto, la disponibilidad de GSH puede ser un factor limitante en la eliminación del plaguicida.

La inyección de NAC facilitó la eliminación del propio diclorvós y los radicales libres generados por él. Así, estas anguilas no sólo tuvieron una mayor supervivencia al plaguicida que las que fueron inyectadas con salino únicamente, sino que también la oxidación y la disminución de los niveles de glutatión, además de la inhibición enzimática, fue menor que en los peces no tratados.

La administración de NAC producía un máximo de cantidad de GSH en el hígado 12 horas después de la inyección, lo que correspondía también con las 9 horas tras la exposición de las anguilas al plaguicida, que es el momento a partir del cual las anguilas empezaron a morir y se obtuvo una mayor mortalidad. Como las anguilas tratadas con NAC contenían niveles más altos de GSH en el hígado, y este órgano es el más importante en la destoxificación de xenobióticos y radicales libres, esto se tradujo en una mayor supervivencia de las anguilas. Esta supervivencia dependía principalmente de la capacidad individual de no perder la homeóstasis del glutatión en el hígado al mantener y aumentar la relación GSH/GSSG hepática y sintetizar GSH por la GCL. La supervivencia era menos dependiente en las actividades AChE y GST musculares y GR hepática como en los animales tratados con salino. A pesar de que las anguilas tratadas con NAC presentaron mayor actividad GST en el hígado, que les permitiría eliminar al plaguicida más eficientemente, esta variable resultó no ser tan importante como las otras para explicar la mortalidad.

Aunque los peces tratados con NAC tuvieron la mitad de probabilidad de morir en las 96 h de seguimiento comparado con los no tratados, la supervivencia al diclorvós estaba condicionada esencialmente por el estado redox del glutatión hepático.

Estos resultados son muy informativos, pues abren la posibilidad del tratamiento y prevención de intoxicaciones con plaguicidas en peces. No obstante, su aplicación es poco práctica ya que la vía intraperitoneal es inviable a escala industrial, por lo que también se ha probado administrar el fármaco mediante baños.

4.5. Efecto de los baños de NAC en la recuperación de las anguilas tras la intoxicación con plaguicidas

En el Artículo V la NAC se administró en forma de baños después de una etapa de exposición al plaguicida diclorvós. La exposición subletal de las anguilas durante 96 h a 0,17 mg/l de diclorvós (1/5 de la CL_{50}, la concentración que elimina a la mitad de la población en 96 h) disminuyó y oxidó el contenido de glutatión muscular, disminuyendo la relación GSH/GSSG, lo cual es indicativo de un proceso de estrés oxidativo. Además, las actividades AChE y GR del cerebro estuvieron altamente inhibidas. Estos resultados son consistentes con los datos de Hai y cols. (1997), que encontraron una disminución del GSH hepático y muscular en la carpa común (*Cyprinus carpio*), e inhibición de la actividad AChE del cerebro después de estar los peces expuestos a 1 y 5 mg/l de diclorvós durante 24 h.

La relación entre la inhibición de la AChE y la mortalidad depende de la especie y la edad, pero normalmente una inhibición de la AChE del cerebro mayor del 70% está asociada a la mortalidad (Fulton y Key, 2001). En el Artículo V la inhibición de la AChE en el cerebro aumentó con el tiempo de exposición del plaguicida hasta el 80% de inhibición respecto a las anguilas no expuestas al diclorvós, pero no se observó mortalidad. Sancho y cols. (1997) encontraron un 64% de inhibición en la AChE del cerebro de las anguilas expuestas a una concentración subletal de fenitrotión. Éste es un inhibidor de las colinesterasas menos potente que el diclorvós porque requiere ser activado para dar lugar a su análogo de oxígeno, fenitrooxón.

La recuperación de la actividad AChE depende del grado de inhibición inicial (Morgan y cols., 1990), ya que, esencialmente, es el resultado de sintetizar nueva proteína enzimática. Así, cuanto mayor sea la inhibición, mayor síntesis de enzima será necesaria (Fulton y Key, 2001). Sancho y cols. (1997) observaron una recuperación de la actividad AChE en el cerebro de las anguilas, que pasaron de tener una inhibición del 64% al 49% y 37%, después de estar 96 y 192 h, respectivamente, recuperándose en agua limpia. En el Artículo V la actividad AChE del cerebro permanecía inhibida un 40% después de 96 h de recuperación en agua limpia, mientras que esta inhibición era tan sólo del 26% en las anguilas recuperadas en la disolución de NAC 0.5 mM. Así, el tratamiento mediante baños de NAC aumentaba drásticamente la recuperación de la AChE después de una exposición a un plaguicida organofosforado, cosa que no se había obtenido previamente si no es mediante la utilización de una oxima para reactivar la AChE inhibida.

Aunque el tratamiento convencional frente a intoxicaciones por plaguicidas consiste en contrarrestar los efectos bioquímicos de la acetilcolina mediante atropina y reactivar la AChE inhibida con una oxima, la NAC se podría utilizar como un antídoto complementario ya que actúa en diferentes sitios diana, aumentando la eliminación del plaguicida y los radicales libres que éste genera. Además, dada la alta solubilidad y bajo precio de la NAC, se podría utilizar en baños terapéuticos para reducir el tiempo necesario de recuperación de peces intoxicados y facilitar la eliminación de plaguicidas, especialmente después de acciones contra los parásitos.

El aumento de la tolerancia de los peces a los plaguicidas mediante la NAC es de gran importancia en el tratamiento de infecciones de parásitos en aguas cálidas ya que en general la temperatura hace aumentar la toxicidad de los plaguicidas. Sería el caso del cultivo de la dorada (*Sparus aurata*) y la lubina (*Dicentrarchus labrax*) en el Mediterráneo, que padecen infecciones de isópodos (Horton y Okamura, 2001), y el uso de los plaguicidas para combatirlos se está fomentando (Papapanagiotou y Trilles, 2001).

5. CONCLUSIONES

Las conclusiones que se pueden extraer de los resultados obtenidos son:

1. Los herbicidas tiocarbamatos molinato y tiobencarb y el plaguicida organofosforado diclorvós disminuyen y oxidan los niveles de glutatión en el hígado de las anguilas.

2. Se ha descubierto que el principal mecanismo de toxicidad de los herbicidas tiocarbamatos es la generación de radicales libres. Éstos producen un estado de estrés oxidativo que sería el responsable de la anemia hemorrágica observada en las anguilas.

3. La tolerancia de las anguilas a los plaguicidas estudiados se puede explicar principalmente como la capacidad individual de mantener e incrementar el estado redox del glutatión y la actividad glutatión reductasa (GR) a nivel hepático. Un descenso en la relación entre el glutatión reducido y oxidado conduce a un excesivo estrés oxidativo, inhibición de la actividad caspasa-3, necrosis y, como consecuencia, la muerte del individuo.

4. La tolerancia de los bivalvos marinos al insecticida organofosforado fenitrotión también está mediada por el mantenimiento del estado redox del glutatión pero principalmente en el músculo aductor.

5. Mientras que la depleción de glutatión (especialmente el muscular) se utiliza como un marcador biológico de exposición a la contaminación, el estado redox del glutatión y las actividades acetilcolinesterasa (AChE), GR, glutamato:cisteína ligasa (GCL) y caspasa-3 se pueden considerar como biomarcadores de efecto y susceptibilidad individual a la mortalidad por los plaguicidas estudiados u otros contaminantes que inducen estrés oxidativo.

6. La disminución de glutatión en los tejidos de los bivalvos marinos también se puede considerar como un biomarcador de exposición a la contaminación, mientras que la relación entre el glutatión reducido y oxidado en el músculo es un potencial biomarcador de efecto y susceptibilidad individual.

7. La *N*-acetilcisteína (NAC) inyectada intraperitonealmente incrementa el contenido de GSH hepático y muscular, la relación GSH/GSSG hepática y las actividades GCL y GR del hígado y la glutatión *S*-transferasa (GST) hepática y muscular.

8. El pretratamiento de las anguilas con la NAC extiende la supervivencia de las anguilas cuando se someten a concentraciones letales del antiparasitario diclorvós mediante una menor depleción y oxidación del glutatión e inhibición enzimática producidas por el plaguicida. Así, la NAC aumenta la tolerancia al estrés oxidativo y necrosis generada por el plaguicida.

9. La NAC administrada en forma de baños aumenta el contenido de GSH muscular, la relación GSH/GSSG y la actividad glutatión reductasa en el cerebro.

10. Los baños de NAC mejoran sustancialmente la recuperación de la actividad acetilcolinesterasa (AChE) del cerebro de las anguilas expuestas a una concentración subletal de diclorvós. Así, la NAC aumenta la recuperación de los peces intoxicados previamente con el organofosforado a través de mejorar el metabolismo del glutatión, disminuyendo la pérdida y oxidación del glutatión y la inhibición enzimática causada por el plaguicida. Por lo tanto, los tratamientos de NAC constituyen un potencial antídoto de fácil administración para tratar las intoxicaciones con plaguicidas en peces.

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8. PUBLICACIONES

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Role of Glutathione in Thiobencarb Resistance in the European Eel *Anguilla anguilla*

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Glutahione-dependent defense against xenobiotic toxicity is a multifaceted phenomenon that has been well characterized in mammals. In the present study, eels of species *Anguilla anguilla* were exposed to 15 ppm of the herbicide thiobencarb (*S*-4-chlorobenzyl diethylthiocarbamate) for 96 h. Eels exposed to the pesticide were grouped in 24-h intervals according to their time of death, while surviving intoxicated eels constituted another group (live animals). Glutathione content (GSx, GSH, GSSG) was determined in liver and muscle tissues of the dead and live (intoxicated) animals and compared to control values (nonexposed eels). The fish that died before 96 h of exposure were considered susceptible to thiobencarb, while those dead after 96 h and the surviving ones were called resistant. Hepatic glutathione (GSH) content in susceptible eels was lower than that in the control fish, while resistant eels presented GSH levels threefold higher than those of controls. Muscle glutathione levels in dead eels were practically unaffected, but there was a significant decrease in GSH levels in the surviving intoxicated eels. These results indicate that the eels that were able to induce glutathione synthesis in the liver due to the presence of thiobencarb in the medium demonstrated a greater longevity than those who lost glutathione homeostasis. © 2000 Academic Press

Key Words: **pesticides; thiocarbamate; glutathione; microplate assay; eel.**

INTRODUCTION

Glutathione (L-γ-glutamyl-cysteinyl-glycine, GSH) is a ubiquitous nonprotein thiol that basically acts as an intracellular reductant and nucleophile. It intervenes directly or indirectly in many important physiological functions. These include the synthesis of proteins and DNA, amino acid transport, maintenance of the thiol-disulfide status (acting as a redox buffer), free radical scavenging (acting synergistically with ascorbate (vitamin C) and vitamin E (α-, β-, γ-, and ∂-tocopherols and tocotrienoles)), signal transduction, as an essential cofactor of several enzymes, as a nontoxic storage form of cysteine, and as a defense against oxidizing molecules and potentially harmful xenobiotics (Meister and Anderson, 1983; Kretzschmar, 1996; Sen and Packer, 1996; Cooper and Kristal, 1997).

Pesticides are widely used chemicals that are designed to be toxic to one or more living organisms and are released intentionally into the environment. They are metabolized by a number of enzymes, including the cytochrome P450-dependent monooxygenase system, the flavin-containing monooxigenase, prostaglandin synthetase, molybdenum hydroxylases, alcohol and aldehyde dehydrogenases, esterases, and a variety of transferases, most notably the glutathione *S*-transferases (Hodgson and Levi, 1996).

The tripeptide glutathione plays a key role in the detoxification of organic xenobiotics in a wide variety of animals. Conjugation of xenobiotics with glutathione (GSH) is a biotransformation process that generally results in the formation of less toxic products (Heath, 1995). In higher organisms, GSH *S*-transferases operate in the detoxification of a large number of chemicals (Dierickx and Vanderwielen, 1986). These enzymes facilitate nucleophilic attack of the sulfhydryl group of GSH on the electrophilic center of a broad spectrum of compounds.

Thiobencarb (*S*-4-chlorobenzyl diethylthiocarbamate) is a thiocarbamate herbicide widely used for weed control in paddy fields (Sabater and Carrasco, 1996). These herbicides undergo volatilization, adsorption, and chemical and microbiological transformations in the environment. However, their application for the control of weeds may also produce adverse effects on aquatic ecosystems in areas near agricultural fields (Sabater, 1994).

The major metabolic pathway for thiocarbamate herbicides in rats involves sulfoxidation and then GST-catalyzed conjugation with GSH. Thiobencarb is oxidized or hydrolytically cleaved, releasing 4-chlorobenzyl-methylthioether and the corresponding sulfone. These have been detected in liver extracts from mice treated with theiobencarb (Staub *et al.*, 1995).

Although glutathione-dependent systems have been characterized in a number of mammalian species, this is not the case for aquatic organisms. This study examined the effect of

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the herbicide thiobencarb on the glutathione content of different eel tissues. The role of glutathione in the expression of resistance or susceptibility of animals against a pesticide exposure was also investigated.

MATERIALS AND METHODS

Test Fish

Eels of the species *Anguilla anguilla* (weight 20–30 g; length 16–20 cm) were obtained form a fish farm (El Palmar, Valencia, Spain) and acclimatized to the laboratory conditions for 1 week before the start of the experiments in 300-L glass tanks (Ferrando, 1990).

Test System

The tanks were supplied with a continuous flow of tap water (temperature: 20 ± 2°C; total hardness: 240 ± 10 ppm as $CaCO_3$ according to the Merck classification, Aquamerck 8039; pH: 7.9 ± 0.2 using a Crison pH meter; alkalinity: 4.1 ± 0.5 mmol/L, Aquamerck 11109). A 12-h photoperiod was maintained (Ferrando, 1990).

Chemicals

Technical grade (98.1% purity) thiobencarb was obtained form ARGOS S.A. (Spain). Stock solutions were prepared by dissolving thiobencarb in acetone (1.3 µL/L). 2-Vinylpyridine was purchased from Aldrich. Glutathione reductase (EC 1.6.4.2) (Type III from Baker's yeast) and all other reagents used in this study were obtained from Sigma–Aldrich Química S.A. (Alcobendas, Madrid, Spain).

Toxicity Test

Experiments were performed for 96 h under static conditions using glass aquaria (25-L volume, with 15 L of water) with a thiobencarb concentration of 15 mg/L (LC_{50} 24 h = 25.7 mg/L; LC_{50} 96 h = 13.23 mg/L; Fernández *et al.*, 1998) and 10 eels in each aquarium. Three replicates of the same experiment were carried out. Ten more eels used as controls were kept in 15 L of clean water with the same concentration of solvent (acetone) (1.3 µL/L) used in the experimental sets.

Previous experiments carried out by Ferrando *et al.* (1992) in the laboratory found the thiobencarb half-life under the experimental conditions used to be 247.66 h and that 85% of the pesticide was found in the water after 96 h.

Eels were periodically checked and each death was registered. Dead fish were immediately removed, weighed, and dissected on an ice-cold glass plate to remove liver and muscle tissues, which were also weighed and frozen (− 85°C). After 96 h, control and surviving intoxicated eels were removed, anesthetized with MS222 in a concentration of 100 mg/L (Van Wardee *et al.*, 1983), weighed, and dissected out quickly. Liver and muscle tissue were also weighed and frozen (− 85°C).

Eels exposed to thiobencarb were grouped in 24-h intervals according to their time of death up to 96 h, while surviving intoxicated eels constituted another group (live animals). The fish that died before 96 h were considered susceptible to 15 ppm of thiobencarb, whereas those that died after 96 h and the surviving ones (live group) were considered resistant.

Sample Preparation

Each fish was analyzed individually at each exposure time. Eel liver and muscle tissues were excised, weighed, and homogenized using a Polytron homogenizer following the procedure of Baker *et al.* (1990) in 5 vol of ice-cold 5% 5-sulfosalicylic acid per gram of wet weight tissue, and further processed by sonication (Vibra-Cell, Sonics & Materials, Inc., Danbury, CT). The homogenates were then centrifuged at 20,000*g* for 10 min at 4°C (Suprafuge 22, Heraeus Sepatech, West Germany) and the supernatant fractions were stored frozen at − 85°C.

Analytical Procedures

Total glutathione content (GSH + GSSG) and oxidized glutathione (GSSG) in eel tissues were determined with a sensitive and specific assay using a recycling reaction of GSH with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in the presence of excess glutathione reductase according to Baker *et al.* (1990) in a microplate reader (Model 3550, Bio-Rad Laboratories, Richmond, CA). 2-Vinylpyridine was used to obtain GSH from the GSSG determination (Griffith, 1980). All measurements were performed at 415 nm using a reference wavelength of 595 nm every 30 s for 3 min at room temperature, as recommended by Cribb *et al.*, (1989). Glutathione concentrations were expressed as GSH equivalents (GSx = [GSH] + 2 × [GSSG]). GSH was calculated by subtracting GSSG levels from the GSx levels determined. Standards and blanks were prepared simultaneously with the samples.

Statistical Analysis

Mean values and standard deviations were calculated for each test group based on the values obtained for each individual tissue (liver and muscle). The results from exposed eels were compared by one-way analysis of variance (ANOVA) and Duncan's significant difference test. Liver and muscle glutathione content from control animals was compared using Student's *t* test. Statistical analysis was performed using the Statistical Analysis System (SPSS +)

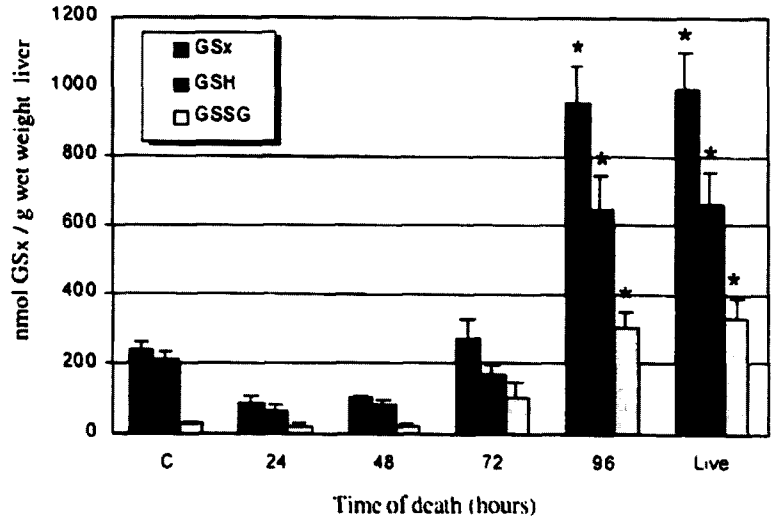

FIG. 1. Hepatic content of total (GSx), reduced (GSH), and oxidized (GSSG) glutathione in the European eel *A. anguilla* exposed to 15 ppm of thiobencarb. Eels are grouped in 24-h intervals according to their time of death up to 96 h, while surviving eels are considered in a separate group. Values are means ± SEM ($n = 6$). (*) $P < 0.05$.

with an IBM computer. The significance level was set at 0.05.

RESULTS

Preliminary toxicity tests were carried out for *A. anguilla* using different concentrations of thiobencarb (5, 12, 15, and 20 ppm). Eel mortality after 96 h of exposure to thiobencarb was 10, 35, 65, and 80%, respectively. On the other hand, those animals exposed to 15 ppm exhibited a mortality that did fit with a normal distribution. It was observed that 6 eels died before 24 h of exposure, 9 between 24 and 48 h, 11 between 48 and 72 h, and 9 between 72 and 96 h, whereas 6 eels were still alive after 96 h. Therefore, 15 ppm was the selected pesticide concentration to carry out the glutathione content evaluation at different times of exposure.

Total hepatic glutathione content (GSx) (Fig. 1) in thiobencarb-resistant eels (96 h and alive) was 4-fold higher than that in control eels, whereas the susceptible ones (24–72 h) has GSx levels 2.5-fold lower. Consequently, resistant eels had 10-fold more total glutathione than the susceptible ones. Hepatic concentrations of GSH and GSSG were significantly different ($P < 0.05$) between control eels and resistant animals (96 h of exposure to the herbicide and live group). In fact, a reduction of 67 and 65% in GSH content compared to GSx was observed in the resistant fish (Fig. 2). There were no significant differences in GSx, GSH, and GSSG between susceptible eels and controls.

The relationship found between GSH and GSSG in the present study (Fig. 2) indicates that the proportion of GSH content decreases as the eels become more resistant to thiobencarb and also the ratio GSH/GSSG decreases. This means that the resistant eels had a higher percentage of GSSG than the susceptible ones, possibly as a consequence of the longer time that they were exposed to the herbicide.

There were significant differences ($P < 0.05$) between glutathione levels in liver and muscle tissues of control eels (Fig. 3). Higher glutathione hepatic levels were found in *A. anguilla* compared to those determined in muscle tissue. On the other hand, liver tissue contains a higher amount of GSH (88%) while eel muscle tissue had lower but similar GSH and GSSG values, 55 and 45%, respectively (Fig. 3).

Muscle total glutathione levels (GSx) in dead eels (24 →96 h) (Fig. 4) were unaffected. However, there was a significant decrease ($P < 0.05$) in GSH content in the eels that died during the first day (24 h), and in the GSx and GSH levels in the surviving eels exposed to thiobencarb (live group).

The relationships between muscular GSH and GSSG (Fig. 5) in eels exposed to thiobencarb was not very clear. Some fluctuations were found during the exposure time to the herbicide. Control eels had a 55% of GSH content in muscle tissue while those fish exposed to the pesticide for 96 h exhibited GSH values of 53 and 55% in dead and live groups, respectively (Fig. 5).

DISCUSSION

The hepatic GSH depletion found in dead eels in the present study during the first 48 h of exposure to thiobencarb was in agreement with the findings reported by several authors. Allen *et al.* (1988) detected a GSH decrease in the liver of the fish *Oreochromis aureus* treated with mercury (1 mg/L $HgCl_2$) for 2 h, but no significant differences were found in the brain, spleen, and intestinal glutathione levels.

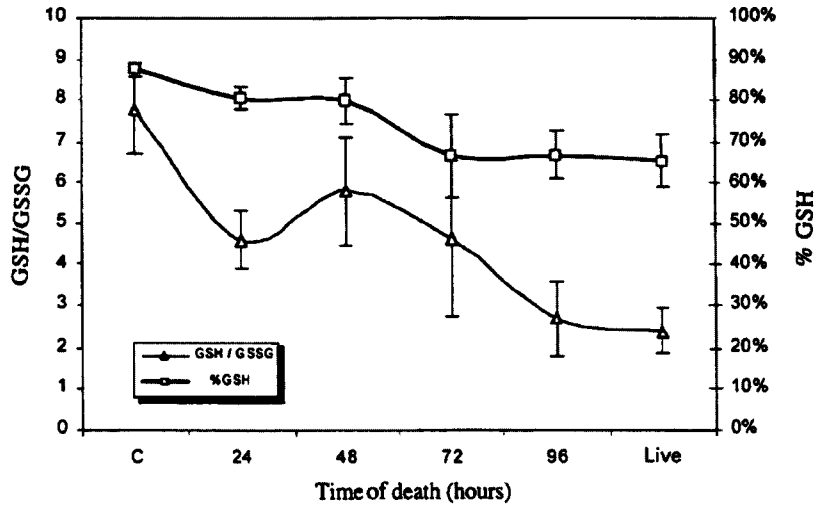

FIG. 2. Relationship between reduced (GSH) and oxidized (GSSG) glutathione in the liver of *A. anguilla* exposed to thiobencarb. Values are means ± SEM ($n = 6$).

In contrast, van der Oost *et al.* (1996) did not find GSH depletion in eels (*A. anguilla*) exposed to organic pollution. In another study, Doyotte *et al.* (1997) reported a GSH depletion in the gills and digestive glands of freshwater mussels (*Unio tumidus*) exposed to the dithiocarbamate thiram for 3 days.

Studies with mammals (rat liver) also revealed a GSH depletion after exposure to the pesticides alachlor, benomyl, and methyl parathion (Della Morte *et al.*, 1994). However, in the same study, the herbicides atrazine, linuron, and propanil did not produce any effect in rat liver GSH under their experimental conditions.

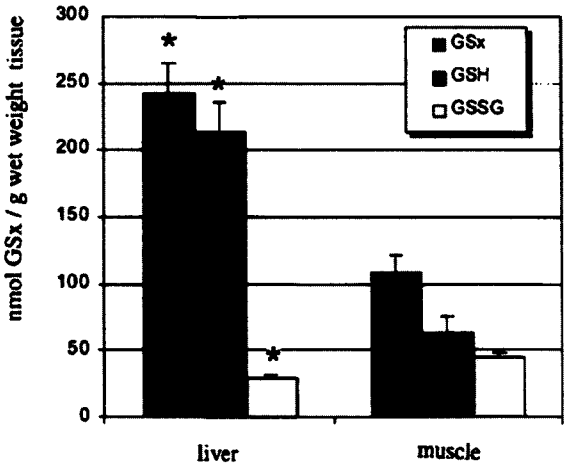

FIG. 3. Total (GSx), reduced (GSH), and oxidized (GSSG) glutathione in liver and muscle tissue of control eels. Values are means ± SEM ($n = 6$) (*) $P < 0.05$.

In the present study, the eels that died after 72 h of exposure to thiobencarb and those that were still alive after 96 h (resistant animals) exhibited a significant increase in hepatic glutathione levels ($P < 0.05$) (Fig. 1). The levels were quantitatively greater than those found by Hasspieler *et al.* (1994) in channel catfish (*Ictalurus punctatus*) and brown bullhead (*Ameriurus nebulosus*) exposed to menadione for 2 days. Glutathione induction was also reported in benthic fish exposed to sediments that were contaminated with polycyclic and polyhalogenated aromatic hydrocarbons (PAHs) (Stein *et al.*, 1992).

Otto *et al.* (1997) injected GSH intraperitoneally into both rainbow trout (*Oncorhynchus mykiss*) and American eels (*Anguilla rostrata*) every day on the third and sixth day of experimentation. Total glutathione content increased in eel liver (4.3- to 7.4-fold), kidney (2-fold), and plasma (51- to 123-fold) but not in gill or muscle tissues. It is likely that the increase in GSH content in eels makes them more resistant to oxidative stress caused by pesticides.

The induction in glutathione content may be interpreted as a defensive response that confers greater protection against certain forms of cellular injury. The results suggest that the eel may be able to respond to thiobencarb with rapid GSH biosynthesis due to higher hepatic activity, but the mechanism underlying herbicide-mediated GSH induction requires further investigation.

Liver tissue plays an important role in the synthesis of GSH released in the blood and bile. Plasma GSH is distributed to different sites, such as kidney, lung, and muscle tissues. Therefore, GSH levels in eel muscle were not altered to the same extent as that the liver because liver is the organ that maintains GSH homeostasis. However, hepatic GSH is not a constant value and it depends on the balance between

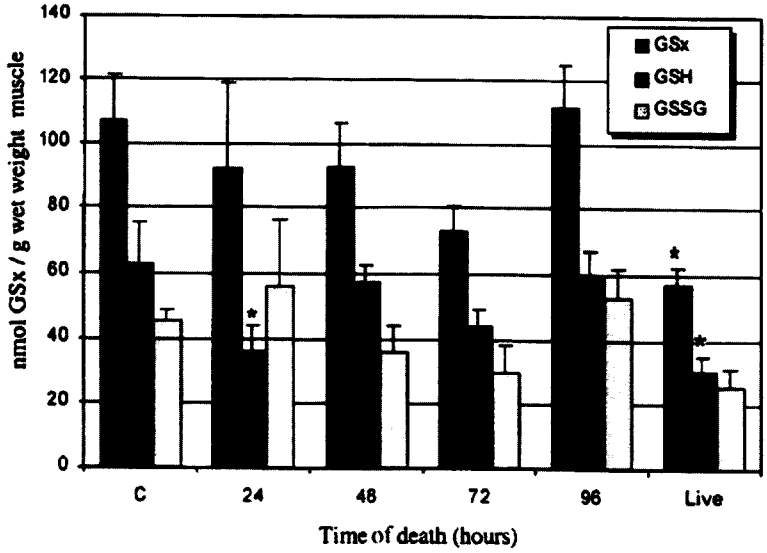

FIG. 4. Muscular content of total (GSx), reduced (GSH), and oxidized (GSSG) glutathione in the European eel *A. anguilla* exposed to 15 ppm of thiobencarb. Eels are grouped in 24-h intervals according to their time of death up to 96 h, while surviving eels are considered in a separate group. Values are means ± SEM ($n = 6$). (*) $P < 0.05$.

synthesis rate (L-γ-glutamylcysteine synthetase, GSH synthetase), GSSG reduction (GSSG reductase), conjugation rate (GSH *S*-transferases), oxidation rate (GSH peroxidase), and transfer to plasma and bile. The GSH synthesis is regulated in part by a nonallosteric competitive feedback inhibition of γ-glutamylcysteine synthetase by GSH (Meister and Anderson, 1983).

When eel hepatocytes detect the presence of electrophilic compounds, they act removing them principally by conjugation with GSH by means of glutathione *S*-transferases, but also by oxidation of GSH to GSSG by glutathione peroxidases, where GSH is recycled by GSSG reductase. Then, as GSSG accumulates, it is translocated outside the cell by specific transporters (Kaplowitz *et al.*, 1996; Keppler *et al.*, 1997) and consequently, glutathione concentration decreases.

Babu *et al.* (1989) demonstrated that repeated thiobencarb treatment in rats was associated with a significant decrease in proteins with a simultaneous increase in free amino acids and specific activity levels of proteases. This suggests an impaired protein synthesis or elevated proteolysis. Therefore, eels treated with thiobencarb could have their

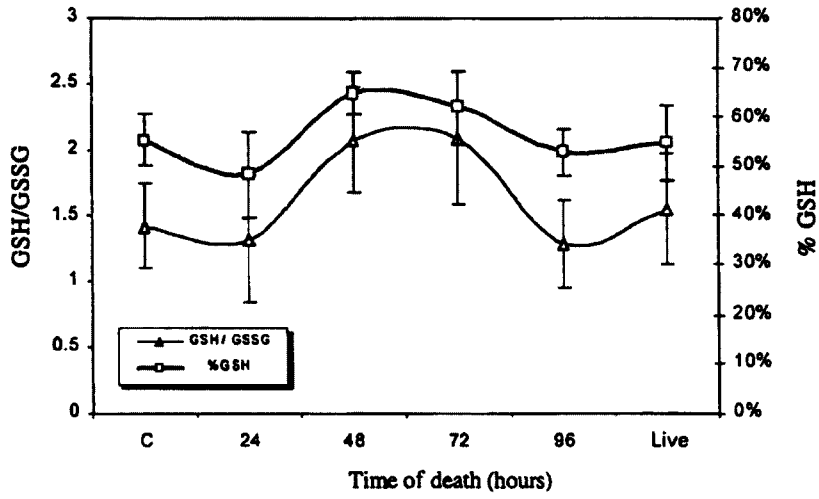

FIG. 5. Relationship between reduced (GSH) and oxidized (GSSG) glutathione in the muscle of *A. anguilla* exposed to thiobencarb. Values are means ± SEM ($n = 6$).

protein metabolism affected, with enzymatic activities altered. Therefore, eels that can keep or induce increases in enzyme-related glutathione activity are more likely to be resistant to high concentrations of thiobencarb than those that suffer glutathione depletion.

CONCLUSIONS

This study illustrates the dynamic nature of the glutathione system in the eel *A. anguilla* and the manner in which its characteristics may differ in different tissues of the same species. Results concerning *A. anguilla* exposure to the herbicide thiobencarb suggest that pesticide-resistant eels have higher hepathic glutathione content than control animals. The natural capacity of eels to induce glutathione synthesis is crucial to the process of detoxifying xenobiotics and preventing cellular injury. Therefore, the differences observed regarding glutathione-dependent defense against herbicides such as thiobencarb may lend insight into possible mechanisms underlying species susceptibility; however, further investigation is necessary.

ACKNOWLEDGMENTS

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ARTÍCULO II

Reimpresión a partir de *Chemosphere*, 45, S. Peña, J.B. Peña, E. Sancho, C. Fernández-Vega y M.D. Ferrando, Glutathione-dependent resistance of the European eel *Anguilla anguilla* to the herbicide molinate, 671-681, Copyright 2001, con permiso de Elsevier Science.

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Glutathione-dependent resistance of the European eel *Anguilla anguilla* to the herbicide molinate

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Abstract

Eels of species *Anguilla anguilla* were exposed to 5/4 LC_{50} (41.8 mg/l) of the herbicide molinate for 96 h in a time to death (TTD) test. Glutathione content (GSx, GSH, GSSG), glutathione reductase (GR) and γ-glutamyl transpeptidase (γ-GT) activities were determined in the liver and muscle tissues of dead and surviving (intoxicated) animals and compared to control values (non-exposed eels). TTD was positively correlated to hepatic GSH, GSH:GSSG ratio, hepatic and muscular GR, but negatively correlated to muscular GSH, which was severely depleted. Furthermore, glutathione and enzyme activities were intercorrelated, especially GSH and GR. These results indicate that eels which were able to induce GR activity, increase GSH and maintain the GSH:GSSG ratio in the liver showed an extended survival under the oxidative stress generated by molinate than those that lost glutathione homeostasis.

Keywords: Pesticide; Oxidative stress; Fish; Reductase; Transpeptidase; TTD

1. Introduction

Molinate (Ordram®, *S*-ethylazepan-1-carbothioate) is a thiocarbamate herbicide widely used for weed control in rice fields. It is toxic to germinating broad-leafed and grassy weeds, possibly by uncoupling plant oxidative phosphorylation (Fang, 1975). This herbicide undergoes volatilization, adsorption, chemical and microbiological transformations in the environment. However, its application for the control of weeds may also produce adverse effects on aquatic ecosystems in areas nearby agricultural fields (Sabater, 1994). This is the case of the Albufera Lake (Valencia, Spain), which is surrounded by rice fields, and where the European eel (*Anguilla anguilla*) lives.

Molinate is not very toxic to rats and mice. The oral LD_{50} for molinate is 369–955 mg/kg in rats, and 530 mg/kg in mice (Hayes and Laws, 1990). However, molinate toxicity in fish is higher and very variable. The 96-h LC_{50} in fish species are 0.21 mg/l for the common carp, 12.1 mg/l for striped bass (Finlayson and Faggella, 1986), 1.3 mg/l for rainbow trout (Meister, 1991) and 30 mg/l for goldfish (Hartley and Kidd, 1983).

Thiocarbamate primary effects are through the inhibition of acetylcholinesterase (AChE) activity, which is an enzyme responsible to finish the transmission of a nerve impulse. However, the mode of action of molinate is more complex because AChE inhibition is not sufficient to explain the severe hemorrhagic anaemia observed in carps (Kawatsu, 1977). Thiocarbamate metabolites inhibit in vivo mitochondrial low-K_m

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aldehyde dehydrogenase ($ALDH_2$; EC 1.2.1.3) in mice liver with similar potency (and homologous structure) to the metabolites of disulfiram (Quistad et al., 1994). Disulfiram is a dithiocarbamate fungicide that alters the glutathione redox status inducing an oxidative stress on the brain of rats (Nagendra et al., 1994). Therefore, a similar mode of action could be expected in eels exposed to molinate.

Glutathione (L-γ-glutamyl-cysteinyl-glycine) is an ubiquitous non-protein thiol that is mainly present in cells in its reduced form (GSH), which basically acts as an intracellular reductant and nucleophile. It intervenes directly or indirectly in many important physiological functions. These include the synthesis of proteins and DNA, amino acid transport, maintenance of the thiol-disulfide status (acting as a redox buffer), free radical scavenging (acting synergistically with ascorbate (vitamin C) and vitamin E (tocopherols and tocotrienols)), signal transduction, as an essential cofactor of several enzymes, as a non-toxic storage form of cysteine, and as a defence against oxidizing molecules and potentially harmful xenobiotics (Meister and Anderson, 1983; Kretzschmar, 1996; Sen and Packer, 1996; Cooper and Kristal, 1997). However, it is also present in its oxidized form (GSSG) where there is a disulfide bond between two molecules of GSH.

Glutathione metabolism is regulated by several well-known enzymes (Meister and Anderson, 1983). Glutathione *S*-transferases (GSTs) facilitate nucleophilic attack of the sulfhydryl group of GSH on the electrophilic centre of a broad spectrum of compounds resulting in the formation of less toxic products (Heath, 1995) and allowing their elimination. Glutathione peroxidase (GPx) catalyses the GSH-dependent reduction of H_2O_2 to H_2O and other peroxides as well, protecting membrane lipids against oxidation. These reductions of peroxides imply the oxidation of GSH to GSSG. Glutathione reductase (GR) is a flavoprotein that catalyses the reduction of GSSG to GSH using NADPH as cofactor. γ-Glutamyl transpeptidase (γ-GT) is the only protease that can cleave intact GSH and GSH conjugate. It is mainly membrane-bound, with its active site oriented on the outer surface of the cell membrane (Horiuchi et al., 1978) enabling resorption of extracellular GSH catabolites from plasma and, therefore, maintaining high-GSH concentrations in extrahepatic tissues (Commandeur et al., 1995). Finally, GSH is synthesized in two consecutive ATP-dependent reactions. γ-Glutamylcysteine synthetase (GCS) catalyses the rate limiting first step in which the amino acid cysteine is linked to glutamate. GSH synthetase catalyses the second step in which glycine is linked to the cysteine carboxyl of γ-glutamylcysteine to yield GSH (DeLeve and Kaplowitz, 1991). This last enzyme is not as important as the first because GCS is regulated by feedback inhibition of GSH (Richman and Meister, 1975). In addition, when GSH is consumed and feedback inhibition is lost, availability of cysteine as a precursor can become the limiting factor in GSH biosynthesis (DeLeve and Kaplowitz, 1991).

The aim of this study was the evaluation of the effects of the herbicide molinate on the glutathione content in the liver and muscle of the European eel *A. anguilla* as well as the activity of two enzymes related to its metabolism, and how they are related to eel survival after exposure to the pesticide.

2. Materials and methods

2.1. Test fish

Eels of the species *A. anguilla* were obtained from a fish farm (Cofradía de Pescadores, El Palmar, Valencia, Spain) and acclimatized to laboratory conditions for one week before the start of the experiments in 300-l glass tanks (Ferrando, 1990).

2.2. Test system

The tanks were supplied with a continuous flow of tap water (temperature: 20 ± 2°C; total hardness: 240 ± 10 ppm as CaCO3 according to the Merck classification, Aquamerck 8039; pH: 7.9 ± 0.2 using a Crison pH-meter; alkalinity: 4.1 ± 0.5 mmol/l, Aquamerck 11109). A 12 h photoperiod was maintained (Ferrando, 1990).

2.3. Chemicals

Technical grade molinate (98% purity) was obtained from ARGOS S.A. (Spain). Stock solutions were prepared by dissolving molinate in acetone (133 μl/l). 2-Vinylpyridine was purchased from Aldrich. GR (EC 1.6.4.2) (Type III from Baker's yeast) and all other reagents used in this study were obtained from Sigma Chemical (St. Louis, MO, USA).

2.4. Toxicity test

Preliminary toxicity tests were carried out in our laboratory following the method described by Ferrando (1990) in order to calculate the 96-h LC_{50} for molinate in *A. anguilla*. Based on these data, 50 randomly selected eels (weight 22–46 g; length 24–30 cm) were exposed to 5/4 LC_{50} of molinate (41.8 mg/l) for 96 h under static conditions (in 15-l aquaria) in order to assure sufficient mortality for statistical comparisons in the time to death (TTD) test. Ten eels used as controls were kept in 15 l of clean water with the same concentration of solvent (133 μl/l acetone) as in the experimental sets.

Eels were periodically checked at approximate 6-h intervals or lower. Dead eels were immediately removed, TTD noted, weighted and dissected out on an ice-cold glass plate to excise liver and muscle tissues, which were weighted and frozen at −85°C. After 96 hr, control and surviving animals were removed, anaesthetized with MS222 in a concentration of 100 mg/l (Van Wardee et al., 1983), weighted and dissected out quickly. Liver and muscle tissues were also excised, weighted and frozen (−85°C).

2.5. Glutathione determination

Eel liver and muscle tissues were homogenized following the procedure of Baker et al. (1990) with five volumes of ice-cold 5% 5-sulfosalicylic acid per gram of wet weight tissue, and further processed by sonication (Vibra-Cell, Sonics and Materials, Danbury, Connecticut, USA). The homogenates were then centrifuged at 4°C and 20,000 *g* for 10 min (Suprafuge 22, Heraeus Sepatech, West Germany) and the supernatant fractions were stored frozen at −85°C.

Total glutathione content (GSH + GSSG) and the oxidized form (GSSG) were determined in a microplate reader (Model 3550, Bio-Rad Laboratories, Richmond, CA) with a sensitive and specific assay using a recycling reaction of GSH with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of excess GR according to Baker et al. (1990). 2-Vinylpyridine was used to conjugate GSH for the GSSG determination (Griffith, 1980). All measurements were done at 415 nm using a reference wavelength of 595 nm every 30 s for 3 min at room temperature as recommended by Cribb et al. (1989). Glutathione concentrations were expressed as GSH equivalents (GSx = [GSH] + 2 x [GSSG]). GSH was calculated by subtracting GSSG levels from the GSx levels determined. GSH:GSSG ratios and GSH percentages were expressed as number of molecules but not moles: [GSH]/[GSSG] = (GSx − GSSG)/(GSSG/2); %[GSH] = 100 x (GSx − GSSG)/(GSx − GSSG/2). Standards and blanks were prepared simultaneously with the samples in the same acidic conditions.

2.6. Enzyme assays

Liver and muscle tissues were homogenized with five and four volumes, respectively, of 100 mM phosphate buffer, pH 7.5, containing 1 mM EDTA. The homogenates were centrifuged at 20,000 *g* (4°C) for 10 min and the resulting supernatants were diluted five-fold with buffer and assayed rapidly for GR and γ-GT activities.

GR (EC 1.6.4.2) activity was assayed by the method of Cribb et al. (1989) with the following final concentrations: [DTNB] = 0.075 mM; [NADPH] = 0.1 mM; [GSSG] = 1 mM according to Smith et al. (1988).

The method of Silber et al. (1986) was used to measure the γ-GT (EC 2.3.2.2) activity. The rate of the substrate analogue γ-glutamyl-*p*-nitroanilide cleavage to form *p*-nitroaniline (*p*-NA) was monitored at 405 nm for 10 min.

2.7. Protein determination

Protein content was determined by the Bio-Rad Protein Assay based on the Bradford dye-binding procedure (Bradford, 1976), using bovine serum albumin as standard. Enzyme activities were calculated as specific activity.

2.8. Statistical analysis

Individual eels exposed to molinate were grouped in four intervals of time according to their death time until 96 h, and survivors constituted another group. Variables were tested for normality (Kolmogorov–Smirnov test with Lilliefors significance correction) and variance homogeneity (Levene test) using SPSS for Windows (SPSS, Chicago, IL, USA). Logarithm transformation was applied to those variables that failed the Levene test. Mean values ± standard error of the mean (SEM) were calculated for each test group and were compared by one-way analysis of variance (ANOVA) followed by Dunnett's test to find different groups to control values. Tukey's honestly significant difference (HSD) test was used to assess differences among survival groups. Pearson correlation coefficients were calculated between individual TTD and variables, and also among variables, for eels exposed to the pesticide. The TTD used for every eel corresponds to the mean between two intervals, that is, between the time the fish was found dead and the latest time the aquarium was checked, except for survivors, which had a censured time at 96 h of exposure to the herbicide.

3. Results

The 96-h LC_{50} for molinate in *A. anguilla* was calculated as 33.46 mg/l, then eels were exposed to 41.8 mg/l (5/4 96-h LC_{50}) of molinate for 96 h. Results showed that there was a 74% of surviving animals at 29 h of exposure, 54% at 48 h, 40% at 72 h, and finally there was a 22% of survivors at 96 h of exposure to the herbicide.

Despite individual weight and length differences among eels, those showed no correlation with any one of the variables studied (data not shown).

The GSH content in the liver of the eels died in the first 29 h (Fig. 1(a)) showed a significant reduction ($P < 0.05$) compared with control values. However, there was an increase in both GSH and GSSG in those eels that died after 48 h and in the survivors to the 96-h TTD test. The relationship between GSH and GSSG in

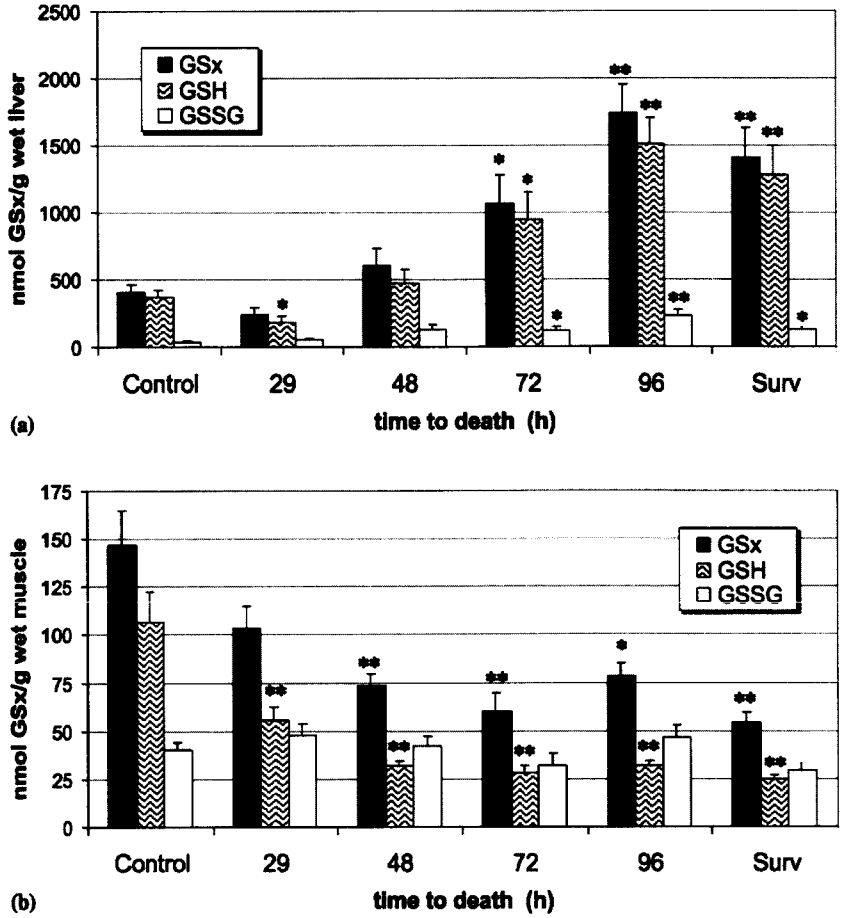

Fig. 1. Total (GSx), reduced (GSH) and oxidized (GSSG) glutathione in the liver (a) and muscle (b) of European eels exposed to 41.8 mg/l of molinate. Eels were grouped in four intervals of time according to their death time until 96 h, where surviving eels were considered as another group. Values are mean ± SEM. (*) $P < 0.05$; (**) $P < 0.01$.

the liver (Fig. 2(A)) was clearly lower ($P < 0.01$) in those eels that died during the first 48 h than in controls. Furthermore, Tukey's test confirmed that those presented lower ($P < 0.01$) GSH:GSSG ratio than survivors (data not shown). Thus, significant correlations were found (Table 1) between the TTD and GSH ($r = 0.76$, $P < 0.001$), GSSG ($r = 0.44$, $P < 0.01$) and the ratio between them ($r = 0.56$, $P < 0.001$).

Every eel exposed to the herbicide presented a serious GSH depletion in the muscle (Fig. 1(b)), however Tukey's test showed significant ($P < 0.05$) differences among these eels (data not shown). Eels that died during the first 29 h contained higher muscular GSH content than those that died between 29 and 96 h, and in all of them, GSH was higher than in survivors. Thus, muscular GSH was negatively correlated to TTD ($r = 0.50$, $P < 0.001$) (Table 1). The GSH:GSSG ratio was markedly lower in all eels exposed to the herbicide (Fig. 2(B)) with no differences among them.

On the other hand, a decrease in both liver and muscle GR activities (30% and 37%, respectively) was detected in those eels that died during the first 29 h of exposure compared to controls (Fig. 3). Nevertheless, survivors to the 96-h TTD test showed a 40% higher hepatic GR activity. These enzymatic activities were strongly correlated with the TTD in the liver ($r = 0.86$, $P < 0.001$) and the muscle ($r = 0.69$, $P < 0.001$).

Hepatic γ-GT activities (Fig. 4) in eels exposed to molinate were not different from those of fishes not exposed to the herbicide (Table 2), but this activity was a 50% higher in the muscle of those eels that died up to 48 h.

Levels of glutathione and glutathione-dependent enzyme activities were intercorrelated (Table 1). For example, the GSH content in eel liver was positively correlated to hepatic GSSG ($r = 0.77$, $P < 0.001$) and GR activity ($r = 0.71$, $P < 0.001$). In addition, hepatic GSH levels were positively correlated to muscular GR activity ($r = 0.49$, $P < 0.001$), but negatively correlated to muscular GSH ($r = 0.46$, $P < 0.001$) and muscular γ-GT activity ($r = 0.46$, $P < 0.01$). GR activities in eel liver and muscle tissues were also correlated ($r = 0.68$, $P < 0.001$).

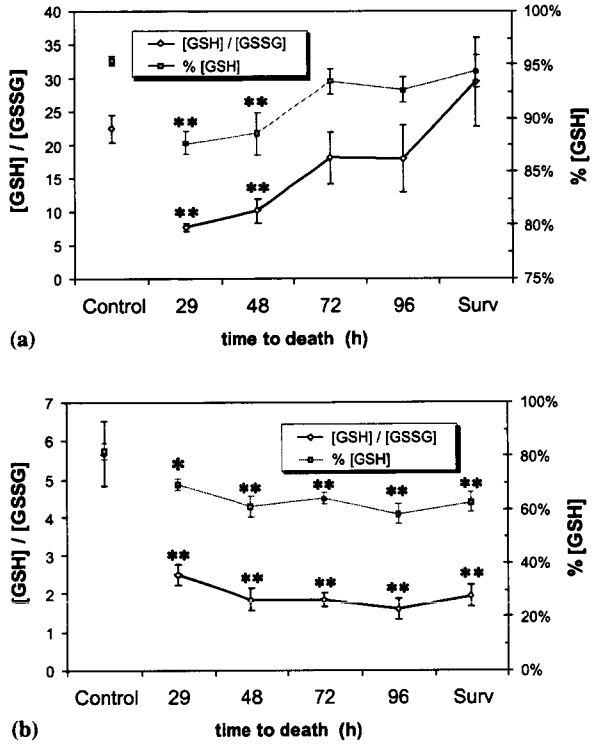

Fig. 2. Relationship between reduced (GSH) and oxidized (GSSG) glutathione in the liver (a) and muscle (b) of eels exposed to 41.8 mg/l of molinate. Eels were grouped in four intervals of time according to their death time until 96 h, and survivors were considered as another group. Values are mean ± SEM. (*) $P < 0.05$; (**) $P < 0.01$.

4. Discussion

Eels exposed to the herbicide molinate presented lethargy and convulsions, surely as a consequence of inhibition in the cholinesterase activity, as was previously demonstrated by Sancho et al. (2000).

Molinate is mainly sulfoxidized by microsomal monooxigenases (Tjeerdema and Crosby, 1987) leading to a potent in vivo rat-liver $ALDH_2$ inhibitor (Hart and Faiman, 1995). Molinate sulfoxide is then conjugated with GSH by GST (Casida et al., 1974), leading to excretion of the mercapturate and releasing the sulfenic acid, which is decomposed to the thiol (Hubbell and Casida, 1977).

However, conjugation of thiocarbamates with GSH does not always lead to their irreversible detoxification. The thiocarbamic acid moiety can be recycled by cysteine conjugate β-lyase, enter the enterohepatic circulation, and then be methylated by *S*-methyl transferases to yield the *S*-methyl thiocarbamate analogue, which inhibits hepatic $ALDH_2$ in mice with potency equal to the parent herbicide (Staub et al., 1995). Hence, the toxicological burden of molinate may be increased.

When eel cells make contact with electrophilic compounds, like molinate sulfoxide, they act removing them principally by conjugation with GSH by means of GSTs, which decreases GSH levels. In addition, the oxidative damage by metabolites of molinate perhaps is mediated by the uncouplement of the mitochondrial oxidative phosphorylation (Fang, 1975), which would generate free radicals or reactive oxygen species (ROS). The detoxification of ROS and hydroperoxides implies the oxidation of GSH to GSSG by GPx. This GSSG is then reduced to GSH by GR at expenses to oxidize NADPH to $NADP^+$, which is recycled by the pentose phosphate pathway. Nevertheless, if generation of GSSG is higher than the reduction back to GSH by GR, then GSSG accumulates and it is translocated outside the cell by specific transporters (Kaplowitz et al., 1996; Keppler et al., 1997) to avoid NADPH exhaustion. Consequently,

Table 1
Pearson correlation coefficients (*r*) among TTD, glutathione and activities of glutathione-dependent enzymes in the liver and muscle from individual eels exposed to molinate[a]

		TTD	Liver					Muscle			
			GSH	GSSG	Ratio	GR	γ-GT	GSH	GSSG	Ratio	GR
Liver	GSH	0.76**									
	GSSG	0.44*	0.77**								
	Ratio	0.56**	0.49**	−0.18							
	GR	0.86**	0.71**	0.41*	0.52**						
	γ-GT	0.15	0.24	0.07	0.26	0.09					
Muscle	GSH	−0.50**	−0.46**	−0.14	−0.51**	−0.56**	−0.04				
	GSSG	−0.27	−0.12	−0.03	−0.14	−0.30	0.04	0.53**			
	Ratio	−0.28	−0.34	−0.09	−0.39*	−0.22	−0.05	0.34	−0.54**		
	GR	0.69**	0.49**	0.25	0.41*	0.68**	−0.02	−0.59**	−0.42*	−0.20	
	γ-GT	−0.36	−0.46*	−0.22	−0.35	−0.41*	0.06	0.29	−0.04	0.29	−0.26

[a] Single asterisk indicated significant ($P < 0.01$) and two asteriks highly significant ($P < 0.001$) correlations with $n = 50$. TTD = time to death; GSH = reduced glutathione; GSSG = glutathione disulfide; ratio = [GSH]/[GSSG]; GR = glutathione reductase activity; γ-GT = γ-glutamyl transpeptidase activity.

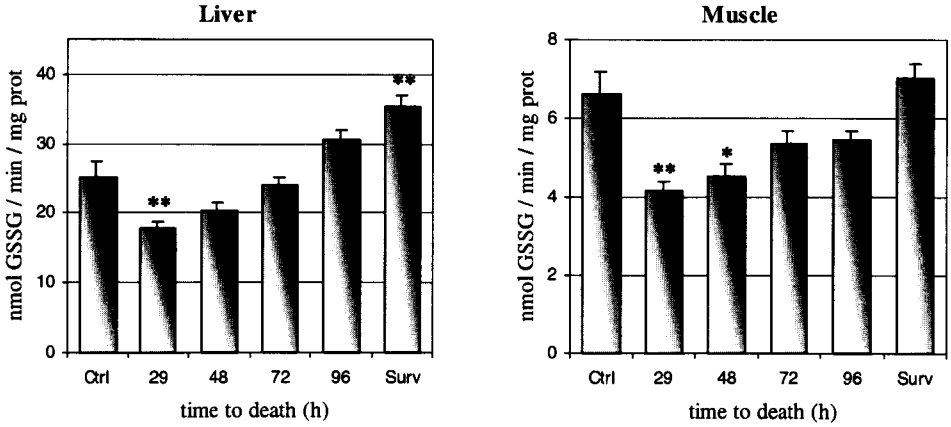

Fig. 3. Glutathione reductase activity (GR) in both liver and muscle tissues of controls and eels exposed to 41.8 mg/l of molinate. Values are means ± SEM. (*) $P < 0.05$; (**) $P < 0.01$.

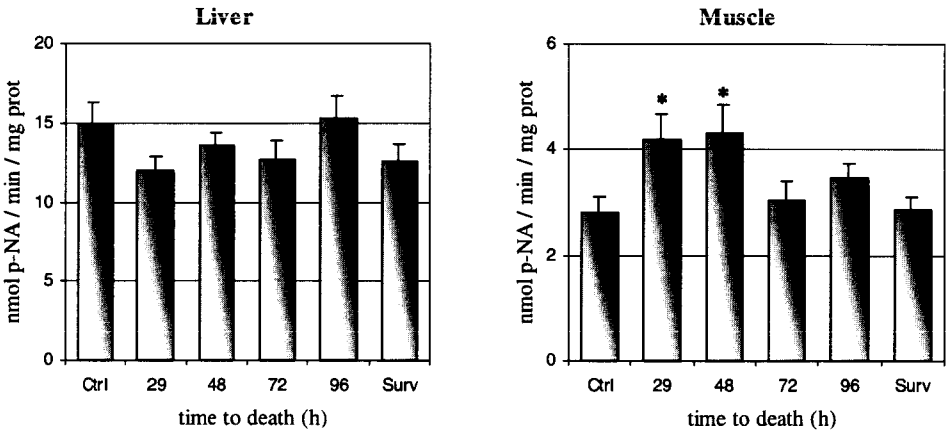

Fig. 4. γ-Glutamyl transpeptidase activity (γ-GT) in both liver and muscle tissues of controls and eels exposed to 41.8 mg/l of molinate. Values are mean ± SEM. (*) $P < 0.05$. p-NA = p-nitroaniline.

this is followed by a depletion of the GSH pool (Kretzschmar, 1996).

On the other hand, mitochondria do not have the enzymes necessary for GSH synthesis (Griffith and Meister, 1985), therefore glutathione must be imported from the cytosol by both high-affinity and high-capacity transporters (Mårtensson et al., 1990). When there is a cytosolic GSH depletion, mitochondrial GSH is retained by an ATP driven uptake of GSH (Mårtensson et al., 1990), but if there is a cytosolic GSH depletion in conjunction with oxidative stress, the GSH pool in mitochondria reduces, thus the antioxidant and detoxifying activities of glutathione diminishes, so oxidative stress prevails inactivating enzymes and producing DNA damage and lipid peroxidation. If toxic exposure continues, then mitochondria become progressively more permeable to small ions and molecules and eventually they lose their function (Cooper and Kristal, 1997) leading to cell death by apoptosis, which is sensitive to redox regulation of glutathione (Jones et al., 1995). Hence, individual differences in enzymatic activities, especially those concerning the synthesis of glutathione and GSSG reduction, were critical in molinate detoxification.

Resistance to the pesticide molinate, considered as differences in the survival time of *A. anguilla* under the 96-h TTD test, could be explained in terms of glutathione metabolism. Surviving eels were characterized by an accrual of the hepatic GSH concentration, but a loss of muscular GSH levels, and an increase in the hepatic GR activity, which raises the GSH:GSSG ratio to the levels of non-exposure to the pesticide. Conversely, those eels in which their GR activity was diminished were not able to restore the GSH:GSSG ratio shifted by the oxidative stress generated by the thiocarbamate herbicide, and thus, glutathione homeostasis was lost.

Table 2
ANOVA results of glutathione levels and activities of glutathione-dependent enzymes in the liver and muscle from eels exposed to molinate and control ones

			Sum of Squares	df	Mean square	F	Sig.
Liver	GSx[a]	Between groups	6.081	5	1.216	14.716	<0.001
		Within groups	4.132	50	8.265×10^{-2}		
		Total	10.214	55			
	GSH	Between groups	6.704	5	1.341	16.219	<0.001
		Within groups	4.134	50	8.267×10^{-2}		
		Total	10.838	55			
	GSSG	Between groups	3.808	5	0.762	6.238	<0.001
		Within groups	6.104	50	0.122		
		Total	9.912	55			
	Ratio	Between groups	2.018	5	0.404	6.685	<0.001
		Within groups	3.019	50	6.037×10^{-2}		
		Total	5.037	55			
	%[GSH]	Between groups	5.001×10^{-2}	5	1.000×10^{-2}	6.205	<0.001
		Within groups	8.060×10^{-2}	50	1.612×10^{-3}		
		Total	0.131	55			
	GR	Between groups	0.691	5	0.138	20.690	<0.001
		Within groups	0.361	54	6.684×10^{-3}		
		Total	1.052	59			
	γ-GT	Between groups	89.553	5	17.911	1.378	0.247
		Within groups	701.977	54	13.000		
		Total	791.530	59			
Muscle	GSx	Between groups	1.113	5	0.223	7.000	<0.001
		Within groups	1.654	52	3.180×10^{-2}		
		Total	2.767	57			
	GSH	Between groups	2.457	5	0.491	13.761	<0.001
		Within groups	1.857	52	3.571×10^{-2}		
		Total	4.314	57			
	GSSG	Between groups	2773.206	5	554.641	2.107	0.079
		Within groups	13,691.010	52	263.289		
		Total	16,464.217	57			
	Ratio	Between groups	1.903	5	0.381	8.648	<0.001
		Within groups	2.288	52	4.400×10^{-2}		
		Total	4.191	57			
	%[GSH]	Between groups	0.354	5	7.076×10^{-2}	6.702	<0.001
		Within groups	0.549	52	1.056×10^{-2}		
		Total	0.903	57			
	GR	Between groups	0.391	5	7.814×10^{-2}	10.095	<0.001
		Within groups	0.418	54	7.741×10^{-3}		
		Total	0.809	59			
	γ-GT	Between groups	0.310	5	6.209×10^{-2}	2.830	0.025
		Within groups	1.141	52	2.194×10^{-2}		
		Total	1.451	57			

[a] GSx = total glutathione; GSH = reduced glutathione; GSSG = glutathione disulfide; ratio = [GSH]/[GSSG]; GR = glutathione reductase activity; γ-GT = γ-glutamyl transpeptidase activity.

Glutathione levels, GR and γ-GT activities in most cases were intercorrelated, even between liver and muscle tissues. However, correlations observed between two variables seemed in several cases to be due to a common cause, the TTD. For example, hepatic GSH levels were positively correlated to hepatic GSH:GSSG ratio,

hepatic and muscular GR activities, but negatively correlated to muscular GSH. TTD could be the cause of these correlations because the highest GSH levels in liver were found in resistant eels to molinate, which, in fact, presented the highest hepatic GSH:GSSG ratio, the highest GR activity in liver and muscle and the lowest muscular GSH levels. Opposite was found in sensitive eels to the herbicide.

Therefore, eel survival to molinate could be dependent on the hepatic glutathione redox status, muscular GSH levels and the activity of GR. This activity, which was strongly correlated to the TTD, is also correlated to the resistance to oxidative stress in plants and animals (Kretzschmar and Klinger, 1990). In fact, brown bullhead (*Ameriurus nebulosus*) presented a markedly lower GR activity compared to channel catfish (*Ictalurus punctatus*) that allowed it to experience greater GSSG loss (via bile or plasma, for example) under conditions of oxidative stress (Hasspieler et al., 1994). Petrivalsky et al. (1997) observed a significant increase of GR activity after treatment of rainbow trout (*Oncorhynchus mykiss*) with xenobiotics that induced oxidative stress. Almar et al. (1998) observed glutathione depletion in the freshwater fishes gudgeon (*Gobio gobio*) and roach (*Rutilus arcasii*) exposed to pollution, but only an increase in GR activity in the roach. In addition, transgenic flies (*Drosophila melanogaster*) overexpressing GR showed a higher survival under elevated oxidative stress but not under normal conditions (Mockett et al., 1999).

The GSH induction in the liver of eels that died after 48 h was in agreement with the results found by Hasspieler et al. (1994) and similar to those found in eels exposed to 15 mg/l of the thiocarbamate herbicide thiobencarb Peña et al. (2000), where glutathione depletion was reviewed. Nevertheless, despite eels died between 72 and 96 h inducing their GSH levels, even higher than survivors, they succumbed possibly because they were not able to restore the GSH:GSSG ratio as well as surviving eels. Only survivors induced significantly the hepatic GR activity, thus, this activity could be crucial to determine life or death under exposure to molinate.

On the contrary, a glutathione depletion results in an increased permeability and a decreased functional intactness of the red blood cell membrane, leading to haemolytic anaemia (Uhlig and Wendel, 1992), as was observed in eels exposed to molinate. Sancho et al. (2000) found a decrease in some eel haematological parameters after exposure to the herbicide molinate, but those effects were only significantly evident 8–9 days after exposure. Kawatsu (1977) reported a severe hemorrhagic anaemia in carps (*Cyprinus carpio*) treated with molinate, but this pathophysiological condition was not found in striped bass (*Morone saxatilis*) and white sturgeon (*Acipenser transmontanus*) because they deactivate molinate more efficiently than carp (Tjeerdema and Crosby, 1988b). Hence, inborn capacities to detoxify molinate sulfoxide and maintain glutathione homeostasis against oxidative stress are probably decisive to explain resistance or susceptibility to molinate in different fish species.

In relation to γ-GT, this enzyme catalyses the removal of the glutamyl moiety in GSH or glutathione-containing compounds. As mentioned before, it is a membrane-bound enzyme with its active site oriented extracellularly, then the breakdown products generated in the intracellular space were reabsorbed back into the cell for further metabolism. Therefore, it facilitates amino acid transport, regulation of intracellular GSH levels, and catalysis of the initial step in the mercapturic acid formation (Gallagher and Di Giulio, 1992). The kidney has the highest γ-GT activity but it is also abundant in other epithelial tissues like intestine, liver and pancreas (Meister and Anderson, 1983).

In this study, γ-GT activity in the liver was not significantly different between eels, and it was 10-fold higher than that found in the American eel (*Anguilla rostrata*) by Otto et al. (1997). However, muscular γ-GT activity was greater in those eels that died until 48 h than in control ones. In fact, γ-GT activity was slightly negatively correlated to TTD ($r = 0.36$, $P < 0.05$; data not shown). Furthermore, despite the severe depletion in GSH levels in the muscle of exposed eels, differences in muscular GSH content among groups were detected, and a negative correlation to TTD was found. A possible explanation to account for these observations is that muscular γ-GT induction is responsible to capture more GSH from plasma, and then leaving less quantity to other extrahepatic tissues like kidneys, intestines, gills and brain. Glutathione levels in this latter are more crucial because brain contains relatively low levels of other antioxidant defences (e.g., catalase) and a high-lipid content (Cooper and Kristal, 1997). Therefore, a higher effect of molinate could be observed in brain, where, in addition, AChE inhibition by molinate could be stronger if the sulfoxide is not properly removed.

The acute toxicity of molinate gives the impression of being the result of an oxidative damage caused by a shift of the glutathione redox status and an impairment in GSH homeostasis. Nevertheless, Pearson correlation coefficients were so low that perhaps implies that there are more variables contributing to glutathione-dependent correlations and TTD. That could be the case of AChE inhibition, which contributes to the toxicology of this thiocarbamate (Sancho et al., 2000), and $ALDH_2$ inhibition as well (Quistad et al., 1994; Hart and Faiman, 1995), but not the case of selected immune parameters (Smialowicz et al., 1985) and haemoglobin carbamylation (Tjeerdema and Crosby, 1988a). However, molinate toxicology is possibly linked to other parameters that are currently unknown. Despite that,

more studies are necessary to understand exactly the role of glutathione metabolism in the detoxification and resistance to pesticides in different tissues.

5. Conclusions

This study demonstrates how glutathione content and enzyme-dependent activities were intercorrelated, and also correlated to the TTD. Consequently, eels that were able to keep or induce the activities of glutathione-dependent enzymes to increase GSH levels and reduce GSSG have more probabilities to resist high concentrations of molinate than those that lose glutathione homeostasis. Information analysed in this paper suggests that glutathione levels and GR activity could be good biomarkers of exposure to oxidative stress caused by molinate.

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Appendix A. Abbreviations

AChE	acetylcholinesterase
$ALDH_2$	mitochondrial low-K_m aldehyde dehydrogenase
GCS	γ-glutamylcysteine synthetase
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione or glutathione disulfide
GST	glutathione *S*-transferase
γ-GT	γ-glutamyl transpeptidase
TTD	time to death

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ARTÍCULO III

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Impaired glutathione redox status is associated with decreased survival in two organophosphate-poisoned marine bivalves

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Abstract

Biomonitoring organophosphate (OP) exposure in marine environments is generally achieved by the measurement of acetylcholinesterase activity in bivalves like mussels. However, there is evidence that indicates that oxidative stress may be implied in OP toxicity. The aim of this study was to evaluate the relationship between survival from the OP insecticide fenitrothion and glutathione levels in marine bivalves. Mussels (*Mytilus galloprovincialis* Lam.) and scallops (*Flexopecten flexuosus* Poli) were exposed, in a time to death test, to their LC_{85} of fenitrothion for 96 h. OP-poisoned mussels showed reduced (GSH) and oxidised (GSSG) glutathione depletion in the digestive gland, muscle and gills. Pectinid spats exposed to this insecticide presented GSH depletion in the digestive gland and mantle, and a reduction of the GSH/GSSG ratio in gills and mantle. Although survival curves were significantly different and mussels withstood twice as much fenitrothion as pectinid spats, muscular GSH/GSSG ratio was highly related to mortality in both species. We suggest that an impairment in the glutathione redox status could result in an induction of the cell death, either by apoptosis or necrosis, leading ultimately to the death of the organism. We conclude that whereas glutathione depletion can be used as a biomarker of exposure, the muscular GSH/GSSG ratio might be used as a biochemical marker of effect and individual susceptibility to mortality of marine bivalves exposed to fenitrothion or other pollutants that induce oxidative stress.

Keywords: Fenitrothion; Pesticides; Oxidative stress; Biomarkers; Mussels; Scallops

1. Introduction

OPs and carbamate pesticides are extensively used in agriculture. Furthermore, the treatment of ectoparasite infestations in the salmon aquaculture implies the discharge of large amounts of OPs or other chemicals in marine fish farms, which may produce a direct environmental impact (Haya et al., 2001). The primary effects of OPs on organisms are through the inhibition of acetylcholinesterase (AChE), the enzyme responsible for terminating the transmission of the nerve impulse. Therefore, the measurement of AChE activity in bivalves is commonly used to diagnose pesticide exposure in environmental monitoring studies (Livingstone, 1993; Escartin and Porte, 1997; Mora et al., 1999; Davies et al., 2001). However, OP toxicity in general implies more than AChE inhibition, since Bagchi et al. (1995) found that different classes of pesticides may induce in vitro and in vivo generation of ROS, such as H_2O_2, superoxide (O_2^-) and the hydroxyl radical (HO·). These free radicals (in particular the hydroxyl radical) are able to react with biological macromolecules and produce enzyme inactivation, lipid peroxidation and DNA damage. Hence, if these potent oxidants are not properly

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Nomenclature

GCS	γ-glutamylcysteine synthetase	OP	organophosphate
GPx	glutathione peroxidase	ROS	reactive oxygen species
GR	glutathione reductase	TTD	time to death
GSH	reduced glutathione	HR	hazard ratio
GSSG	oxidised glutathione or glutathione disulfide	RR	relative risk
GST	glutathione S-transferase	CI	confidence interval

removed by antioxidant defences, they can lead to oxidative stress (Winston, 1991). Other studies showed that OP toxicity is also mediated by an impairment of antioxidant defences due to the generation of ROS (Hai et al., 1997; Poovala et al., 1998; Banerjee et al., 1999).

It is well established that the mitochondria are the main source of ROS within the cell. ROS are generated due to physiological (by the respiration chain) or pathological (by determined diseases or pathogens) or toxicological (by inducers of oxidative stress) conditions. However, almost the only defence against ROS in this organelle is the tripeptide glutathione, which largely maintains the cellular redox status catalysed by the enzyme GPx (DeLeve and Kaplowitz, 1991). Glutathione is a ubiquitous non-protein thiol that participates in numerous cellular functions, such as the detoxification of electrophilic xenobiotics and the scavenging of ROS (Meister and Anderson, 1983). It is predominantly present in cells in its active and reduced form (GSH). However, as a consequence of oxidising conditions, two molecules of GSH are linked by a disulfide bond to comprise a molecule of oxidised glutathione (GSSG). Evidence for a ROS-mediated toxicity by oxidation of glutathione levels has been demonstrated in previous studies with fishes exposed to thiocarbamate herbicides (Peña et al., 2000; Peña-Llopis et al., 2001).

Bivalve molluscs, particularly mussels are used as 'sentinel' or 'indicator' organisms in environmental monitoring programmes throughout the world because they are widely distributed, sedentary, relatively tolerant to a wide range of environmental conditions and filter—feeders that pump large volumes of water and bioconcentrate many chemicals in their tissues (Widdows and Donkin, 1992). The aim of this study was to evaluate the association between the glutathione levels in tissues of marine bivalves and survival upon exposure to the OP pesticide fenitrothion (*O,O*-dimethyl *O*-4-nitro-*m*-tolyl phosphorothioate), which is an insecticide used extensively in agriculture for crop protection. For this purpose, mussels of the species *Mytilus galloprovincialis* (Lam.) were obtained from a marine farm of the Fangar Bay (Ebro Delta, Spain), which is exposed to a seasonal variety of pesticides from rice crops, as was reported by Escartin and Porte (1997), with fenitrothion being the most abundant OP pesticide detected (Barceló et al., 1990, 1991). Furthermore, in order to generalise the results obtained, the same TTD tests were applied to scallops of the species *Flexopecten flexuosus* (Poli). They live among seaweed and on sandy and muddy bottoms and are distributed in the central and western part of the Mediterranean, and the adjacent area of the Atlantic (Wagner, 1991).

Other survival test studies have principally focused on genetic variation in resistance to pollutants, such as heavy metals and pesticides (e.g. Hawkins et al., 1989; Moraga and Tanguy, 2000), but not physiological parameters such as antioxidants like glutathione. To our knowledge, no previous attempts have been made to evaluate the independent value of prognostic factors on mortality of marine bivalves to OP poisoning.

2. Materials and methods

2.1. Animals

Adult mussels (*M. galloprovincialis*) were collected from a suspended culture system of the Fangar Bay (Ebro Delta, Tarragona, Spain) while pectinid spats of the species *F. flexuosus* were obtained by means of filamentous collectors deployed for three months (from June to September of 1998) close to the Cabanes artificial reef off the coast of Castellón (western Mediterranean; 40° 07′ N, 0° 13′ E) at a depth of 20 m, according to Peña et al. (1998). Both species were acclimatised to laboratory conditions in 150-l aquaria of seawater (salinity: 38 g/l; drawn up from 300 m offshore and filtered through a 1-μm net) for at least three weeks at a temperature of 18 ± 1 °C with continuous aeration. Acclimation and selection of bivalves for acute toxicity tests were carried out according to US Environmental Protection Agency guidelines (USEPA, 1996a,b). Organisms were fed with a daily diet of microalgae composed mainly by *Isochrysis galbana* and *Tetraselmis suecica*, but were starved 48 h prior to the start of experiments.

2.2. Chemicals

Sumithion 50 (Sumitomo Chemical Co.), which contained 50% fenitrothion, was purchased from Argos

(Valencia, Spain). Stock solutions were prepared by dissolving Sumithion 50 in acetone (6.4 μl/l). 2-Vinylpyridine was acquired from Aldrich. GR (EC 1.6.4.2) (Type III from Baker's yeast) and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Toxicity tests

Preliminary tests were carried out in order to estimate the 96-h LC_{50} and LC_{85} for fenitrothion to use in the 96-h TTD tests in accordance with USEPA (1996a,b). Thirty-six randomly selected *M. galloprovincialis* (66–70 mm length) and 40 *F. flexuosus* (19–20 mm length) were exposed to their 96-h LC_{85} (12 and 4.7 mg/l, respectively) of fenitrothion (Sumithion 50) for 96 h under static conditions using glass aquaria (40-l volume, with 35 l of filtered seawater). Water temperature was maintained at 18 ± 1 °C. Ten mussels and 10 pectinids used as controls were kept in 35 l of clean seawater with the same concentration of solvent (acetone) (6.4 μl/l).

Bivalves were periodically inspected from 3 to 6-h intervals and were considered dead if touching the gaping shell produced no reaction. However, the mechanical stimulation of the mantle was reduced as much as possible to avoid stressing the organisms. Dead animals were immediately removed, TTD noted, weighed, length measured with a Digimatic caliper (Mitutoyo Corp., Japan) and the digestive gland, adductor muscle, gills and mantle (only in *F. flexuosus*) were excised, weighed and frozen at −85 °C. Height was also measured in pectinids because of their rounded shape. At 96 h, survivors and control bivalves were sacrificed and processed equally.

2.4. Glutathione determination

Tissue samples were homogenised following Baker et al. (1990) with five volumes of ice-cold 5% 5-sulfosalicylic acid per gram of wet weight tissue, and processed by sonication (Vibra-Cell, Sonics & Materials Inc., Danbury, CT, USA). Homogenates were then centrifuged in a Suprafuge 22 (Heraeus Sepatech, Germany) at 4 °C and 20 000 g for 10 min, and supernatant fractions were stored frozen at −85 °C.

Total glutathione content (tGSx) and oxidised glutathione (GSSG) were determined with a sensitive and specific assay using a recycling reaction of GSH with 5,5′dithiobis(2-nitrobenzoic acid) in the presence of excess GR according to Baker et al. (1990) in a microplate reader (Model 3550, Bio-Rad Laboratories, Richmond, CA, USA). Absorbance at 415 nm with a reference wavelength of 595 nm was measured every 30 s for 3 min at room temperature in accordance with Cribb et al. (1989). Samples for GSSG determination were carefully checked in a pH paper 5.5–9.0 (Merck, Darmstadt, Germany) to avoid exceeding pH 7.5. Glutathione concentrations were expressed as nmol of GSH equivalents (GSx) per gram of wet weight tissue (nmol GSx/g wwt) (GSx = [GSH] + 2[GSSG]). GSH was calculated by subtracting GSSG levels from the tGSx levels determined. GSH/GSSG ratio was expressed as number of molecules but not moles: [GSH]/[GSSG] = (tGSx − GSSG)/(GSSG/2). Standards and blanks were prepared simultaneously with the samples in the same background of buffer and acid as them.

2.5. Statistics

The end point of this study was mortality of bivalves exposed to their 96-h LC_{85} of fenitrothion during the 96 h of follow-up. However, there were bivalves still alive at the end of the TTD test. As their true survival time was greater than 96 h, such observations were said to be censored. Standard ANOVA-type and common multivariate regression methods cannot be used for survival data because of the presence of censored observations and skewing of the data (Piegorsch and Bailer, 1997).

The 96-h LC_{50} and LC_{85} were determined with the USEPA Probit Analysis Program 1.5. The SPSS 9.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for all other statistical analyses. Survival curves for each species of bivalves exposed to their 96-h LC_{85} were constructed using the Kaplan–Meier method (Kaplan and Meier, 1958) and compared by the log-rank χ^2 statistics.

Variables were checked for normality with the Kolmogorov–Smirnov test with Lilliefors significance correction, and data not normally distributed were log transformed. Comparisons between bivalves exposed to fenitrothion and controls were made using the independent samples t test. Pearson correlation coefficients were calculated among biometrical parameters and glutathione levels in different tissues of bivalves in order to measure the strength of a linear association between two variables. Bonferroni correction was applied to multiple significance tests to avoid spurious significant differences (Sokal and Rohlf, 1994).

The Cox proportional hazards regression model (Cox, 1972) was used to determine the relationship between fenitrothion mortality and glutathione levels in several tissues of marine bivalves. Unadjusted HRs were obtained from Cox proportional hazard models with only each variable as a predictor. Adjusted HRs were obtained from significant prognostic factors determined using a stepwise forward selection procedure from all covariates based on conditional parameter estimates. $P \leqslant 0.05$ and $P > 0.10$ were set, respectively, as limits for variable inclusion and exclusion. Covariates were treated as continuous variables, but also were dichotomised at the median (recoded as 0 if were lower than the

median and 1 if were greater). The assumption of proportional hazards was ensured by visual inspection of the log-minus-log survival plots of the covariates modelled as binary variables (median split).

3. Results

Preliminary acute toxicity tests, exposing mussels and pectinids to fenitrothion, allowed the estimation of the 96-h LC_{50} of 8.4 and 3.9 mg/l, and the 96-h LC_{85} of 12.01 and 4.7 mg/l in *M. galloprovincialis* and *F. flexuosus*, respectively. Therefore, 12 and 4.7 mg/l were the concentrations of fenitrothion used in the TTD test for mussels and pectinids, respectively, to ensure a low percentage of survivors at 96 h, allowing statistical comparisons within intervals of time.

Although both species of bivalves were exposed to their 96-h LC_{85}, a 67% and 77.5% mortality was observed among mussels and pectinids, respectively. Survival curves were different (log-rank $\chi^2 = 7.7$, $P < 0.01$) (Fig. 1), showing that *M. galloprovincialis* had higher resistance to fenitrothion than *F. flexuosus*.

When OP-poisoned mussels were compared to controls (Table 1), they showed GSH depletion in the muscle and the gills ($P < 0.001$). GSH depletion in the digestive gland was significant at the single test level ($P = 0.005$) but not after the Bonferroni correction ($P > 0.0045$). GSSG was also diminished in the digestive gland, muscle and gills ($P < 0.001$), with no changes in GSH/GSSG ratios. Pectinid spats exposed to fenitrothion (Table 2) showed GSH depletion in the digestive gland and mantle ($P < 0.001$), and a reduction of GSH/GSSG ratio in the mantle ($P < 0.001$). GSH levels were slightly less in the gills ($P < 0.05$), GSSG in mantle ($P < 0.05$) and GSH/GSSG ratio in the digestive gland ($P < 0.05$) and gills ($P < 0.01$), although none of these values were significant at the table-wide Bonferroni level ($P > 0.0033$). This could be due to the low statistical power resulting from small sample sizes.

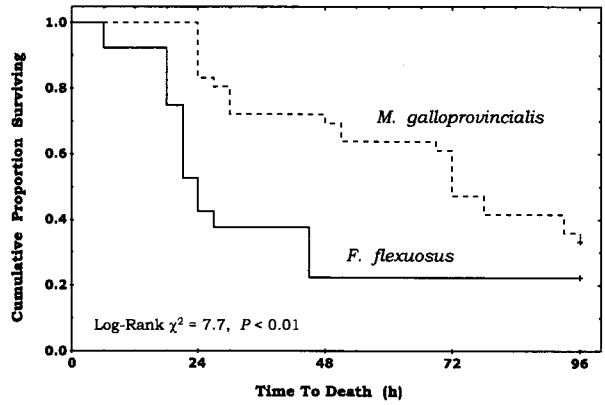

Fig. 1. Survival of bivalves exposed to their 96-h LC_{85} of fenitrothion according to the Kaplan–Meier method. Censored observations at 96 h were designated by crosses.

3.1. Bivariate and partial correlations

Mussels showed a negative correlation between GSH/GSSG ratio in the digestive gland and the muscular GSSG ($r = 0.45$, $P < 0.01$) (Table 3), hence, those

Table 1
Descriptive statistics of mussels (*M. galloprovincialis*) exposed to the 96-h LC_{85} of fenitrothion ($n = 36$) and compared to non-exposed ones ($n = 10$) by the t test[a]

Variables	Controls Mean ± SE	96-h TTD exposed mussels		
		Mean ± SE	95% CI	Median
Length (mm)	65 ± 1	68 ± 1	66–70	68
Weight (g)	13.2 ± 0.6	14.6 ± 0.5	13.7–15.6	14.5
Digestive gland				
GSH	796 ± 138	414 ± 51*	310–518	279
GSSG	168 ± 9	*97 ± 9***	80–115	88
Ratio	10.0 ± 2.4	9.2 ± 1.3	6.5–11.9	6.5
Muscle				
GSH	722 ± 83	*412 ± 32***	347–476	380
GSSG	121 ± 9	*74 ± 3***	68–80	77
Ratio	13.4 ± 2.8	11.4 ± 0.9	9.7–13.1	10.9
Gills				
GSH	228 ± 50	*52 ± 6***	38–65	42
GSSG	80 ± 10	*28 ± 2***	23–32	26
Ratio	6.2 ± 1.4	4.0 ± 0.4	3.2–4.8	4.0

*$P = 0.005$, **$P < 0.001$. Italic means indicate significant values corrected by the Bonferroni method.
[a] Ratio = [GSH]/[GSSG]. GSH and GSSG were expressed in nmol GSx/g wwt.

Table 2
Descriptive statistics of pectinid spats (*F. flexuosus*) exposed to the 96-h LC_{85} of fenitrothion ($n = 40$) and compared to non-exposed ones ($n = 10$) by the t test[a]

Variables	Controls Mean ± SE	96-h TTD exposed pectinids		
		Mean ± SE	95% CI	Median
Height (mm)	19.8 ± 0.4	19.3 ± 0.2	18.9–19.7	19.2
Length (mm)	20.8 ± 0.4	19.9 ± 0.2	19.4–20.3	20.1
Weight (g)	1.50 ± 0.16	1.10 ± 0.04	1.02–1.17	1.12
Digestive gland				
GSH	990 ± 88	*590 ± 33***	523–657	577
GSSG	531 ± 58	519 ± 25	470–569	481
Ratio	4.37 ± 0.78	2.26 ± 0.07*	2.11–2.40	2.19
Muscle				
GSH	497 ± 82	360 ± 24	311–409	329
GSSG	98 ± 17	90 ± 8	74–106	74
Ratio	11.36 ± 1.58	9.49 ± 0.71	8.05–10.9	8.28
Gills				
GSH	244 ± 31	149 ± 15*	119–180	129
GSSG	220 ± 12	191 ± 12	166–216	182
Ratio	2.18 ± 0.26	1.52 ± 0.10**	1.32–1.71	1.54
Mantle				
GSH	301 ± 34	*67 ± 7****	53–82	60
GSSG	255 ± 21	188 ± 12*	164–212	169
Ratio	2.36 ± 0.22	*0.67 ± 0.05****	0.56–0.77	0.68

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Italic means indicate significant values corrected by the Bonferroni method.
[a] Ratio = [GSH]/[GSSG]. GSH and GSSG were expressed in nmol GSx/g wwt.

mussels that presented a higher GSH/GSSG ratio in the digestive gland, tended to have less GSSG in the muscle, and vice versa. Length and weight were negatively correlated to muscular GSH ($r = -0.40$ and -0.44, respectively, $P < 0.05$), but the partial correlations controlling for TTD were non-significant (data not shown). This suggests that the correlation between muscular GSH and length and weight were related to

Table 3
Pearson correlation coefficients (r) among biometric parameters and glutathione levels from several tissues of *M. galloprovincialis* ($n = 36$) exposed to the 96-h LC_{85} of fenitrothion[a]

	Length	Weight	Digestive gland			Muscle			Gills	
			GSH	GSSG	Ratio	GSH	GSSG	Ratio	GSH	GSSG
Weight	**0.77****									
Digestive gland										
GSH	−0.002	0.04								
GSSG	0.12	0.11	**0.59****							
Ratio	0.20	0.16	**−0.66****	0.16						
Muscle										
GSH	−0.40*	−0.44*	0.24	0.13	−0.33					
GSSG	−0.37*	−0.41*	0.25	−0.09	−0.45**	0.40*				
Ratio	−0.11	−0.29	0.17	0.27	−0.10	**0.77****	−0.20			
Gills										
GSH	0.07	−0.24	0.11	0.07	−0.17	0.17	0.24	0.14		
GSSG	0.04	−0.11	0.15	0.11	−0.19	0.28	0.11	0.23	**0.61****	
Ratio	0.004	−0.23	−0.05	−0.06	−0.05	−0.05	0.19	−0.05	**0.61****	−0.16

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Bold correlation coefficients indicate significant values corrected by the Bonferroni method.
[a] Ratio = [GSH]/[GSSG].

TTD. Muscular GSSG was also negatively correlated to length ($r = -0.37$, $P = 0.041$) and weight ($r = -0.41$, $P = 0.025$), but these values were not significant after Bonferroni correction was applied.

Glutathione levels in *F. flexuosus* were highly intercorrelated (Table 4). GSH content in the digestive gland was correlated to muscular GSH ($r = 0.55$, $P < 0.001$), GSH and GSSG in the gills ($r = 0.55$ and 0.66, respectively, $P < 0.001$) and GSH and GSSG in the mantle ($r = 0.64$ and 0.74, $P < 0.001$). GSSG in the digestive gland was correlated to GSSG in gills ($r = 0.58$, $P < 0.001$) and GSH and GSSG in the mantle ($r = 0.60$ and $0.71, P < 0.001$). Muscular GSH was related to GSH and GSSG in mantle ($r = 0.56$ and $0.73, P < 0.001$). GSH in gills was correlated to GSSG in mantle ($r = 0.58$, $P < 0.001$) and GSSG in gills was positively correlated to GSH and GSSG in mantle ($r = 0.57$ and $0.65, P < 0.001$).

Other relationships were weaker and some of them seemed to be related to a common factor, TTD. That was the case of correlations between weight and GSH/GSSG ratio in the digestive gland ($r = 0.35$, $P < 0.05$) and muscle ($r = 0.39$, $P < 0.05$), between GSH/GSSG ratio in the digestive gland and GSSG content in the gills ($r = 0.42, P < 0.01$) and mantle ($r = 0.35, P < 0.05$), and between muscular GSH/GSSG ratio and GSSG in gills ($r = 0.39$, $P < 0.05$), which showed non-significant partial correlations when TTD was fixed (data not shown).

3.2. Cox proportional hazards models

The Cox proportional hazards model is a multivariate regression method used to evaluate the effect of multiple predictor independent variables (covariates) on the survival curve. The HR (or relative hazard) estimates the percentage change in risk with each unit change in the covariate. For dichotomous variables, the HR represents the RR of death between the two classes of the variable, 0 and 1. When RR > 1, risk of death rises when the covariate increases; when RR < 1, risk of death decreases when the covariate increases.

In unadjusted Cox proportional hazards models, muscular GSH and GSH/GSSG ratio in mussels (Table 5) were significantly related to survival (unadjusted RR = 0.20 and 0.24, $P = 0.0005$ and 0.0014, respectively). Mussels that presented a muscular GSH content higher than the median (380 nmol GSx/g wwt) or a GSH/GSSG ratio greater than 10.9 were 5.0 and 4.2 times, respectively, less prone to die within the 96 h of exposure to fenitrothion than mussels with these glutathione levels lower than the median. Similarly, when considered as continuous variables, these covariates were associated to survival as well ($P < 0.0001$). The wide CIs were due to the small number of mussels used in the TTD test.

Glutathione redox status in the muscle and digestive gland of pectinids (Table 6) were independent prognostic factors of survival when no other covariates were considered in the model (unadjusted RR = 0.29 and 0.31, $P = 0.0017$ and 0.0022, respectively). Similar results were obtained when they were treated as continuous variables ($P = 0.0016$ and 0.0018 for GSH/GSSG ratio in the muscle and digestive gland, respectively). GSH in the digestive gland was highly associated to survival when was treated as a continuous variable ($P = 0.0014$), but when the covariate was dichotomised at the median it became non-significant (unadjusted RR = 0.41, $P = 0.019$). Therefore, those *F. flexuosus* with muscular GSH/GSSG ratio higher than 8.28, or a GSH/GSSG ratio in the digestive gland higher than 2.19, or a GSH content in the digestive gland greater than 577 nmol GSx/g wwt were 3.4, 3.2 and 2.4 times, respectively, more likely to survive during the 96-h TTD test than those pectinids with these covariates lower than their median.

Weight of pectinid spats seemed to be related to survival (unadjusted RR = $0.47, P = 0.043$) even though, when treated as a continuous variable, this relationship was non-significant ($P = 0.11$). The same happened to GSSG content in gills, which was non-significant as a binary variable (unadjusted RR = 0.57, $P = 0.13$) and non-significant when it was treated as a continuous variable ($P = 0.021$) and corrected by the Bonferroni method. Again, this might reflect a reduced statistical power due to small sample sizes.

When all biometric parameters and glutathione levels of several tissues of bivalves were entered in a stepwise forward selection procedure (Table 7), muscular GSH and GSSG were the only significant covariates related to fenitrothion mortality in *M. galloprovincialis* ($P < 0.0001$ and $P = 0.024$, respectively) when were treated as continuous variables. When covariates were dichotomised at the median, muscular GSH exclusively was the significant prognostic factor of survival of mussels (RR = 0.20, $P = 0.0005$). On the other hand, only GSH in the digestive gland and GSH/GSSG ratio in the muscle of pectinids were associated to survival when were considered as continuous variables ($P = 0.0083$ and 0.0060, respectively). However, when covariates were treated as binary variables, only muscular GSH/GSSG ratio was significantly related to TTD in pectinids (RR = 0.29, $P = 0.0017$).

4. Discussion

Exposure of bivalves to the pesticide fenitrothion resulted, in general, in a GSH depletion in the digestive gland, muscle and gills of OP-exposed mussels and in the digestive gland and mantle of poisoned pectinids. These GSH depletions were in agreement with those reported by many authors in bivalves stressed by other chemical or natural contaminants (Suteau et al., 1988;

Table 4
Pearson correlation coefficients (r) among biometric parameters and glutathione levels from several tissues of *F. flexuosus* ($n = 40$) exposed to the 96-h LC_{85} of fenitrothion[a]

	Height	Length	Weight	Digestive gland			Muscle			Gills			Mantle	
				GSH	GSSG	Ratio	GSH	GSSG	Ratio	GSH	GSSG	Ratio	GSH	GSSG
Length	**0.89*****													
Weight	**0.59*****	**0.55*****												
Digestive gland														
GSH	0.02	0.05	0.17											
GSSG	−0.19	−0.14	0.01	**0.88*****										
Ratio	0.38*	0.32*	0.35*	**0.57*****	0.13									
Muscle														
GSH	−0.05	−0.09	0.03	**0.55*****	**0.54*****	0.26								
GSSG	−0.11	−0.10	−0.26	0.21	0.27	0.02	**0.66*****							
Ratio	0.13	0.05	0.39*	0.31	0.19	0.30	0.10	**−0.66*****						
Gills														
GSH	0.06	−0.04	0.14	**0.55*****	0.44**	0.43**	0.39*	0.20	0.20					
GSSG	0.02	−0.03	0.19	**0.66*****	**0.58*****	0.42**	0.42**	0.06	0.39*	**0.72*****				
Ratio	0.15	0.03	0.07	0.17	0.07	0.27	0.23	0.19	0.01	**0.78*****	0.20			
Mantle														
GSH	−0.18	−0.22	−0.05	**0.64*****	**0.60*****	0.29	**0.56*****	0.44**	−0.02	0.50**	**0.57*****	0.22		
GSSG	−0.09	−0.05	−0.03	**0.74*****	**0.71*****	0.35*	**0.73*****	0.51**	0.05	**0.58*****	**0.65*****	0.28	**0.75*****	
Ratio	−0.15	−0.24	0.07	0.40*	0.37*	0.17	0.31	0.24	0.05	0.40*	0.44**	0.13	**0.84*****	0.37*

*$P < 0.05$ level, **$P < 0.01$, ***$P < 0.001$. Bold correlation coefficients indicate significant values corrected by the Bonferroni method.
[a] Ratio = [GSH]/[GSSG].

Table 5
Unadjusted HRs relating survival under fenitrothion exposure to biometric parameters and glutathione levels in mussels[a]

Covariates	Continuous		Dichotomised	
	HR (95% CI)	*P*	RR (95% CI)	*P*
Length	1.055 (0.948–1.175)	0.33	1.62 (0.64–4.13)	0.31
Weight	1.145 (0.952–1.378)	0.15	1.90 (0.74–4.88)	0.18
Digestive gland				
GSH	1.000 (0.999–1.002)	0.66	0.64 (0.28–1.45)	0.28
GSSG	0.997 (0.989–1.005)	0.45	0.77 (0.34–1.72)	0.52
Ratio	1.044 (0.990–1.101)	0.11	0.74 (0.33–1.66)	0.47
Muscle				
GSH	0.991 (0.987–0.995)	*<0.0001*	0.20 (0.08–0.49)	*0.0005*
GSSG	0.992 (0.969–1.015)	0.48	0.56 (0.25–1.30)	0.18
Ratio	0.675 (0.558–0.816)	*<0.0001*	0.24 (0.10–0.57)	*0.0014*
Gills				
GSH	1.002 (0.991–1.014)	0.71	0.71 (0.32–1.59)	0.41
GSSG	0.992 (0.964–1.022)	0.61	1.23 (0.55–2.75)	0.61
Ratio	1.100 (0.906–1.336)	0.34	1.03 (0.46–2.30)	0.95

[a] Covariates were treated as continuous variables or dichotomised at the median (0 if lower than median, 1 if greater). Ratio = [GSH]/[GSSG]. Italic *P* values indicate significant ones corrected by the Bonferroni method.

Table 6
Unadjusted HRs relating survival under fenitrothion exposure to biometric parameters and glutathione levels in pectinids[a]

Covariates	Continuous		Dichotomised	
	HR (95% CI)	*P*	RR (95% CI)	*P*
Height	1.016 (0.759–1.361)	0.91	0.85 (0.42–1.72)	0.65
Length	1.006 (0.774–1.307)	0.97	0.72 (0.35–1.46)	0.36
Weight	0.283 (0.061–1.311)	0.11	0.47 (0.23–0.98)	0.043
Digestive gland				
GSH	0.996 (0.994–0.999)	*0.0014*	0.41 (0.20–0.86)	0.019
GSSG	0.997 (0.995–1.000)	0.044	0.79 (0.39–1.61)	0.52
Ratio	0.158 (0.050–0.502)	*0.0018*	0.31 (0.14–0.65)	*0.0022*
Muscle				
GSH	0.999 (0.996–1.002)	0.38	0.86 (0.42–1.75)	0.68
GSSG	1.006 (0.999–1.014)	0.10	2.05 (0.98–4.30)	0.056
Ratio	0.847 (0.765–0.939)	*0.0016*	0.29 (0.14–0.63)	*0.0017*
Gills				
GSH	0.997 (0.993–1.002)	0.19	0.63 (0.31–1.29)	0.21
GSSG	0.993 (0.987–0.999)	0.021	0.57 (0.28–1.17)	0.13
Ratio	1.005 (0.572–1.766)	0.99	0.92 (0.45–1.86)	0.82
Mantle				
GSH	0.993 (0.984–1.002)	0.15	0.65 (0.32–1.33)	0.24
GSSG	0.996 (0.990–1.001)	0.12	0.69 (0.34–1.40)	0.30
Ratio	0.434 (0.139–1.356)	0.15	0.56 (0.27–1.16)	0.12

[a] Covariates were treated as continuous variables or dichotomised at the median (0 if lower than median, 1 if greater). Ratio = [GSH]/[GSSG]. Italic *P* values indicate significant ones corrected by the Bonferroni method.

Viarengo et al., 1990; Regoli and Principato, 1995; Doyotte et al., 1997; Regoli et al., 1997; Canesi et al., 1999; Cossu et al., 2000). We have also found that glutathione levels among mussel tissues were scarcely intercorrelated. In pectinids, glutathione content (particularly GSH and GSSG) showed abundant significant correlations among different tissues. Although some of the weak correlations were due to the TTD, the strongest correlations indicate that glutathione levels in every pectinid were balanced among tissues. Thus, a

Table 7
Adjusted HRs from the most related covariates to fenitrothion survival in *M. galloprovincialis* and *F. flexuosus*[a]

Covariates	Continuous		Dichotomised	
	HR (95% CI)	*P*	RR (95% CI)	*P*
Mussel				
Muscle				
GSH	0.988 (0.983–0.993)	<0.0001	0.20 (0.08–0.49)	0.0005
GSSG	1.033 (1.004–1.062)	0.024		
Pectinid				
DG GSH	0.997 (0.995–0.999)	0.0083		
Muscle ratio	0.869 (0.786–0.961)	0.0060	0.29 (0.14–0.63)	0.0017

[a] Covariates were treated as continuous variables or dichotomised at the median (0 if lower than median, 1 if greater). Ratio = [GSH]/[GSSG]. DG, digestive gland.

scallop with high GSH content in the digestive gland will probably have high GSH levels in the muscle, gills and mantle, and vice versa, although this linearity was not so clear in mussels. This lack of interorgan glutathione homeostasis in mussels is unclear and may be related to a variable turnover of GSH within these bivalves.

Glutathione levels were also correlated to TTD. Muscular GSH and GSH/GSSG ratio were related to the survival of fenitrothion-exposed mussels when each covariate was considered solely in the Cox regression model. Therefore, the higher the muscular GSH or GSH/GSSG ratio is, the longer mussels are expected to live under fenitrothion exposure. However, when all covariates were taken together following a stepwise forward selection method, muscular GSH and GSSG levels (as continuous variables) or muscular GSH (as dichotomised variables) were the only significant covariates related to survival. The muscular GSH/GSSG ratio was discarded from the model because muscular GSH had the lowest significance level for inclusion in the model and entered first in the equation. As the contribution of each variable to the regression model is determined in the context of the contribution of all other variables in the model, the inclusion of GSH has the effect of excluding the GSH/GSSG ratio in muscle since they are not independent covariates. Hence, muscular GSH/GSSG ratio can also be considered as significantly related to survival in mussels. Correspondingly, muscular GSH/GSSG ratio and GSH or GSH/GSSG ratio in the digestive gland were the most related covariates to fenitrothion survival of pectinids.

Mussels exhibited twofold greater 96-h LC_{50} and LC_{85} values than pectinids, and when both species of bivalves were exposed to their LC_{85} of fenitrothion for 96 h, pectinids exhibited a higher and earlier mortality than mussels. These different tolerances to fenitrothion have three possible explanations. Firstly, these can be due to differences in the inborn capacity of each species to detoxify pollutants, e.g. through different basal activities of key enzymes, as was found in two benthic fishes by Hasspieler et al. (1994). Secondly, the differences in the development stage and size between adult mussels and pectinid spats can contribute to fenitrothion metabolisation. Thirdly, as mussels had been living under a seasonal exposure of OPs, they may present a degree of adaptation to OP pesticides, as was suggested by Hoare et al. (1995) in copper exposed mussels. Nevertheless, although both species of bivalves exhibited a different tolerance to fenitrothion, they presented the muscular GSH/GSSG ratio as one of the most prognostic factors of fenitrothion survival.

Our study has two main limitations: Firstly, the rather small number of individuals used in TTD tests. Perhaps for that reason only the strongest covariates related to survival were detected whereas weaker ones might have not been perceived. Secondly, the fact that glutathione levels were measured on dead organisms that were processed at intervals of time instead of every real death time. Although tissues analysed in this study were not especially rich in the enzyme γ-glutamyl transferase, which is the only protease that cleaves GSH and GSSG, total glutathione levels may decrease if they are not processed rapidly (Anderson, 1985). Furthermore, since GSH oxidises non-enzymatically, a diminution in glutathione levels and autooxidation of GSH are expected in bivalves from their death time to the time their tissues were excised and frozen at −85 °C. We performed an extra experiment in order to test the effect of mortality on possible glutathione loss and oxidation. We compared the glutathione levels in one piece of the digestive gland and adductor muscle of five mussels sampled immediately after death with other pieces of tissues of the same mussels maintained in clean seawater for 3, 6 and 9 h after being sacrificed. We found no significant differences in the digestive gland, but GSH and GSSG were diminished in the muscle of mussels maintained in seawater after death (Dunnet's test, $P < 0.05$; data not shown), whereas glutathione redox

status was slightly oxidised at 9 h but not significantly different. Therefore, it seems that the interval of time from the death of the bivalve to its dissection results in a decrease in the muscular GSH and GSSG levels, but not a significant oxidation of GSH, while the digestive gland was practically unaffected. These effects of mortality on glutathione levels may add some noise to the correlations obtained but it does not appear to alter them essentially. Despite these limitations, this study is the first demonstration relating antioxidants to the survival of marine bivalves poisoned with OPs like fenitrothion. More studies need to be conducted to discover other biomarkers related to the survival from pollutants that induce oxidative stress to finally use them in biological monitoring.

Biomonitoring studies are complicated by the existence of seasonal variations in pollutant concentrations and natural factors, such as temperature, food availability and reproductive status, which affect the biochemical processes involving specific bioindicator molecules (Sheehan and Power, 1999). GSH follows a seasonal variation in the digestive gland of mussels, as well as other antioxidants, that seems to be at their lowest in winter when there is less food available and gonad resting (Viarengo et al., 1991; Porte et al., 2000).

Another complication with GSH levels is that glutathione metabolism is regulated by several well-known enzymes (Meister and Anderson, 1983). Hence, cellular GSH content is not a constant value and it depends on the balance between synthesis rate (by L-GCS and glutathione synthase), conjugation rate (by GSTs), oxidation rate (nonenzymatically or by GPx) and the GSSG reduction to GSH (by GR). The GSH synthesis is regulated in part by a non-allosteric competitive feedback inhibition of GCS by GSH (Richman and Meister, 1975). Therefore, GSH consumption produces an enzymatic activation of GCS to restore its decrease, but if this activation is maintained several days, that could increase GSH levels even higher than non-exposed individuals, as was found by Yan et al. (1997) in the green mussel *Perna viridis* in response to heavy metals. This GSH induction was also found in fish studies (Stein et al., 1992; Hasspieler et al., 1994; Peña et al., 2000; Peña-Llopis et al., 2001). The conjugation of GSH to electrophilic compounds like fenitrothion by GSTs leads to the detoxification and excretion of the pesticide, but if this process is not associated with GSH synthesis de novo by GCS, GSH could be depleted. In addition, if there is oxidative stress, GSH oxidises to GSSG by GPx or non-enzymatically to remove ROS and hydroperoxides. This GSSG is then reduced to GSH by GR at the expense of oxidising NADPH to $NADP^+$, which is recycled by the pentose phosphate pathway. However, if the generation of GSSG is higher than the reduction back to GSH by GR, GSSG accumulates within the cytosol and then is translocated outside the cell by specific transporters (Sies et al., 1972; Kaplowitz et al., 1996) to protect the cell from a shift in the redox equilibrium. Consequently, this fact also implies a depletion of the glutathione pool.

As was discussed previously (Peña-Llopis et al., 2001), an exclusively cytosolic GSH depletion does not injure cells because GSH is retained in mitochondria by high affinity transporters (Mårtensson et al., 1990). Nonetheless, if there is a cytosolic GSH depletion in conjunction with oxidative stress, the GSH pool in mitochondria oxidises and decreases. As the mitochondrion is unable to export GSSG (Olafsdottir and Reed, 1988) and synthesise GSH (Griffith and Meister, 1985), this organelle may be more susceptible than the rest of the cell to the consequences of a shift in the redox state and GSH depletion (DeLeve and Kaplowitz, 1991). That was confirmed by Conners and Ringwood (2000) who reported that oysters that were glutathione depleted by the GCS inhibitor buthionine sulfoximine showed non-toxic effects whereas when this GSH depletion was accompanied by an exposure to copper (a ROS generator), there was an increase in the lipid peroxidation and indices of lysosomal membrane destabilization in the digestive gland of these bivalves.

Nevertheless, the molecular processes from the exposure to the OP pesticide to the death of the organism are still unclear. Carlson et al. (2000) suggested that cytotoxicity of OP compounds could be due to induction of programmed cell death (apoptosis) by multifunctional pathways, including mitochondrial permeability pores (the opening of which can lead to mitochondrial depolarisation and dysfunction), muscarinic receptor activation, receptor-mediated caspase activation, or serine protease activation. Furthermore, Domenicotti et al. (2000) found an apoptotic pathway dependent on glutathione depletion: a shift in cellular redox state activates protein kinase C novel isoforms and then induces nuclear binding of the redox-sensitive transcription factor, activator protein-1 (AP-1), leading to apoptosis. In fact, many cellular signalling molecules (such as protein tyrosine and serine/threonine kinases and phosphatases, Ca^{2+} channels and transporters, and transcription factors like NF-κB, *p*53 and AP-1) are regulated by the redox state of cells, although signalling systems regulate the cellular redox state as well (Kamata and Hirata, 1999). That relationship between tyrosine kinase-mediated signal transduction and cellular redox balance was also suggested by Canesi et al. (2000) in *M. galloprovincialis* cells. Therefore, an important oxidation of the glutathione redox status of bivalves by fenitrothion might alter transcriptional responses to induce cell death, possibly by an apoptotic mechanism though a necrotic mechanism is more likely at higher doses of ROS because the machinery necessary for a programmed cell death is overcome (Jones et al., 1995). This glutathione redox status can be indicative of the bio-

logical status of the cell, as was reviewed by Schafer and Buettner (2001). Increasing the half-cell reduction potential (E_{hc}) of the GSSG/2GSH couple may move cells through different biological stages, such as proliferation, differentiation, apoptosis and, at very high values of E_{hc}, necrosis. Hence, as muscular glutathione redox status was highly related to the TTD of bivalves, it could be used as a biomarker of the oxidative stress effects.

On the other hand, correlations of TTD with glutathione levels, especially muscular glutathione redox status, suggest that those marine bivalves that were not able to maintain glutathione homeostasis were more susceptible to die under fenitrothion exposure than those that presented a higher muscular GSH/GSSG ratio. This could be explained by the presence of different polymorphisms among the populations of mussels and scallops. Resistant bivalves are more likely to maintain glutathione homeostasis because they may have increased synthesis of GSH by GCS and/or increased GR activity and/or higher capacity to supply NADPH by the pentose phosphate pathway. In fact, Voehringer et al. (2000), using DNA microarrays, found that cells resistant to apoptosis preserved high intracellular pools of GSH by enhancing pathways for establishment and maintenance of high intracellular redox status. Therefore, the muscular glutathione redox status could also be used as a biochemical marker of susceptibility to mortality in heavily polluted areas.

Although GSH depletion can be used as a biomarker of exposure, its levels might be conditioned by extrinsic factors, as mentioned before. However, the GSH/GSSG ratio is less likely to be influenced by seasonal variations since its levels are balanced by the glutathione metabolism. When this metabolism is impaired and is not rapidly restored, oxidative stress prevails and may finally lead to the death of the organism. The potential of muscular glutathione redox status as a biomarker of effect and susceptibility is presented. However, more studies are necessary to validate it to be used in combination with a battery of biomarker techniques that are currently used to monitor marine environments (Davies et al., 2001).

5. Conclusions

Marine bivalves studied showed an impairment of their muscular GSH/GSSG ratio that was highly related to fenitrothion TTD, possibly through an apoptotic or necrotic process. In addition, this association seemed to be independent of the species, possible adaptation to OPs and developmental stage of mussels (*M. galloprovincialis*) and scallops (*F. flexuosus*). In summary, as glutathione determinations are considerably fast and inexpensive, muscular GSH/GSSG ratio might be included in biological monitoring programmes as a biochemical marker of effect and susceptibility of bivalves to oxidative stress generated by fenitrothion contamination and, by extension, to complex mixtures of oxidant stressors when the biomonitoring area is heavily polluted.

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ARTÍCULO IV

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Fish tolerance to organophosphate-induced oxidative stress is dependent on the glutathione metabolism and enhanced by *N*-acetylcysteine

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Abstract

In previous studies we have demonstrated the importance of glutathione (GSH) metabolism in the resistance of the European eel (*Anguilla anguilla* L.) to thiocarbamate herbicides. The present work studied the effects of the antioxidant and glutathione pro-drug *N*-acetyl-L-cysteine (NAC) on the survival of a natural population of *A. anguilla* to a lethal concentration of the organophosphorus (OP) insecticide dichlorvos (2,2-dichlorovinyl dimethyl phosphate, DDVP), through the physiological point of view of the glutathione metabolism and the activities of acetylcholinesterase and caspase-3 as biomarkers of neurotoxicity and induction of apoptosis, respectively. Fish pre-treated with NAC (1 mmol kg^{-1}, ip) and exposed to 1.5 mg l^{-1} (the 96-h LC_{85}) of dichlorvos for 96 h in a static-renewal system achieved an increase of GSH content, GSH/GSSG ratio, hepatic glutathione reductase, glutathione *S*-transferase, glutamate:cysteine ligase, and γ-glutamyl transferase activities, which ameliorated the glutathione loss and oxidation, and enzyme inactivation caused by the OP pesticide. Although NAC-treated fish presented a higher survival and were two-fold less likely to die within the 96 h of follow-up, Cox proportional hazard models showed that hepatic GSH/GSSG ratio was the best explanatory variable related to survival. Then, tolerance to a lethal concentration of dichlorvos can be explained by the individual capacity to maintain and improve the hepatic glutathione redox status. Impairment of the GSH/GSSG ratio can lead to excessive oxidative stress and inhibition of caspase-3-like activity, inducing cell death by necrosis, and ultimately resulting in the death of the organism. As NAC increased the tolerance to dichlorvos, it could be a potential antidote for OP poisoning, complementary to current treatments.

Keywords: dichlorvos, organophosphorus pesticide, necrosis, glutathione redox status, biomarkers, time-to-death test.

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1. Introduction

Dichlorvos (2,2-dichlorovinyl dimethyl phosphate; DDVP) is a relatively non-persistent organophosphate (OP) compound that undergoes fast and complete hydrolysis in most environmental compartments and is rapidly degraded by mammalian metabolism (WHO, 1989). These characteristics made it attractive for worldwide use to control insects on crops, household, stored products, and treat external parasitic infections of farmed fish, livestock, and domestic animals. In fact, dichlorvos has extensively been used to treat sea lice infestations (by the copepod parasites *Lepeophtheirus salmonis* and *Caligus elongatus*) in the Atlantic salmon (*Salmo salar*) culture.

The primary effects of dichlorvos and other OPs on organisms are through the inhibition of the enzyme acetylcholinesterase (AChE), which is responsible for terminating the transmission of the nerve impulse. However, OP toxicity in general implies more than AChE inhibition, since Bagchi et al. (1995) found that different classes of pesticides may induce *in vitro* and *in vivo* generation of reactive oxygen species (ROS). They are mainly hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and hydroxyl radical (HO·), which are able to react with biological macromolecules (specially the hydroxyl radical) and produce enzyme inactivation, lipid peroxidation and DNA damage. The balance between ROS production and antioxidant defences determines the degree of oxidative stress. In fact, dichlorvos was shown to induce oxidative stress effects in two species of fish (Hai et al., 1997).

In previous studies we demonstrated that thiocarbamate herbicides induced oxidative stress in the European eel (*Anguilla anguilla* L.) and we highlighted the importance of glutathione metabolism in the tolerance to these pesticides (Peña-Llopis et al., 2000; 2001). Glutathione is a ubiquitous thiol-containing tripeptide that is involved in numerous processes that are essential for normal biological function, such as DNA and protein synthesis (Meister and Anderson, 1983). It is predominantly present in cells in its active and reduced form (GSH), which contributes to the removal of reactive electrophiles (like those activated metabolites formed by the cytochrome P-450 system) by glutathione *S*-tranferases (GSTs). GSH also scavenges ROS, catalysed by glutathione peroxidase (GPx) or non-enzymatically, through oxidation of two molecules of GSH to a molecule of glutathione disulphide (GSSG). Then, the GSH/GSSG ratio or glutathione redox status is considered an index of the cellular redox status and a biomarker of oxidative damage, because glutathione maintains the thiol-disulphide status of proteins, acting as a redox buffer.

Glutathione levels are regulated by several enzymes (Meister and Anderson, 1983), but mainly depend on the balance between GSH synthesis rate (by glutamate:cysteine ligase [GCL], also known as γ-glutamylcysteine synthetase, which catalyses the rate limiting step in which the amino acid L-cysteine is linked to L-glutamate), conjugation rate (by GSTs), oxidation rate (non-enzymatically or by GPx), and GSSG reduction to GSH by glutathione reductase (GR) through oxidation of NADPH to $NADP^+$, which is recycled by the pentose phosphate pathway. In extrahepatic tissues, high GSH concentrations are also maintained by γ-glutamyl transferase (γGT, also known as γ-glutamyl transpeptidase), which is the only protease that can cleave intact GSH and GSH-conjugates (Curthoys and Hughey, 1979), because, as it is mainly a membrane-bound enzyme with its active site oriented on the outer surface of the cell membrane (Horiuchi et al., 1978), it enables resorption of extracellular GSH catabolites from plasma. Therefore, as we found previously that those eels that enhanced GR activity, increased GSH, and maintained the GSH/GSSG ratio in liver showed a higher survival upon herbicide exposure (Peña-Llopis et al., 2001), a drug that could increase the GSH content and act as a reductant would improve the survival of OP-poisoned fish.

N-Acetyl-L-cysteine (NAC) is a known antioxidant and free radical scavenger that can easily be deacetylated to L-cysteine, the limiting amino acid for glutathione synthesis. NAC is used clinically to treat several diseases related to oxidative stress and/or glutathione deficiency such as paracetamol (acetaminophen) overdose (Prescott et al., 1977), VIH infection (De Rosa et al., 2000), and lung (Gillissen and Nowak, 1998) and heart (Sochman, 2002) diseases. It has also shown usefulness in acute heavy metal (Ballatori et al., 1998; Gurer and Ercal, 2000) and paraquat (Hoffer et al., 1996) poisoning.

OPs are also capable to induce programmed cell death (apoptosis) by multifunctional pathways (Carlson et al., 2000). Apoptosis is a complex

process characterised by a cell shrinkage, chromatin condensation, and internucleosomal DNA fragmentation that allows unwanted or useless cell removal by phagocytosis, preventing an inflammatory response to the intracellular components. Caspases are a family of cysteine proteases that are present in cytosol as inactive pro-enzymes but become activated when apoptosis is initiated, playing an essential role at various stages of it (Cohen, 1997). Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many structural and regulatory proteins (e.g., poly[ADP-ribose] polymerase [PARP], lamins, cytokeratins, etc.). However, at higher stressing conditions cellular impairment is so high that apoptosis is suppressed, leading to cell death by necrosis, which is characterised morphologically by cellular swelling and ultimately rupture of plasma membrane, causing further tissue damage and intense inflammatory response.

Tolerance to pollutants generally follows a log-normal distribution (as is assumed in the Probit model for dose-response relationships). Two hypotheses have been proposed to explain it. Gaddum (1953) introduced the concept of individual tolerance or individual effective dose, where every individual has an innate background to tolerate a pollutant until it receives a dose equal to or greater than its individual effective dose, and then dies. Conversely, mortality can be the result of stochastic processes occurring within all individuals with the chance of death being essentially the same for all individuals receiving a dose. Although the first hypothesis remained universally accepted until recently (Newman and McCloskey, 2000), tolerance to pollutants and/or oxidative stress have principally been focused on studies about genetic variations of natural populations (e.g., Sullivan and Lydy, 1999) or antioxidant defences of different species (Hasspieler et al., 1994; Hansen et al., 2001) or strains (Mathews and Leiter, 1999), but not the effect of antioxidant defences on the survival of a natural population exposed lethally to a pollutant.

Evidence demonstrates great similarities in the types of oxidative damage and defences between mammalian and lower vertebrates like fish (Kelly et al., 1998). This fact allows them to be a more 'ethically sound' alternative system to perform oxidative stress studies. The aim of this study is to evaluate the effect of the antioxidant NAC on dichlorvos survival of a genetically diverse population of European eels through the physiological point of view of the glutathione metabolism, in addition to the use of AChE and caspase-3 activities as biomarkers of neurotoxicity and induction of apoptosis, respectively.

2. Materials and methods

2.1. Animals

Young yellow eels of the species *Anguilla anguilla* (5-15 g weight), which were sexually undifferentiated at this stage of development, were used to avoid the effects of sex variation and minimise hormonal interactions in toxicity assays. These European eels were obtained from a fish farm (Valenciana de Acuicultura S.A., Spain) free of any disease. Acclimation and selection of fish for acute toxicity tests were carried out according to OECD guidelines (1992). Animals were kept for two weeks before the starting of experiments in aerated and filtered dechlorinated freshwater (total hardness: 192 ± 5 mg l^{-1} as $CaCO_3$; pH: 7.5 ± 0.1; dissolved oxygen: 7.2 ± 0.1 mg l^{-1}) at 24.0 ± 0.5 °C, and with a 12-h photoperiod.

2.2. Chemicals

Hexipra Solucion®, an emulsifiable concentrate which contained 40% of dichlorvos, 8% of emulgators, and 47% of non-toxic solvents, composed principally by 2-propanol, was obtained from Laboratorios Hipra S.A. (Girona, Spain). 2-vinylpyridine was acquired from Aldrich. NADPH was purchased from Applichem (Darmstadt, Germany). *N*-Acetyl-L-cysteine and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless mentioned otherwise.

2.3. N-Acetylcysteine supplementation assay

Fish received a single ip injection of either 1 mmol kg^{-1} NAC or its vehicle (physiological saline). This amount of NAC was used in order to induce GSH synthesis beyond physiological levels. Five animals were removed from water at 3, 12, 24, 48, 72, and 96 h after the injection, and were anaesthetised in ice instead of using a chemical anaesthesia because it could interfere with

glutathione metabolism (Brigelius et al., 1982). They were then weighed, the lengths measured, and were euthanised by decapitation. The liver and muscle were excised, weighed and stored frozen at –80 °C until the biochemical determinations were performed.

2.4. Time-to-death (TTD) static-renewal tests

Mortality within 96 h was the main end point of this study. In order to ensure a low percentage of survivors at 96 h in the TTD tests, preliminary acute toxicity tests were performed in accordance with OECD guidelines (1992) to estimate the lethal concentration that causes 85% mortality at 96 h (96-h LC_{85}). Fish were exposed to different nominal concentrations of dichlorvos at 24.0 ± 0.5 °C in a static-renewal system, where water and pesticide were completely replaced every 24 h in 40-l glass aquaria. These concentration-effect experiments indicated that the median lethal concentration at 96 h (96-h LC_{50}) for dichlorvos in the European eel was 0.852 mg l^{-1} (95% Confidence Interval [CI], 0.735 - 0.957), and the 96-h LC_{85} was 1.498 mg l^{-1} (95% CI, 1.378 - 1.774). This latter concentration (1.5 mg l^{-1}) was then used in the TTD tests to expect a mortality of 85%. This nominal concentration of dichlorvos includes 1.7 mg l^{-1} of 2-propanol, but as the 96-h LC_{50} of this solvent for freshwater fish ranged from 4,200 to 11,130 mg l^{-1} (WHO, 1990), the toxicity of Hexipra Solucion® observed was virtually due exclusively to dichlorvos.

A hundred randomly selected eels were separated into two groups. Fifty ice-anaesthetised fish were injected ip with 1 mmol kg^{-1} NAC, whereas the other fifty were only injected with the same amount of saline and were assigned to four 40-l tanks, receiving 25 fish each. After 3 h of recovery in clean water, the fish were exposed to 1.5 mg l^{-1} of dichlorvos for 96 h in semi-static conditions as mentioned before, where water and pesticide were completely replaced once a day. Water temperature was recorded every 3 h and maintained at 24.0 ± 0.5 °C in all tanks during the experiment. Fish were continually inspected at 3-h intervals, but during the first 24 h they were checked every 90 minutes because a higher mortality was expected. Dead animals were immediately removed, the TTD noted, weighed, the length measured and the liver and muscle were excised, weighed and stored frozen at –80 °C. At 96 h, survivors were anaesthetised with ice and processed as previously described. The same TTD experiment was replicated again in order to have 100 NAC-treated and 100 non-treated fish, and then gain statistical power.

2.5. Glutathione determination

Tissue samples were homogenised with 5 volumes of ice-cold 5% 5-sulfosalicylic acid per gram of wet weight tissue, and further processed by sonication (Vibra-Cell, Sonics & Materials Inc., Danbury, CT, USA). Homogenates were then centrifuged at 20,000*g* for 10 minutes at 4°C. Total glutathione content (tGSx) and oxidised glutathione (GSSG) were determined in supernatant fractions with a sensitive and specific assay using a recycling reaction of GSH with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of excess glutathione reductase according to Baker et al. (1990) in a microplate reader (Model 3550, Bio-Rad Laboratories, Richmond, CA, USA) as previously described (Peña-Llopis et al., 2001). Glutathione concentrations were expressed as nmol of GSH equivalents (GSx) per mg of protein (GSx = [GSH] + 2 · [GSSG]). GSH was calculated by subtracting GSSG levels from the tGSx levels determined. GSH/GSSG ratio was expressed as number of molecules but not moles: GSH/GSSG = (tGSx - GSSG) / (GSSG / 2).

2.6. Kinetic Enzyme Assays

Liver and muscle tissues were homogenised with 5 and 4 volumes, respectively, of Henriksson stabilising medium (Henriksson et al., 1986), which contained 50% glycerol, 20 mM phosphate buffer pH 7.4, 0.5 mM EDTA, and 0.02% defatted bovine serum albumin. β-Mercaptoethanol was not included because it interferes with the GR assay. Homogenates were centrifuged at 20,000*g* for 10 minutes at 4 °C, and the resulting supernatants were diluted 5 or 10-fold with buffer and assayed rapidly for enzyme activities.

2.6.1. AChE (EC 3.1.1.7) activity. AChE activity was determined at 415 nm with acetylthiocholine as substrate in accordance to an adaptation of the Ellman method (Ellman et al., 1961) to microtiter plates by Doctor et al. (1987), but with 0.1 M phosphate buffer, pH 7.27 and 1 mM EDTA as recommended by Riddles et al. (1979). Eel

cholinesterase activity detected in muscle was considered as true AChE as was previously characterised (Lundin, 1962; Ferenczy et al. 1997).

2.6.2. GR (EC 1.6.4.2) activity. The method of Cribb et al. (1989) was used to assay the GR activity through the increase of absorbance at 415 nm with reference wavelength at 595 nm. The final concentrations of 0.075 mM DTNB, 0.1 mM NADPH, and 1 mM GSSG were used in accordance with Smith et al. (1988).

2.6.3. GST (EC 2.5.1.18) activity. GST activity was measured by the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) according to Habig et al. (1974). The assay mixture contained 100 mM potassium phosphate buffer, pH 6.5, 1 mM CDNB in ethanol, and 1 mM GSH. The formation of the adduct of CDNB, *S*-2,4-dinitrophenyl glutathione, was monitored by measuring the rate of increase in absorbance at 340 nm with a Multiskan Ascent microplate reader.

2.6.4. γGT (EC 2.3.2.2) activity. γGT activity was determined by the method of Silber et al. (1986). The rate of the substrate analogue γ-glutamyl-*p*-nitroanilide cleavage to form *p*-nitroaniline (pNA) by transfer of a glutamyl moiety to glycylglycine was monitored at 405 nm for at least ten minutes.

2.6.5. GCL (EC 6.3.2.2) activity. GCL activity assay was adapted to microtiter plates from the indirect method of Seelig and Meister (1985), which utilises the coupled reaction of pyruvate kinase (PK) and lactate dehydrogenase (LDH) to determine the rate of formation of ADP by GCL through the oxidation of NADH. Each well contained 0.1 M Tris–HCl buffer, pH 8, 150 mM KCl, 2 mM EDTA, 20 mM $MgCl_2$, 5 mM ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 0.2 mM NADH, 7 U/ml PK, and 10 U/ml LDH. Enzyme activity was evaluated by following the decrease in the absorbance of NADH at 340 nm at 25 °C with the Multiskan Ascent microplate reader.

A calibration curve of known activities of purified enzymes was used on every 96-well plate to avoid miscalculations that stem from an ill-defined path length. AChE (type V) from electric eel, GR (type III) from baker's yeast, GST from equine liver, and γGT (type I) from bovine kidney were used as standards, whose activities were determined in quartz cuvettes using a Hitachi U-2001 UV/Vis spectrophotometer (Hitachi Instruments Inc., USA). A molar absorption coefficient at 412 nm (ε_{412}) of 14.150 was used for the dianion of DTNB (TNB^{2-}) as Riddles et al. (1979) determined. As no purified GCL enzyme was available, several samples were used as standards and their activity were validated by the spectrophotometer. Reliability of AChE and γGT assays was verified with the standard ACCUTROL™ Normal. Specific enzyme activities were expressed as nmoles of substrate hydrolysed per min per milligram of protein (mU mg^{-1} prot).

2.7. Caspase-3 assay

Caspase-3 activity was measured in 96-well plates using the Sigma caspase-3 colorimetric assay kit according to the manufacturer's instructions. The hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) to release pNA was monitored at 405 nm and calculated using a pNA calibration curve, whose concentrations were determined with a spectrophotometer. Sealed and light preserved microplates were incubated at 25°C for several days in order to detect extremely low enzyme activities. Pseudo-zero-order kinetics was ensured by plotting absorbance against time on every well. Recombinant human caspase-3 was used as a positive control to validate results. Specific enzyme activity was expressed as pmoles of Ac-DEVD-pNA hydrolysed per min per mg protein (μU mg^{-1} prot). The DEVDase activity measured was considered as caspase-3-like because caspase-7 is another key executioner of apoptosis that has similar function and substrate specificity to caspase-3 (Fernandes-Alnemri et al., 1995).

2.8. Protein Determination

Protein content was determined by the Bio-Rad Protein Assay kit (Bio-Rad Laboratories GmbH, Munich, Germany) based on the Bradford dye-binding procedure, using bovine serum albumin as standard.

2.9. Statistics

The 96-h lethal concentrations (LC_{50} and LC_{85}) were determined with the Probit Analysis procedure using the SPSS 10.0 statistical software package (SPSS Inc, Chicago, IL, USA), which was used for

all the other statistical analyses. Survival curves were constructed using the Kaplan-Meier method (Kaplan and Meier, 1958) and compared by the log-rank χ^2 statistics. Two-factor analysis of variance (ANOVA) with the type III sum-of-squares method was used to investigate the effect of pre-treatment and pesticide exposure and their interaction on studied variables. The time dependence of variables after NAC injection in controls was also tested by the two-way ANOVA. *A priori* contrasts between selected single levels of factors were made to compare means. Variables with heterogeneity of variances, according to the Levene test, were properly transformed. Pearson correlation coefficients were calculated among all studied parameters of dichlorvos-exposed eels in order to measure the strength of a linear association between two variables. These relationships were also tested after removing the effect of TTD by means of partial correlations. These variables were checked for normality with the Kolmogorov-Smirnov test with Lilliefors significance correction, and data not normally distributed were appropriately transformed. Sequential Bonferroni correction was applied to multiple significance tests to avoid spurious significant differences (Rice, 1989). As standard ANOVA-type and common multivariate regression methods cannot be used for survival data because of the presence of censored observations and skewing of the data (Piegorsch and Bailer, 1997), the Cox proportional hazards regression model (Cox, 1972) was used to determine the relationship between dichlorvos mortality and studied variables. Unadjusted hazard ratios were obtained from univariate Cox proportional hazard models. Adjusted hazard ratios were obtained from significant explanatory variables determined using a multivariate stepwise forward selection procedure from all covariates based on conditional parameter estimates. $p \leq 0.05$ and $p > 0.10$ were set, respectively, as limits for variable inclusion and exclusion. These covariates were then adjusted for the effect of length and weight. The assumption of proportional hazards was ensured by visual inspection of the smoothed plots of scaled Schoenfeld residuals (Schoenfeld, 1982) versus survival time, in accordance with Hess (1995), and the plots of martingale residuals against the covariates.

3. Results

3.1. Dichlorvos mortality

Mortality observed upon exposure to the 96-h LC_{85} of dichlorvos was 92% for eels pre-treated with saline in the first replicate, whereas it was of 86% in NAC-pre-treated fish. In the second replicate of the experiment a 90 and 84% of mortality, respectively, was found. Replicates showed no different survival curves when compared stratifying for treatment (log-rank $\chi^2 = 0.6$, $p = 0.43$). Aquaria also did not affect survival in saline- (log-rank $\chi^2 = 0.6$, $p = 0.90$) nor NAC-treated fish (log-rank $\chi^2 = 1.8$, $p = 0.60$), thus data of replicates and aquaria were pooled. Then, only nine of the 100 fish injected with the vehicle survived within the 96 h of follow-up, with a mean survival of 25 h (95% CI, 20 - 30), while 15 of the 100 fish pre-treated with NAC survived, with a mean survival of 34 h (95% CI, 28 - 41). Therefore, eels pre-treated with 1 mmol kg^{-1} of NAC presented a 66.7% higher survival than non-treated ones (log-rank $\chi^2 = 7.8$, $p < 0.005$; Fig. 1), which was more evident within the first 24 h.

3.2. Effect of NAC and/or dichlorvos on biochemical parameters

Basically, fish exposure to the 96-h LC_{85} of dichlorvos resulted in a decrease of the hepatic and muscular GSH levels ($p < 0.001$; Table 1), but a

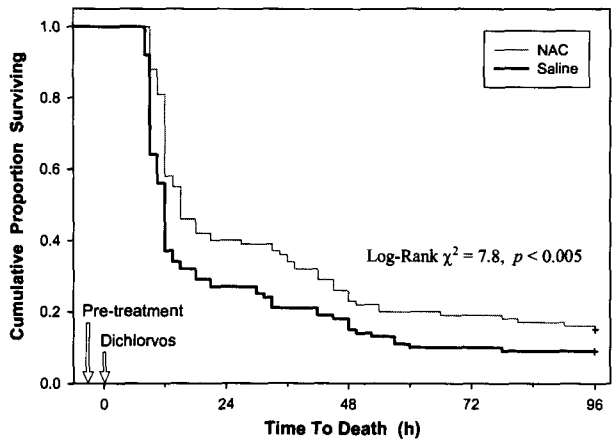

Fig. 1. Kaplan-Meier estimates of survival of eels ip injected either with 1 mmol kg^{-1} NAC or its vehicle (saline) and, after 3 h, were exposed to 1.5 mg l^{-1} (the 96-h LC_{85}) of dichlorvos. Censored observations at the end of the observation time were represented by crosses.

Table 1
Effect of NAC pre-treatment and/or exposure to the 96-h LC_{85} of dichlorvos on biochemical parameters of the European eel (*A. anguilla*)

	Controls (n=30)		96-h TTD exposed eels to dichlorvos (n=100)						Main Effects		
	Saline	NAC	Saline			NAC					
Variables	Mean ± SE	Mean ± SE	Mean ± SE	95% CI	Median	Mean ± SE	95% CI	Median	T	D	TxD
Length (cm)	18.8 ± 0.3	18.1 ± 0.5	18.2 ± 0.3	17.7 - 18.7	18.1	18.7 ± 0.2	18.3 - 19.2	18.5			
Weight (g)	9.4 ± 0.4	8.9 ± 0.8	8.9 ± 0.4	8.1 - 9.7	8.4	9.3 ± 0.3	8.7 - 10.0	9.2			
Liver											
GSH	68 ± 2.7	94.7 ± 4.3 ^{aaa}	32.4 ± 1.2 ^{aaa}	30.0 - 34.8	31.2	51.6 ± 1.9 $^{aaa, bbb, ccc}$	47.9 - 55.3	48.4	***	***	
GSSG	3.2 ± 0.2	3.1 ± 0.2	3.2 ± 0.2	2.9 - 3.6	2.7	3.5 ± 0.2	3.0 - 3.9	2.6			
GSH/GSSG	46.2 ± 2.7	70.8 ± 5.9 ^{aaa}	31.9 ± 2.7 ^{aa}	26.4 - 37.3	19.6	44.9 ± 2.6 $^{bbb, ccc}$	39.8 - 50.0	41.4	***	***	
GR	45.5 ± 1.1	50.8 ± 1.5 ^{aa}	31.3 ± 0.9 ^{aaa}	29.6 - 33.0	31.3	38.8 ± 0.7 $^{aaa, bbb, ccc}$	37.4 - 40.3	37.8	***	***	
GST	1834 ± 72	2158 ± 76 ^{aa}	1351 ± 27 ^{aaa}	1297 - 1404	1329	1463 ± 27 $^{aaa, bbb, cc}$	1408 - 1517	1502	***	***	
γGT	15.4 ± 0.8	15.8 ± 0.9	10.1 ± 0.4 ^{aaa}	9.4 - 10.8	9.7	13.4 ± 0.5 $^{a, b, ccc}$	12.5 - 14.4	13.0	**	***	*
GCL	182 ± 5	238 ± 14 ^{aaa}	121 ± 5 ^{aaa}	111 - 131	114	174 ± 7 $^{bbb, ccc}$	159 - 188	150	***	***	
Caspase-3	16.8 ± 0.6	16.5 ± 1.1	7.7 ± 0.4 ^{aaa}	6.9 - 8.5	7.0	9.1 ± 0.4 $^{aaa, bbb, c}$	8.3 - 9.9	9.0		***	
Muscle											
GSH	17.2 ± 0.7	19.7 ± 0.7 ^{a}	10.3 ± 0.3 ^{aaa}	9.6 - 10.9	10.4	11.9 ± 0.4 $^{aaa, bbb, cc}$	11.0 - 12.7	11.7	***	***	
GSSG	1.49 ± 0.09	1.48 ± 0.08	1.88 ± 0.07 ^{a}	1.73 - 2.02	1.83	1.69 ± 0.09	1.51 - 1.87	1.72		**	
GSH/GSSG	26.1 ± 2.1	28.3 ± 1.5	12.5 ± 0.6 ^{aaa}	11.2 - 13.7	10.7	17.1 ± 0.9 $^{aaa, bbb, ccc}$	15.4 - 18.8	14.8	**	***	
GR	5.96 ± 0.2	5.99 ± 0.14	6.35 ± 0.17	6.02 - 6.68	6.16	6.56 ± 0.14	6.29 - 6.84	6.44			
GST	39.7 ± 2.1	46.2 ± 1.8 ^{a}	48.5 ± 1.2 ^{aaa}	46.1 - 50.9	47.2	49.4 ± 1.1 ^{aaa}	46.6 - 51.2	47.6	*	***	
γGT	1.08 ± 0.1	0.87 ± 0.06	0.61 ± 0.04 ^{aaa}	0.53 - 0.70	0.50	0.67 ± 0.04 $^{aaa, b}$	0.59 - 0.75	0.61		***	*
AChE	184 ± 8	185 ± 8	18.2 ± 0.5 ^{aaa}	17.3 - 19.1	17.7	18.5 ± 0.4 $^{aaa, bbb}$	17.7 - 19.2	17.9		***	

Effects were tested by two-way ANOVA. GSH and GSSG levels were expressed in nmol GSx mg^{-1} prot. GR, GST, γGT, GCL, and AChE activities were expressed in mU mg^{-1} prot, and caspase-3-like activity in μU mg^{-1} prot.

$^{a, aa, aaa}$ $p < 0.05$, 0.01, and 0.001, respectively, compared to the saline-control group.

$^{b, bb, bbb}$ $p < 0.05$, 0.01, and 0.001, respectively, compared to the NAC-control group.

$^{c, cc, ccc}$ $p < 0.05$, 0.01, and 0.001, respectively, compared to the saline-dichlorvos group.

*, **, *** Significantly different at 0.05, 0.01, and 0.001 p-level, respectively, of the main effects of treatment (T), exposure to dichlorvos (D), and the interaction between both of them (TxD).

muscular GSSG increase ($p < 0.01$) that lowered the GSH/GSSG ratio in the muscle ($p < 0.001$). The glutathione redox status was also decreased in the liver ($p < 0.001$). The activities of hepatic GR and GST, hepatic and muscular γGT, hepatic GCL and caspase-3-like, and especially muscular AChE were also diminished ($p < 0.001$), whereas GST activity increased in the muscle ($p < 0.001$). Conversely, NAC treatment achieved an increase of the GSH content and GSH/GSSG ratio in the liver ($p < 0.001$) and muscle ($p < 0.001$ and $p < 0.01$, respectively), in addition to an enhancement of hepatic GR, GST, GCL ($p < 0.001$), and γGT ($p < 0.01$) activities, and muscular GST ($p < 0.05$). Interactions of treatment and dichlorvos exposure were only found on the hepatic and muscular γGT activities ($p < 0.05$).

The single ip injection of 1 mmol kg^{-1} NAC increased the levels of hepatic and muscular GSH by 39% and 14% ($p < 0.001$ and 0.05, respectively), hepatic GSH/GSSG ratio by 53% ($p < 0.001$), hepatic GR activity by 12%, hepatic and muscular GST activity by 18% and 16% ($p < 0.01$ and 0.05, respectively), and hepatic GCL activity by 31% ($p < 0.001$). However, GSH content in the liver was time-dependent (Fig. 2B). Three hours after the injection, GSH raises ($p < 0.05$) and reaches a two-fold increase at 12 h ($p < 0.001$), that returns to baseline after 48 h. In addition, the administration of NAC enhanced the hepatic GSH/GSSG ratio by 134% (Fig. 3B) and GR activity by 26% (Fig. 3D) at the first 3 h ($p < 0.001$ and 0.01, respectively), but were not very different afterwards, except for GR activity at 96 h ($p < 0.01$).

The decrease of glutathione levels and enzyme activities found in dichlorvos-exposed eels were ameliorated with NAC pre-treatment (Table 1). Hepatic and muscular GSH levels of NAC-treated animals were 59% and 16% higher ($p < 0.001$ and 0.01, respectively), as can be observed in Figs. 2A and 5A, which resulted in 29% and 9% lower GSH diminution than non-treated fish compared to non-exposed ones. The GSSG increase in muscle as a consequence of dichlorvos exposure was 13% lower in NAC-treated fish. This fact raised the GSH/GSSG ratio by 37% ($p < 0.001$) in the muscle (Fig. 5B), which was an 18% less shifted toward the oxidised state compared to controls. Glutathione redox status in the liver of NAC-treated fish (Fig. 3A) was also 41% superior. Activities of hepatic GR (Fig. 3C), GST (Fig. 4A), γGT (Fig. 4B), GCL (Fig. 4C), and caspase-3-like (Fig. 4D) were increased by 24% ($p < 0.001$), 8% ($p < 0.01$), 33% ($p < 0.001$), 44% ($p < 0.001$), and 18% ($p < 0.01$), respectively, than non-treated fish. When compared to those fish not exposed to the pesticide, these activities were 16%, 6%, 22%, 29%, and 8%, respectively, less inhibited than saline-treated ones. However, no significant differences of enzyme activities were found in the skeletal muscle.

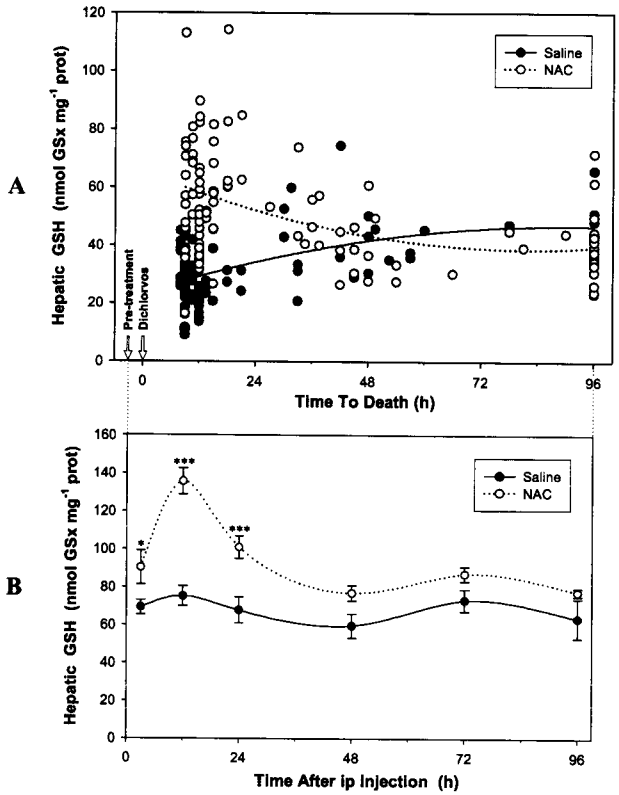

Fig. 2. Effect of NAC treatment on hepatic GSH levels. (A) Eels received a single ip injection of 1 mmol kg^{-1} NAC, or its vehicle, 3 h before that were exposed to dichlorvos (1.5 mg l^{-1}, the 96-h LC_{85}). Data represent individual GSH levels at mortality time or at the end of the experiment (96 h). (B) Fish were given a single dose of NAC (1 mmol kg^{-1} ip), or saline, and were maintained in clean water. Data are means ± SE (n = 5). *, **, *** $p < 0.05$, 0.01 and 0.001, respectively, compared to controls at the same post-injection time. Vertical dotted lines indicate overlapping times for ip injection.

As glutathione levels and enzyme activities were measured on dead fish, the time since the fish death to when its tissues were excised and frozen at –80 °C might allow the autooxidation of GSH, the cleavage of GSH and GSSG by γGT (Anderson, 1985), and protein degradation (Gallenkamp et al., 1981). However, although eel liver and muscle were not especially rich in γGT compared to other tissues (Tate and Meister, 1981) or species (Sulakhe and Lautt, 1985), and intervals of time were inferior to 3

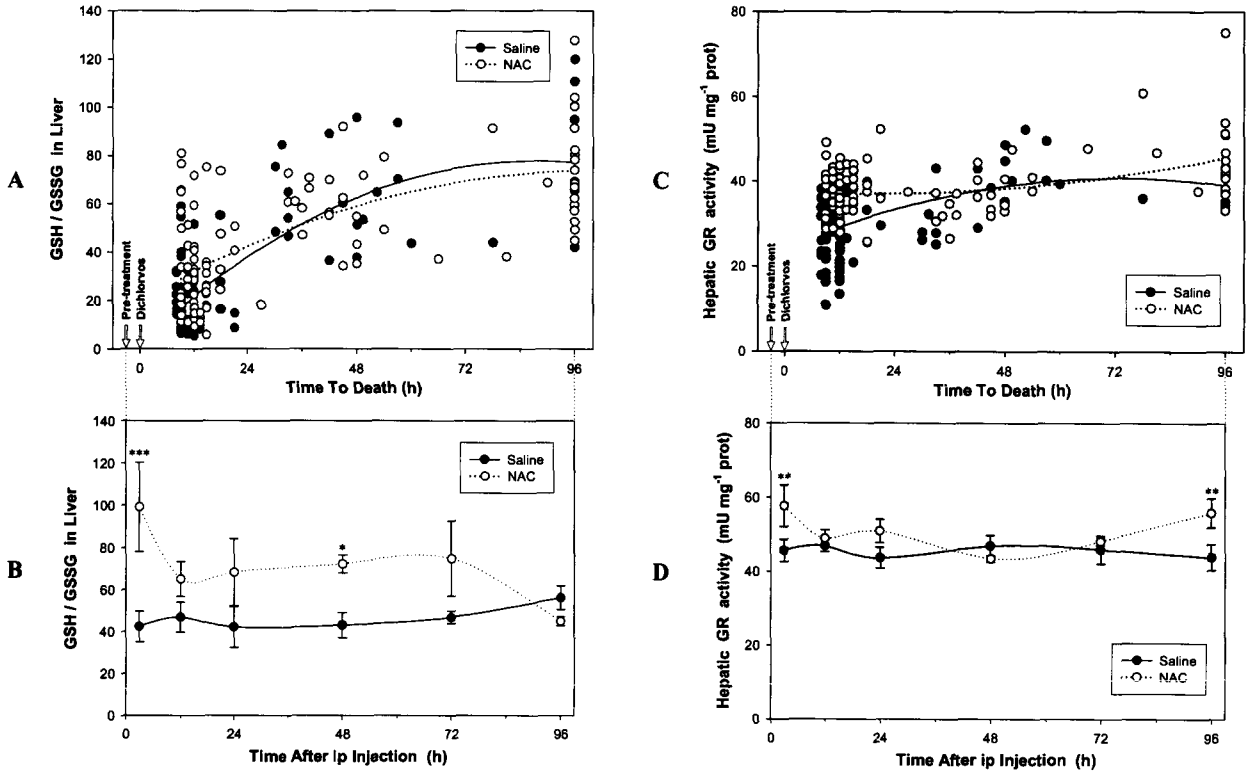

Fig. 3. Effect of NAC treatment on hepatic glutathione redox status and GR activity. (A, C) Fish received a single ip injection of 1 mmol kg^{-1} NAC, or its vehicle, 3 h before that were exposed to dichlorvos (1.5 mg l^{-1}, the 96-h LC_{85}). Data represent individual GSH/GSSG levels (A) and GR activity (C) at mortality time or at the end of the experiment (96 h). (B, D) Fish were given a single dose of NAC (1 mmol kg^{-1} ip), or saline, and were maintained in clean water. Data are means ± SE (n = 5) of GSH/GSSG ratio (B) and GR activity (D). *, **, *** $p < 0.05$, 0.01, and 0.001, respectively, compared to controls at the same post-injection time. Vertical dotted lines indicate overlapping times for ip injection.

h, we performed an extra experiment in order to test the effect of mortality on possible glutathione loss and oxidation, and protein degradation. We compared the glutathione levels and enzyme activities in the liver and muscle of five eels excised immediately after being killed by a blow on the head with other fish kept in clean water at 24 °C for 1.5, 3, and 6 h after being euthanised. There were no significant differences among fish sampled immediately than those sampled 1.5 and 3 h after death, but GSH was slightly diminished and oxidised and GR inhibited (Dunnet's test, $p < 0.05$; data not shown) in the liver and muscle of fish maintained for 6 h in water after death. Therefore, the effect of post-mortem delay on glutathione levels and enzyme activities in this study seemed to be negligible.

In summary, fish mortality started about 9 h after dichlorvos exposure (Fig. 1), which corresponded with the 12 h post-injection, and then, the highest GSH levels in liver of NAC-treated fish (Fig. 2B). Hence, within the first 24 h, NAC-treated fish presented higher GSH levels and GSH/GSSG ratios in the liver and muscle, less inhibited GR, GST, and GCL activities in the liver, and higher GST activity in the muscle that allowed them to detoxify dichlorvos and the ROS generated more efficiently, and therefore, improve tolerance.

3.3. Bivariate and Partial Correlations

Tables 2 and 3 show correlations among parameters in fish exposed to dichlorvos. Hepatic GSH content was correlated to the length and weight of non-treated eels ($r = 0.40$ and 0.35, respectively). Caspase-3-like activity was linearly correlated with the hepatic GSH/GSSG ratio of saline- and NAC-treated eels ($r = 0.52$ and 0.55, respectively). Furthermore, the pattern of individual caspase-3-like activity against TTD (Fig. 4B) was very similar to that of hepatic GSH/GSSG ratio (Fig. 3A), which suggests that the caspase-3-like activity may be regulated by the glutathione redox status as previously stated Ueda et al. (1998). Caspase-3-like

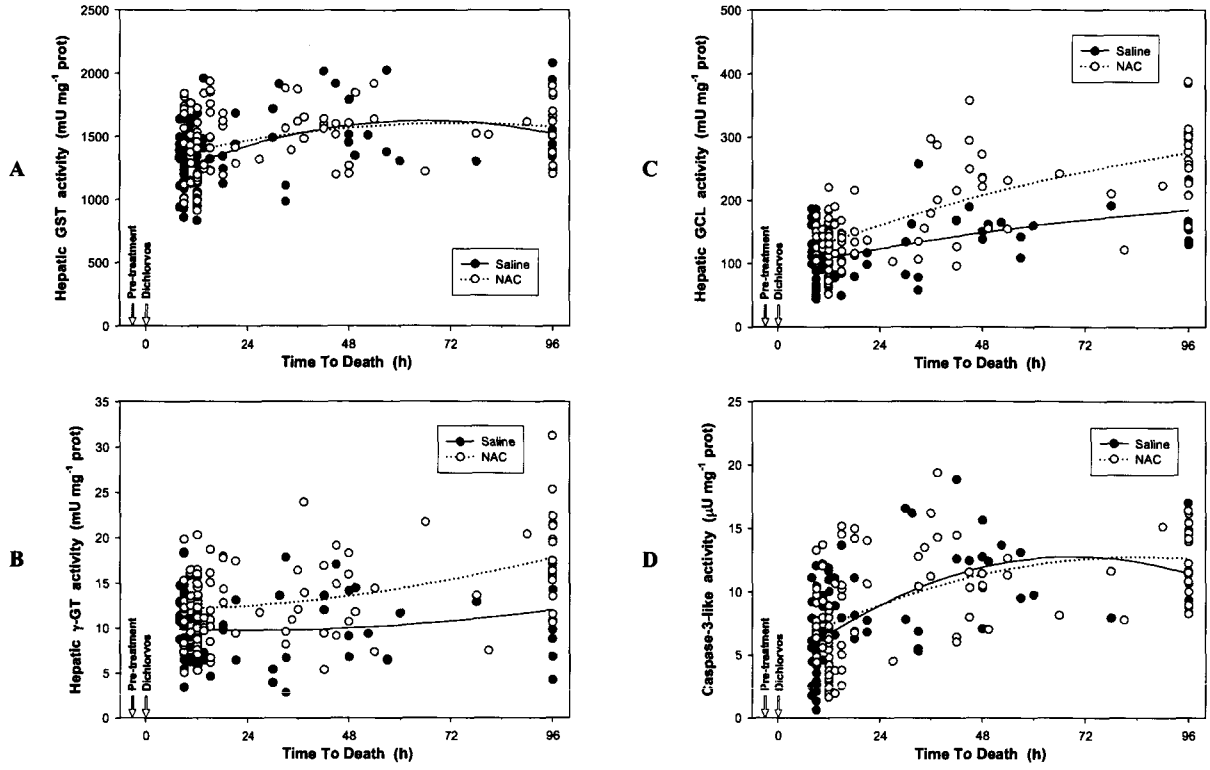

Fig. 4. Effect of NAC treatment on hepatic GST (A), γGT (B), GCL (C), and caspase-3-like (D) activities. Fish received a single ip injection of 1 mmol kg^{-1} NAC, or its vehicle, 3 h before that were exposed to dichlorvos (1.5 mg l^{-1}, the 96-h LC_{85}). Data represent individual values at mortality time or at the end of the experiment (96 h).

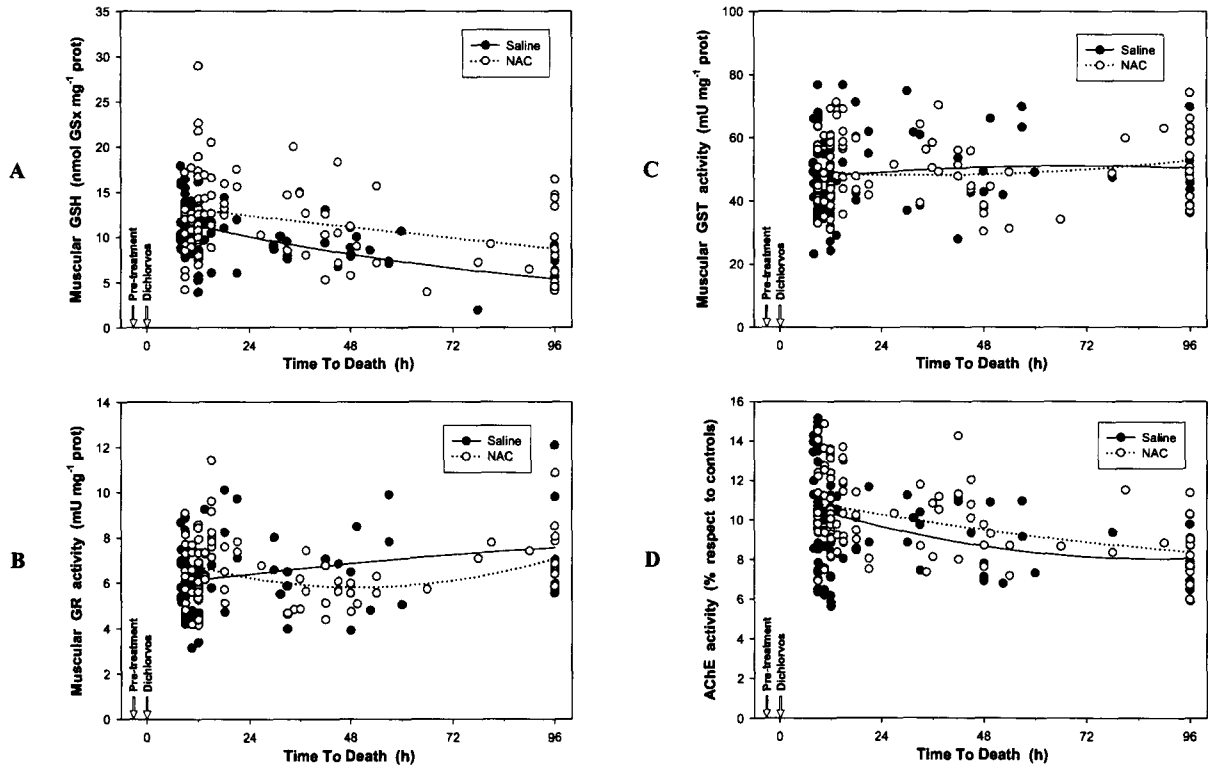

Fig. 5. Effect of NAC treatment on muscular GSH content (A), GR (B), GST (C), and AChE activities (D). Fish received a single ip injection of 1 mmol kg^{-1} NAC, or its vehicle, 3 h before that were exposed to dichlorvos (1.5 mg l^{-1}, the 96-h LC_{85}). Data represent individual values at mortality time or at the end of the experiment (96 h).

Table 2
Pearson correlation coefficients (r) among studied variables of eels pre-treated with saline and exposed to dichlorvos (n = 100)

			Liver								Muscle					
	Length	Weight	GSH	GSSG	Ratio	GR	GST	γGT	GCL	Casp-3	GSH	GSSG	Ratio	GR	GST	γGT
Weight	**0.78*****															
Liver																
GSH	**0.40*****	**0.35*****														
GSSG	-0.12	-0.06	-0.29**													
Ratio	0.30**	0.24*	**0.68*****	**-0.89*****												
GR	0.14	0.16	**0.38*****	-0.34***	**0.44*****											
GST	0.13	0.124	**0.39*****	-0.31**	**0.40*****	**0.34*****										
γGT	0.12	0.003	0.03	-0.20*	0.16	0.20*	0.12									
GCL	0.16	0.12	**0.43*****	-0.42***	**0.54*****	**0.61*****	**0.35*****	**0.50*****								
Casp-3	0.18	0.17	**0.46*****	-0.43***	**0.52*****	**0.50*****	**0.50*****	0.23*	**0.40*****							
Muscle																
GSH	-0.01	0.02	-0.15	**0.38*****	-0.35***	-0.07	-0.13	-0.06	-0.17	-0.23*						
GSSG	-0.09	-0.11	-0.21*	**0.35*****	-0.35***	-0.09	-0.09	-0.06	-0.12	-0.20*	**0.37*****					
Ratio	0.04	0.08	0.10	-0.07	0.09	0.00	-0.01	-0.03	-0.08	0.07	**0.43*****	**-0.62*****				
GR	0.01	0.07	0.15	-0.16	0.19	0.32**	0.11	0.18	**0.37*****	0.11	-0.13	0.05	-0.22*			
GST	0.10	0.05	0.18	-0.09	0.17	0.20*	-0.03	0.12	0.21*	0.02	0.02	-0.12	0.10	**0.47*****		
γGT	-0.09	-0.10	0.04	-0.23*	0.19	0.02	-0.13	**0.36*****	0.25*	0.15	-0.07	-0.26**	0.19	0.07	0.08	
AChE	-0.23*	-0.25*	-0.09	0.02	-0.05	-0.04	-0.14	0.19	0.16	-0.17	0.19	0.20	-0.02	**0.36*****	**0.36*****	0.20*

Bold correlation coefficients indicate significant p values at $\alpha = 0.05$ after sequential Bonferroni correction for multiple tests. Correlations with $p < 0.05$ that were non-significant after controlling for TTD were represented in boxes. Ratio indicates GSH/GSSG.

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 3
Pearson correlation coefficients (r) among studied variables of eels pre-treated with NAC and exposed to dichlorvos ($n = 100$)

			Liver								Muscle					
	Length	Weight	GSH	GSSG	Ratio	GR	GST	γGT	GCL	Casp-3	GSH	GSSG	Ratio	GR	GST	γGT
Weight	**0.92*****															
Liver																
GSH	0.21*	0.16														
GSSG	-0.13	-0.17	**0.49*****													
Ratio	0.25*	0.27**	-0.07	**-0.89*****												
GR	0.13	0.13	-0.07	-0.24*	0.24*											
GST	0.27**	0.22*	0.08	-0.14	0.22*	0.18										
γGT	0.18	0.19	-0.17	-0.33***	0.30**	**0.39*****	0.06									
GCL	0.10	0.11	-0.24*	**-0.46*****	**0.43*****	0.25*	0.22*	**0.44*****								
Casp-3	0.19	0.24*	-0.03	**-0.49*****	**0.55*****	0.30**	**0.34*****	**0.42*****	**-0.46*****							
Muscle																
GSH	-0.13	-0.10	0.19	**0.39*****	**-0.37*****	-0.16	-0.14	-0.25*	-0.27**	-0.26**						
GSSG	-0.12	-0.08	-0.06	0.26**	-0.34***	0.02	0.12	-0.03	-0.01	-0.04	**0.41*****					
Ratio	0.07	0.03	0.25*	0.02	0.09	-0.15	-0.19	-0.13	-0.17	-0.14	0.27**	**-0.74*****				
GR	-0.12	-0.12	0.03	0.11	-0.09	0.28**	0.19	0.02	0.02	0.07	0.15	**0.39*****	-0.31**			
GST	0.06	0.03	-0.03	0.07	-0.07	0.07	0.25*	0.22*	0.05	0.10	-0.08	0.17	-0.17	0.26**		
γGT	-0.03	-0.08	-0.09	-0.11	0.08	-0.01	-0.11	-0.05	-0.05	0.05	0.05	-0.27**	0.33***	-0.02	-0.01	
AChE	-0.28**	-0.31**	0.06	0.33***	**-0.35*****	-0.23*	-0.12	-0.24*	-0.27**	**-0.38*****	0.20*	0.17	-0.004	0.18	0.13	0.12

Bold correlation coefficients indicate significant p values at $\alpha = 0.05$ after sequential Bonferroni correction for multiple tests. Correlations with $p < 0.05$ that were non-significant after controlling for TTD were represented in boxes. Ratio indicates GSH/GSSG.

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

activity was also related to hepatic GSH (r = 0.46), GR (r = 0.50), and GST (r = 0.50) activity of saline-treated eels. GCL activity was correlated with hepatic GSH (r = 0.43), GSH/GSSG ratio (r = 0.54), γGT activity (r = 0.50), and hepatic and muscular GR activity (r = 0.61 and 0.37, respectively). Other significant correlations found in saline-treated eels were between GST activity and GSH in the liver (r = 0.39), between the γGT activity in the liver and muscle (r = 0.36), and between muscular AChE and GR (r = 0.36), and GST (r = 0.36) activities. However, many relationships between two variables were non-significant when the linear effect of the TTD is removed, because both variables were related to the TTD. Conversely, after adjusting for TTD, the hepatic and muscular GSH/GSSG ratios were correlated in saline- and NAC-treated eels (r = 0.31 and 0.31, respectively, $p < 0.01$; data not shown).

3.4. Cox Proportional Hazards Models

The Cox proportional hazards model is a semi-parametric multivariate regression method used to estimate the effects of several explanatory variables (covariates) on survival. Although this model does not assume a particular mathematical distribution for the survival times, it assumes that the effects of the different covariates on survival do not change over time. The hazard ratio (HR) evaluates the percentage change in risk with each unit change in the covariate. When HR is higher than 1, the risk of death rises when the covariate increases; when HR is lower than 1, the risk of death decreases when the covariate increases.

Univariate Cox models (Table 4) show that hepatic GSH, GSH/GSSG ratio, GR, GST, GCL, and caspase-3-like activities were significantly associated with dichlorvos survival in saline-treated fish when each covariate was considered solely on the model ($p = 2{\cdot}10^{-5}$, $8{\cdot}10^{-9}$, $5{\cdot}10^{-5}$, 0.0014, 0.0005, and $3{\cdot}10^{-6}$, respectively). Each increase in one nmol GSx mg^{-1} prot of GSH or GSH/GSSG unit or mU mg^{-1} prot of GR, GST, or GCL activity or one μU mg^{-1} prot of caspase-3-like activity decreases independently the risk of death of these fish by 4.3%, 3.6%, 5.3%, 0.13%, 0.8%, and 14%, respectively. In contrast, every increase in one nmol GSx mg^{-1} prot of hepatic GSSG or muscular GSH or GSSG or one mU mg^{-1} prot of AChE in saline-treated fish increases the risk of death by 32% ($p = 9{\cdot}10^{-7}$), 27% ($p = 10^{-9}$), 59% (p = 0.0004), and 12% ($p = 2{\cdot}10^{-5}$), respectively. In the case of NAC-treated fish, every increase in one nmol GSx mg^{-1} prot of hepatic GSH or GSSG or muscular GSH or one mU mg^{-1} prot of AChE increases the hazard of death by 2.3% ($p = 5{\cdot}10^{-5}$), 34% ($p = 10^{-10}$), 7.2% (p = 0.003) or 16% ($p = 10^{-6}$), respectively. Every increase in the hepatic glutathione redox status or one mU mg^{-1} prot of GR, GST, γGT, GCL or one μU mg^{-1} prot of caspase-3-like activity decreases the risk of death by 3.5% ($p = 2{\cdot}10^{-9}$), 5.8% (p = 0.0008), 0.12% (p = 0.008), 8.7% (p = 0.0002), 1.3% ($p = 10^{-10}$), or 14% ($p = 6{\cdot}10^{-7}$), respectively.

Dichlorvos mortality can be explained as a consequence of the effects of several covariates (Table 5). The best explanatory variables associated

Table 4
Unadjusted hazard ratios relating survival under dichlorvos exposure to studied variables of fish pre-treated with saline or 1 mmol kg^{-1} NAC

	Saline		NAC	
Covariates	HR (95% CI)	p	HR (95% CI)	p
Length	0.925 (0.856-1.009)	0.047	0.882 (0.797-0.977)	0.016
Weight	0.951 (0.902-1.006)	0.07	0.918 (0.858-0.983)	0.014
Liver				
GSH	0.957 (0.938-0.983)	*$2{\cdot}10^{-5}$*	1.023 (1.012-1.034)	*$5{\cdot}10^{-5}$*
GSSG	1.320 (1.181-1.299)	*$9{\cdot}10^{-7}$*	1.342 (1.227-1.468)	*10^{-10}*
GSH/GSSG	0.964 (0.953-0.983)	*$8{\cdot}10^{-9}$*	0.965 (0.954-0.977)	*$2{\cdot}10^{-9}$*
GR	0.947 (0.922-0.973)	*$5{\cdot}10^{-5}$*	0.942 (0.910-0.976)	*0.0008*
GST	0.999 (0.998-0.999)	*0.0014*	0.999 (0.998-1.000)	*0.008*
γGT	0.979 (0.929-1.032)	0.43	0.913 (0.869-0.958)	*0.0002*
GCL	0.992 (0.987-0.996)	*0.0005*	0.987 (0.984-0.991)	*10^{-10}*
Caspase-3	0.864 (0.813-0.934)	*$3{\cdot}10^{-6}$*	0.856 (0.805-0.910)	*$6{\cdot}10^{-7}$*
Muscle				
GSH	1.271 (1.177-1.372)	*10^{-9}*	1.072 (1.023-1.122)	*0.003*
GSSG	1.593 (1.230-2.063)	*0.0004*	0.974 (0.787-1.205)	0.81
GSH/GSSG	1.011 (0.982-1.040)	0.46	1.010 (0.988-1.033)	0.37
GR	0.881 (0.778-1.016)	0.044	0.947 (0.804-1.116)	0.51
GST	0.994 (0.976-1.012)	0.50	0.989 (0.973-1.006)	0.21
γGT	1.070 (0.669-1.287)	0.78	1.163 (0.661-2.049)	0.60
AChE	1.123 (1.065-1.124)	*$2{\cdot}10^{-5}$*	1.158 (1.091-1.230)	*10^{-6}*

Italic p values indicate significant ones at $\alpha = 0.05$ after sequential Bonferroni correction for multiple tests. HR indicates hazard ratio and CI confidence interval.

Table 5
Adjusted hazard ratios of the best explanatory variables related to dichlorvos mortality of European eels pre-treated with saline or 1 mmol kg^{-1} NAC

Covariates	HR (95% CI)	p
Saline-treated fish		
Hepatic GSH/GSSG	0.972 (0.959-0.984)	10^{-5}
Hepatic GR	0.968 (0.939-0.997)	0.033
Muscular GSH	1.266 (1.153-1.390)	$8 \cdot 10^{-7}$
Muscular GR	0.860 (0.739-0.999)	0.049
Muscular AChE	1.134 (1.071-1.202)	$2 \cdot 10^{-5}$
NAC-treated fish		
Hepatic GSH	1.031 (1.017-1.045)	$2 \cdot 10^{-5}$
Hepatic GSH/GSSG	0.972 (0.959-0.985)	$3 \cdot 10^{-5}$
Hepatic GR	0.960 (0.923-1.000)	0.048
Hepatic GCL	0.992 (0.988-0.996)	0.0002
Muscular GST	0.977 (0.957-0.996)	0.019
Muscular AChE	1.115 (1.033-1.205)	0.006
All the individuals		
Treatment	0.493 (0.326-0.746)	0.0008
Hepatic GSH	1.020 (1.009-1.032)	0.0006
Hepatic GSH/GSSG	0.967 (0.957-0.977)	$4 \cdot 10^{-11}$
Hepatic GCL	0.994 (0.991-0.998)	0.0007
Muscular GSH	1.085 (1.040-1.132)	0.00018
Muscular GR	0.815 (0.730-0.909)	0.0002
Muscular AChE	1.133 (1.084-1.185)	$4 \cdot 10^{-8}$

A stepwise forward selection procedure from all covariates was used on each model to obtain those covariates more associated with survival. Length and weight were added to these models in order to adjust for them. HR indicates hazard ratio and CI confidence interval.

with survival in non-treated eels were muscular GSH ($p = 8 \cdot 10^{-7}$), hepatic GSH/GSSG ratio ($p = 10^{-5}$), muscular AChE ($p = 2 \cdot 10^{-5}$), and hepatic and muscular GR activity ($p = 0.033$ and 0.049, respectively). Hence, fish with higher hepatic glutathione redox status or hepatic or muscular GR activity or lower muscular GSH or AChE activity are expected to have 2.8%, 3.2%, 14%, 27% or 13% less probability of dying, respectively, on each unit change of these parameters, after adjustment for the other variables in the model. When fish were pre-treated with NAC, survival was much more dependent on the availability of GSH ($p = 2 \cdot 10^{-5}$) and maintenance of the glutathione redox status ($p = 3 \cdot 10^{-5}$) than on the AChE activity ($p = 0.006$). In addition, in excess of glutathione, the activities of hepatic GCL ($p = 0.0002$) and GR ($p = 0.048$), and muscular GST ($p = 0.019$) were determinant for prolonging survival.

The Cox regression analysis was also carried out in all animals exposed to the pesticide to determine the net weight of the treatment after adjusting for the other explanatory variables in the model. As a result, pre-treatment with NAC decreased the risk of death by 51% ($p = 0.0008$). Hepatic glutathione redox status ($p = 4 \cdot 10^{-11}$), GCL activity ($p = 0.0007$), and muscular GR activity ($p = 0.0002$) were independently related to higher survival, whereas muscular ($p = 0.00018$) and hepatic ($p = 0.0006$) GSH, and AChE activity ($p = 4 \cdot 10^{-8}$) were negatively associated with survival.

4. Discussion

The present work demonstrates that NAC injected ip improved eel survival upon dichlorvos exposure. A lethal concentration of this OP pesticide (1.5 mg l^{-1}, the 96-h LC_{85}) decreased and oxidised glutathione levels and inhibited enzyme activities in a higher degree than that observed in fish exposed to a sublethal concentration of dichlorvos (Peña-Llopis et al., submitted). The oxidation of GSH and inhibition of GR indicated the presence of oxidative processes, and was in agreement with Hai et al. (1997), who found a GSH diminution in the carp liver and muscle, and AChE inhibition after being exposed to 1 and 5 mg l^{-1} of dichlorvos for 24 h. Dichlorvos also decreased glutathione levels and inhibited AChE and GPx activities in several tissues of rats (Julka et al., 1992).

Glutathione depletion is considered a biomarker of environmental stress as was observed in fish stressed either by chemical or natural pollutants (e.g., Chatterjee and Bhattacharya, 1983; Allen et al., 1988; Gallagher et al., 1992; Hasspieler et al., 1994; Almar et al., 1998; Vaglio and Landriscina, 1999; Peña-Llopis et al., 2001). Nevertheless, hepatic GSH synthesis can be induced as a consequence of the pollutant interaction with GCL, which is the rate limiting enzyme of GSH biosynthesis, and then increase GSH beyond control levels, as was observed in several fish studies (Stein et al., 1992; Hasspieler et al., 1994; Peña-Llopis et al., 2000; 2001). Conversely, diminution of muscular GSH content seems to be an intrinsic factor of pesticide exposure, as the longer the exposure, the lower its values. Glutathione levels in muscle depend largely on GSH capture from plasma by γGT (Griffith and Meister, 1979). Then, as the liver is the

main source of plasma GSH (Kaplowitz et al., 1985), if there is hepatic GSH depletion, less glutathione would be exported to plasma, and less would be available for extrahepatic tissues, allowing muscular GSH consumption. Thus, as GSH replenishment in the muscle is less likely than in the liver, its depletion could be a better biochemical marker of pollutant exposure than the latter tissue.

Muscular AChE is an established biomarker of OP and carbamate exposure that is more reliable than residue analysis for the diagnosis of poisoning, because it accounts for a physiological effect directly linked to the compound toxic mode of action and persists much longer than pesticides in environmental samples (Fulton and Key, 2001). AChE inhibition in dichlorvos-exposed fish increased over time and was about 90% of the control activity, even in survivors at 96 h. Fernandez-Vega et al. (2002) reported a muscular 53-70% AChE inhibition of eels (*A. anguilla*) exposed to a sublethal concentration of the thiocarbamate herbicide thiobencarb, which is a cholinesterase inhibitor less potent than dichlorvos. Although the relationship between AChE inhibition and mortality is species- and/or age-specific, a brain AChE inhibition higher than 70% is normally associated with mortality (Fulton and Key, 2001).

The processes from OP toxicity to fish death, and how are regulated by the glutathione metabolism and modulated by NAC are summarised in Fig. 6. The inhibition of brain AChE activity by OP pesticides blocks the hydrolysis of the neurotransmitter acetylcholine (ACh), which can accumulate and activate muscarinic receptors. The overstimulation of cholinergic neurones initiates a process of hyperexcitation and convulsive activity that progresses rapidly to status epilecticus, which leads to profound structural brain damage or the death of the organism if the muscarinic ACh receptor antagonist atropine is not rapidly administered. These OP-induced seizures allow the release of excitatory amino acids (EAA), such as glutamate and aspartate, prolonging the convulsive activity, and making atropine treatment ineffective (Shih & McDonough 1997). High concentrations of EAA can activate the *N*-methyl-D-aspartate (NMDA) receptor, leading to intracellular influx of Ca^{2+}, which triggers the activation of proteolytic enzymes and the generation of free radicals (Beal 1995).

Dichlorvos is metabolised mainly in the liver *via* two enzymatic pathways: one, producing desmethyldichlorvos, is glutathione dependent, while the other, resulting in dimethyl-phosphate and dichloroacetaldehyde, is glutathione independent (Dicowsky and Morello, 1971). Hence, GSH availability can result in a limiting factor for dichlorvos elimination. Although *de novo* synthesis of glutathione by GCL is regulated by feedback inhibition of GSH (Richman and Meister, 1975), the single ip injection of NAC enhanced the GCL activity and increased transiently the hepatic GSH levels two-fold. This drug also increased the muscular GSH and hepatic glutathione redox status, in addition to the activities of GR in the liver and GST in the liver and muscle. Hepatic γGT activity was less inhibited in NAC-treated fish, enabling to degrade circulating GSH more efficiently, and producing abundant catabolites for maintenance of GSH levels.

Mortality in non-treated fish was dependent primarily on muscular GSH, hepatic GSH/GSSG ratio, muscular AChE activity, and hepatic and muscular GR activity. This was in concurrence with previous results of *A. anguilla* exposed to the herbicide molinate (Peña-Llopis et al., 2001). On the other hand, as GSH was in excess within the first 24 h of dichlorvos exposure in NAC-treated fish, survival relied principally on the individual capacity to maintain and enhance the hepatic GSH/GSSG ratio and synthesise GSH by GCL. Survival was less dependent on muscular AChE and GST activities and hepatic GR activity. Paradoxically, hepatic GST activity, which is directly linked to dichlorvos detoxification and was related to the survival of treated and non-treated fish when considered solely in the model, was not among the best explanatory variables of dichlorvos mortality. NAC-treated fish presented higher GST activity in the liver that could allow them to eliminate the pesticide, but other variables were more important to explain mortality.

Although NAC-treated fish were twice less likely to die within the 96 h of follow-up, survival to dichlorvos exposure was mainly dependent on hepatic glutathione redox status. As this dependence was greater than that of the muscular AChE activity, the glutathione redox status represents a better biomarker of effect and individual susceptibility than the muscular AChE activity. In addition, the genetic background was more important than the effect of the treatment to explain survival. Then, those fish that were able to preserve or increase the GSH/GSSG ratio were expected to live longer upon

dichlorvos exposure than those that lost redox homeostasis. Similarly, we found that mortality of two marine bivalves exposed to the OP insecticide fenitrothion was also associated with impaired glutathione redox status (Peña-Llopis et al., 2002). Raising the GSH/GSSG ratio by reducing GSSG to two molecules of GSH is energetically less costly than synthesising GSH *de novo*. Despite the relevance of GR in the tolerance to oxidative stress (Peña-Llopis et al., 2001), its activity also depends on NADPH availability. Then, the individual capacity to enhance enzyme activities of gluconeogenesis and the pentose phosphate pathway could also determine tolerance to the OP. In fact, resistance to oxidative stress and GSH levels were found to be modulated by several of these enzymes, such as glucose-6-phosphate dehydrogenase (Salvemini et al., 1999), transaldolase (Banki et al., 1996), and cytosolic $NADP^+$-dependent isocitrate dehydrogenase (Lee et al., 2002). In addition, Godon et al. (1998), using a proteomic approach, established that over 100 proteins whose levels changed after oxidative stress in yeast (*Saccharomyces cerevisiae*), a quarter were involved in the repression of glucolysis and the tricarboxylic acid cycle, and redirection of carbohydrate metabolism toward the regeneration of NADPH at expense of ATP. Voehringer et al. (2000), using DNA microarrays, found that cells resistant to apoptosis preserved high intracellular pools of GSH by enhancing pathways for establishment and maintenance of high intracellular redox potential.

Many cellular signalling molecules (such as protein tyrosine and serine/threonine kinases and phosphatases, Ca^{2+} channels and transporters, and transcription factors like NF-κB, *p*53 and AP-1) are regulated by the redox state of cells, although signalling systems regulate the cellular redox state as well (Kamata and Hirata, 1999). Therefore, an important oxidation of the hepatic glutathione redox status of fish by dichlorvos can alter transcriptional responses to induce programmed cell death (Esteve et al., 1999).

Dichlorvos-exposed fish showed marked caspase-3-like inhibition compared to non-exposed ones, whose values were strongly correlated to the hepatic glutathione redox status. Caspase-3-like activity was also highly related to survival when considered solely in the model. However, it was not among the best explanatory variables when adjusted to the other covariates, because the contribution of each variable to the regression model is determined in the context of the contribution of all other variables in the model. Then, as caspase-3-like activity was correlated to GSH/GSSG ratio, the latter entered first in the equation excluding the caspase-3-like. If the model were constructed without the glutathione redox status, caspase-3-like activity would be among the best explanatory variables (data not shown). Hence, caspase-3-like can also be considered as significantly related to survival.

Caspases have cysteine residues in the catalytic domain (Wilson et al., 1994) that are prone to oxidation or thiol alkylation, and therefore, are regulated by the oxidative stress and intracellular redox state (Ueda et al., 1998). The inhibition of caspase-3-like activity induced by excessive oxidation may cause a shift from apoptosis to necrosis in the liver as described in cell cultures (Lemaire et al., 1998; Samali et al., 1999). Hence, our results suggest that fish individual differences in the homeostasis of the hepatic glutathione redox status can determine the degree of dichlorvos-induced oxidative stress and caspase-3-like inhibition, resulting in hepatic cell death by necrosis. Schafer and Buettner (2001) found that the glutathione redox status can be indicative of the biological status of the cell, because increasing the half-cell reduction potential (E_{hc}) of the GSSG/2GSH couple may shift cells through different biological stages, such as proliferation, differentiation, apoptosis and, at very high values of E_{hc}, necrosis. In our study, NAC treatment diminished caspase-3-like inhibition, allowing higher fish tolerance to dichlorvos. Evidence indicates that NAC can decrease apoptosis and necrosis in numerous systems (e.g., Atkins et al., 2000; Rojas et al., 2002).

According to the impairment-disability concept of Depledge (1989), an initial perturbation caused by a toxic exposure will generate responses in order to compensate the cellular and tissular homeostasis of an organism. This compensatory phase will be established with higher stress-induced disturbance, but when further repair and/or compensation are impossible, the organism dies. Newman and McCloskey (2000) found that the tolerance to a lethal concentration of a toxicant is neither due to innate qualities of individuals nor stochastic processes. Hence, according to information provided in this study we propose an intermediate hypothesis to explain this kind of tolerance. Tolerance to a

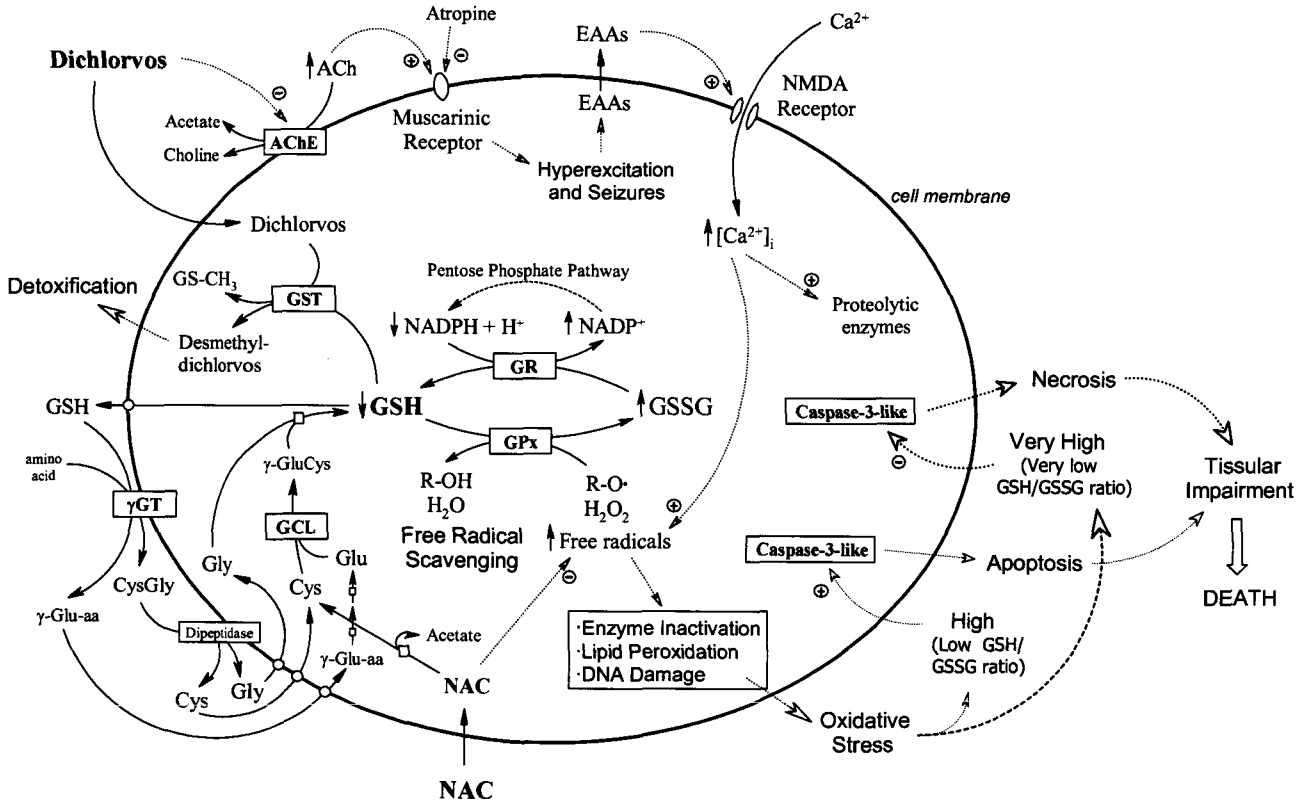

Fig. 6. Proposal of the involvement of the glutathione metabolism and NAC in the tolerance to dichlorvos mortality. The OP inhibits the AChE activity, leading to excessive accumulation of ACh and overstimulation of muscarinic receptors. This process produces neuronal hyperexcitation and seizures that allow the release of EAA, such as Glu and Asp, which activate the NMDA receptor. Excessive activation of NMDA receptors triggers the influx of large amounts of extracellular Ca^{2+}, which activates proteolytic enzymes and leads to formation of free radicals. Then, GSH oxidises to GSSG to remove ROS, catalysed by GPx or non-enzymatically. GSSG is subsequently reduced to GSH by GR at expense of oxidising NADPH to $NADP^+$, which is recycled by the pentose phosphate pathway. In addition, GSH can be conjugated to dichlorvos by GST allowing the detoxification of the pesticide. The antioxidant NAC acts as a reductant and free radical scavenger that can also be deacetylated to Cys, the limiting amino acid for glutathione synthesis, which is regulated by GCL. Extracellular GSH is degraded to its catabolites by γGT and dipeptidase, enabling intracellular GSH synthesis by the γ-glutamyl cycle. Excessive ROS can lead to high oxidative stress, which activates caspases and induces cell death by apoptosis. However, when oxidative stress is very high - like in this study - caspases are inhibited leading cell death by necrosis. Ultimately, these tissular damages can cause the death of the organism.

lethal concentration of dichlorvos primarily depends on maintaining and raising the hepatic glutathione redox status. Then, individual regulations to synthesise GSH, enhance GR activity or other redox systems, or derive carbohydrate metabolism toward NADPH regeneration, will modulate intracellular redox potentials. When glutathione metabolism is impaired and is not rapidly restored, oxidative stress generated by the OP prevails and may finally lead to hepatic cell death by necrosis, resulting in tissular dysfunction, and eventually fish death.

Although cellular redox potential is predominantly maintained by the reducing equivalents of glutathione, other enzyme systems such as thioredoxin and glutaredoxin are also involved (Björnstedt et al., 1997). Furthermore, redox status can be indirectly modulated by other antioxidant enzymes like superoxide dismutase and catalase, and by the redox couple $NADP^+$/NADPH, which is dependent on enzymes implicated in NADPH synthesis, such as glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, transaldolase, isocitrate dehydrogenase and malic enzyme. Therefore, as maintenance of cellular redox status is multifactorial, more studies are necessary to comprehend the intimate regulation of metabolism for establishment of cellular and interogan homeostasis in order to design better drugs or combinations of them to treat oxidative stress-related diseases and poisonings.

As OP pesticides are extensively used in widespread applications, poisoning by these

compounds represents a serious public health problem, especially since OPs are used as agents of chemical warfare (Goozner et al., 2002). In fact, dichlorvos was among the several OPs used during the Persian Gulf War (Cecchine et al., 2000). Although the standard treatment against OP poisoning consists of reactivation of the inhibited AChE with an oxime and reversal of the biochemical effects of acetylcholine with atropine (Kwong, 2002), the present study demonstrates that NAC may be a complementary antidote for OP poisoning, acting at different target sites. In addition, NAC characteristics, such as its low toxicity, low price, and high solubility makes it suitable for treatment of OP intoxications in fish, especially prophylactically before antiparasitic actions.

5. Conclusions

- Tolerance to the OP dichlorvos can be explained as the individual capacity of maintaining and increasing the hepatic GSH/GSSG ratio through the glutathione metabolism. An impairment of the glutathione redox status could lead to excessive oxidative stress, hepatic necrosis, and eventually the death of the organism.
- NAC improves fish survival to a lethal concentration of dichlorvos through enhancing the glutathione metabolism and decreasing the glutathione loss and oxidation, and enzyme inactivation caused by the OP pesticide. Therefore, NAC increases the tolerance to dichlorvos-induced oxidative stress and necrosis, being a potential antidote for OP poisoning and complementary to current treatments.
- Muscular GSH depletion was a better biomarker of pollutant exposure than hepatic GSH. Hepatic glutathione redox status, GR, GCL, and caspase-3-like activities, in addition of muscular AChE activity, can be used as biochemical markers of effect and individual susceptibility to dichlorvos mortality or other pollutants that induce oxidative stress.

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Appendix

Abbreviations: AChE, acetylcholinesterase; EAA, excitatory amino acids; GCL, glutamate:cysteine ligase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidised glutathione or glutathione disulfide; GST, glutathione *S*-transferase; γGT, γ-glutamyl transferase; NAC, *N*-acetyl-L-cysteine; NMDA, *N*-methyl-D-aspartate; OP, organophosphate; ROS, reactive oxygen species; TTD, time to death.

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ARTÍCULO V

DISEASES OF AQUATIC ORGANISMS
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Increased recovery of brain acetylcholinesterase activity in dichlorvos-intoxicated European eels (*Anguilla anguilla*) by bath treatment with *N*-acetylcysteine

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ABSTRACT: Organophosphate (OP) pesticides are widely used as antiparasitic medicines in fish aquaculture. However, current antidotes cannot be applied to treat intoxicated fish. We showed in previous studies the importance of glutathione (GSH) metabolism in pesticide resistance of the European eel (*Anguilla anguilla* L.). The present work studied the effects of the antioxidant and glutathione pro-drug *N*-acetyl-L-cysteine (NAC) on the recovery of European eels exposed for 96 h to a sublethal concentration of the OP insecticide dichlorvos (2,2-dichlorovinyl dimethyl phosphate, DDVP). This pesticide decreased muscular GSH content and increased oxidised glutathione (GSSG), lowering the GSH/GSSG ratio, which is indicative of a condition of oxidative stress. Acetylcholinesterase (AChE) and glutathione reductase (GR) activities in the brain, which were biomarkers of neurotoxicity and oxidative stress, respectively, were also highly inhibited. NAC treatment ameliorated muscular GSH depletion, GSH/GSSG ratio, and the inhibition of brain AChE and GR activities. Hence, this is the first evidence of improved recovery of organophosphate-poisoned fish by bath treatments.

KEY WORDS: Detoxification, organophosphorus pesticide, organophosphate poisoning, cholinesterase inhibitor, oxidative stress, *N*-acetyl-L-cysteine, AChE, glutathione metabolism.

INTRODUCTION

The rapid growth and development of intensive aquaculture has been concomitant to the use of chemicals to deal with animal health. Parasitic infestations cause fish stress and susceptibility to secondary infections, producing significant losses to the industry. Among the existing medicines, the OP insecticide dichlorvos has been extensively used to treat sea lice infestations (by the copepod parasites *Lepeophtheirus salmonis* and *Caligus elongatus*) in the Atlantic salmon (*Salmo salar*) culture because it is relatively non-persistent and undergoes fast and complete hydrolysis in most environments, being rapidly metabolised (WHO, 1989).

The primary effects of dichlorvos and other OPs on organisms are through the inhibition of the enzyme AChE, which is responsible for terminating the transmission of the nerve impulse. Hence, AChE inhibition is an established biomarker of OP and carbamate exposure that is more reliable than the analytical determination of pesticide content for the diagnosis of poisoning, because it accounts for a physiological effect directly linked to the compound toxic mode of action and persists much longer than pesticides in environmental samples (Fulton & Key 2001). However, OP toxicity in general implies more than AChE inhibition, since Bagchi et al. (1995) found that different classes of pesticides may induce *in vitro* and *in vivo* generation of reactive oxygen species (ROS). They are mainly hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and hydroxyl radical ($HO\cdot$), which are able to react with biological macromolecules (specially the hydroxyl radical) and cause enzyme inactivation, lipid peroxidation and DNA damage. The balance between ROS production and antioxidant defences determines

the degree of oxidative damage. In fact, dichlorvos induced oxidative stress effects in the carp (*Cyprinus carpio*) and catfish (*Ictalurus nebulosus*) (Hai et al. 1997).

In previous studies we demonstrated that thiocarbamate herbicides induced oxidative stress in the European eel (*Anguilla anguilla* L.) and we highlighted the importance of glutathione metabolism in the tolerance to these pesticides (Peña-Llopis et al. 2000, 2001). Glutathione is a ubiquitous thiol-containing tripeptide that is involved in numerous processes that are essential for normal biological function, such as the detoxification of electrophilic xenobiotics and free-radical scavenging (Meister & Anderson, 1983). It is predominantly present in cells in its active and reduced form (GSH), but as a consequence of oxidising conditions, two molecules of GSH are linked by a disul□de bond to comprise a molecule of oxidised glutathione (GSSG). Then, the GSH/GSSG ratio or glutathione redox status is considered an index of the cellular redox status and a biomarker of oxidative damage, because glutathione maintains the thiol-disulphide status of proteins, acting as a redox buffer.

As we found previously that those eels that enhanced GR activity, increased GSH, and maintained the GSH/GSSG ratio in liver showed a higher survival upon herbicide exposure (Peña-Llopis et al. 2001), a drug that could increase the GSH content and act as a reductant would improve the tolerance of OP-poisoned fish. NAC is a known antioxidant and free radical scavenger that can easily be deacetylated to L-cysteine, the limiting amino acid for glutathione synthesis. This antioxidant extended the survival of *A. anguilla* exposed to a lethal concentration of dichlorvos because enhanced the glutathione metabolism (Peña-Llopis et al. submitted).

The main objective of this work was to evaluate the effect of the antioxidant NAC on the recovery of European eels intoxicated with a sublethal concentration of dichlorvos.

MATERIALS AND METHODS

Experimental Animals

Young yellow eels of the species *Anguilla anguilla* (10-20 g weight), which were sexually undifferentiated at this stage of development, were used to avoid the effects of sex variation and minimise hormonal interactions in toxicity assays. These European eels were obtained from a fish farm (Valenciana de Acuicultura S.A., Spain) free of any disease. Animals were acclimatised to laboratory conditions, according to OECD guidelines (1992), for two weeks before the starting of experiments in aerated and filtered dechlorinated freshwater (total hardness: 198 ± 5 mg l^{-1} as $CaCO_3$; pH: 7.4 ± 0.1; dissolved oxygen: 7.2 ± 0.1 mg l^{-1}) at 24.0 ± 0.5°C, and with a 12-h photoperiod. Fish did not respond to feeding attempts during the acclimatisation period (Van Waarde et al. 1983; Ferrando, 1990).

Chemicals

Dichlorvos (2,2-dichlorovinyl dimethyl phosphate; DDVP) was obtained from Laboratorios Hipra S.A. (Girona, Spain) as an emulsifiable concentrate which contained 40% of the parent pesticide (Hexipra Solucion®). 2-vinylpyridine was acquired from Aldrich. NADPH was purchased from Applichem (Darmstadt, Germany). *N*-Acetyl-L-cysteine and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless mentioned otherwise.

Experimental Procedure

Initial experiments were performed in order to choose the best concentrations of the pesticide and the antioxidant for the study of exposure and recovery. Previous concentration-effect experiments indicated that the median lethal concentration at 96 h (96-h LC_{50}) for dichlorvos in the European eel was 0.852 mg l^{-1} (95% Confidence Interval, 0.735 - 0.957) (Peña-Llopis et al. submitted). A sublethal concentration of dichlorvos of 0.17 mg l^{-1}, which corresponded to 1/5 of the 96-h LC_{50}, was then selected for all the experiments. As the pesticide formulation contained 8% of emulgators and 47% of non-toxic solvents, composed principally by 2-propanol (Laboratorios Hipra S.A., personal communication), controls were exposed to similar solvent conditions, that is, 0.2 mg l^{-1} of 2-propanol. This concentration is 40,000 times lower than the 96-h LC_{50}s for freshwater fish, which ranged from 4,200 to 11,130 mg l^{-1} (WHO 1990), thus the inclusion of another control group without the solvent was unnecessary.

Two experimental procedures were conducted to choose the NAC concentration for the recovery experiment. Firstly, fish were exposed for 48 h to several concentrations of NAC (0, 0.1, 1, and 10 mM) in order to compare the GSH levels in the liver and muscle. Secondly, fish were exposed to a sublethal concentration of dichlorvos (0.17 mg l^{-1}, 1/5 96-h LC_{50}) for 48 h and then transferred to 0, 0.1, 0.5, and 1 mM NAC solutions for 48 h. As discussed below in the results section, the most suitable concentration of NAC was 0.5 mM (81.6 mg l^{-1}).

Five groups of animals were used:

1. *Controls*. These animals received no treatment but were maintained in the same solvent environment (2-propanol, 0.2 mg l^{-1}) for 96 h.

2. *NAC*. Fish were exposed to 0.5 mM NAC and 0.2 mg l^{-1} of 2-propanol for 96 h.
3. *Dichlorvos*. Fish were exposed to a sublethal concentration of dichlorvos (0.17 mg l^{-1}, 1/5 96-h LC_{50}) for 96 h.
4. *D + Water*. Fish were exposed to 0.17 mg l^{-1} dichlorvos for 96 h and then transferred to clean water for 96 h.
5. *D + NAC*. Fish were exposed to 0.17 mg l^{-1} dichlorvos for 96 h and then transferred to a 0.5 mM NAC solution for 96 h.

All groups were maintained per duplicate in 40-L glass aquaria at 24.0 ± 0.5 °C in a static-renewal system, in accordance with OECD guidelines (1992), where water and either the pesticide or NAC were completely replaced every 24 h. No mortality was observed during the experiment but fish exposed to the pesticide showed convulsions, tremors and erratic swimming. Five to six animals were removed from each group at 3, 6, 12, 24, 48, and 96 h after the treatment, which corresponded to the 99, 102, 108, 120, 144, and 192 h for the D + Water and D + NAC groups, and were anaesthetised in ice instead of using chemical anaesthesia because it could interfere with glutathione metabolism (Brigelius et al. 1982). They were then weighed, the length measured, and euthanised by decapitation. The brain and muscle were excised, weighed and stored frozen at -80 °C until the biochemical determinations were performed.

Glutathione Determination

Liver and muscle were homogenised with 5 volumes of ice-cold 5% 5-sulfosalicylic acid per gram of wet weight tissue, and further processed by sonication (Vibra-Cell, Sonics & Materials Inc., Danbury, CT, USA). Homogenates were then centrifuged at 20,000*g* for 10 minutes at 4 °C. Total glutathione content (tGSx) and GSSG were determined in supernatant fractions with a sensitive and specific assay using a recycling reaction of GSH with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of excess glutathione reductase according to Baker et al. (1990) in a microplate reader (Model 3550, Bio-Rad Laboratories, Richmond, CA, USA) as previously described (Peña-Llopis et al. 2001). Glutathione concentrations were expressed as nmol of GSH equivalents (GSx) per mg of protein (GSx = [GSH] + 2 · [GSSG]). GSH was calculated by subtracting GSSG levels from the tGSx levels determined. The GSH/GSSG ratio was expressed as number of molecules but not moles: GSH/GSSG = (tGSx - GSSG) / (GSSG / 2).

Enzyme Assays

Brain tissues were homogenised with 10 volumes of Henriksson stabilising medium (Henriksson et al. 1986), which contained 50% glycerol, 20 mM phosphate buffer pH 7.4, 0.5 mM EDTA, and 0.02% defatted bovine serum albumin. β-Mercaptoethanol was not included because it interferes with the GR assay. Homogenates were centrifuged at 20,000*g* for 10 minutes at 4°C, and the resulting supernatants were diluted 5-fold with buffer and assayed rapidly for enzyme activities. AChE (EC 3.1.1.7) activity was determined with acetylthiocholine as substrate in accordance to an adaptation of the Ellman method (Ellman et al. 1961) to microtiter plates by Doctor et al. (1987), but with 0.1 M phosphate buffer, pH 7.27 and 1 mM EDTA as recommended by Riddles et al. (1979). Eel cholinesterase activity detected was considered as true AChE as was previously characterised (Ferenczy et al. 1997). GR (EC 1.6.4.2) activity was assayed by the method of Cribb et al. (1989) with the following final concentrations: [DTNB] = 0.075 mM; [NADPH] = 0.1 mM; [GSSG] = 1 mM according to Smith et al. (1988). A calibration curve of known activities of purified enzymes was used on every 96-well plate to avoid miscalculations that stem from an ill-defined path length. AChE (type V) from electric eel and GR (type III) from baker's yeast were used as standards, whose activities were determined in quartz cuvettes using a Hitachi U-2001 UV/Vis spectrophotometer (Hitachi Instruments Inc., USA). A molar absorption coefficient at 412 nm (ε_{412}) of 14.150 was used for the dianion of DTNB (TNB^{2-}) as Riddles et al. (1979) d etermined. Specific enzyme activities were expressed as nmoles of substrate hydrolysed per min per mg protein (mU mg^{-1} prot).

Protein Determination

Protein content was determined by the Bio-Rad Protein Assay kit (Bio-Rad Laboratories GmbH, Munich, Germany) based on the Bradford dye-binding procedure, using bovine serum albumin as standard.

Statistical Analyses

NAC solutions in preliminary experiments were compared to controls by the Dunnett's test using the SPSS 10.0 statistical software package (SPSS Inc, Chicago, IL, USA), which was used for all the other statistical analyses. The two-factor analysis of variance (ANOVA) with the type III sum-of-squares method by means of multivariate general linear models (GLM) were used to investigate the effect of time and exposure to dichlorvos and/or

NAC and their interaction on biochemical parameters. *A priori* contrasts between selected levels of factors were made to compare means at specific exposure times. The GSH/GSSG ratios were log-transformed when showed heterogeneity of variances according to the Levene test.

RESULTS

Preliminary Experiments

Early experiments were performed to choose the most suitable NAC concentration for recovery from OP intoxication. Fish that were exposed to a 0.1 mM NAC solution for 48 h did not alter their glutathione levels in the liver or muscle, whereas those that were treated with 1 mM concentration increased significantly the muscular GSH content ($p < 0.05$; Table 1). However, all eels that were exposed to a 10 mM NAC solution died within the 48 h but glutathione levels at post-mortem time did not change significantly. These mortalities might be due to the excessive ROS generated by NAC, because very high doses of low-molecular-weight thiols are pro-oxidants instead of antioxidants (Sprong et al. 1998).

When fish were exposed to a sublethal concentration of dichlorvos (0.17 mg l^{-1}, 1/5 96-h LC_{50}) for 48 h and were transferred to a 0.1 mM NAC solution for 48 h, they did not present significant differences in glutathione levels in the liver or muscle compared to fish recovered in clean water (Table 2). Conversely, fish recovered in 0.5 and 1 mM NAC solutions increased muscular GSH content ($p < 0.05$). Furthermore, the 0.5 mM NAC concentration increased the hepatic glutathione redox status (GSH/GSSG) ($p < 0.05$). Therefore, a 0.5 mM NAC concentration offered a better ratio of benefits/risks compared to 1 mM and was selected for the subsequent experiments.

Effect of NAC exposure

The effect of the treatment of European eels with a 0.5 mM NAC solution was an increase of muscular GSH content ($p = 0.006$; Table 3) and GSH/GSSG ratio ($p = 0.05$) without any significant change to GSSG levels ($p = 0.65$) nor brain AChE activity ($p = 0.55$). NAC treatment also positively influenced the GR activity ($p = 0.012$).

When NAC-treated eels were compared to controls according to the sampling time, the GSH content in the muscle was increased by 29% from 48 to 96 h (Table 4), and the GSH/GSSG ratio was raised by 24% at 96 h. However, NAC treatment produced no effect on GSSG levels or AChE activity (Table 5), but increased significantly the GR activity by 29% from 48 to 96 h.

Table 1. Glutathione levels in the liver and muscle of the European eel after being exposed to different concentrations of NAC for 48 h[a].

[NAC] (mM)	Liver			Muscle		
	GSH	GSSG	GSH/GSSG	GSH	GSSG	GSH/GSSG
0	63.0 ± 4.1	2.9 ± 0.4	46.0 ± 5.4	14.7 ± 1.4	1.20 ± 0.13	25.2 ± 2.5
0.1	68.6 ± 6.1	3.3 ± 0.4	43.4 ± 4.8	17.1 ± 2.7	1.31 ± 0.27	27.3 ± 2.6
1	78 ± 2.9	2.8 ± 0.2	56.9 ± 4.7	22.4 ± 1.6*	1.41 ± 0.07	31.8 ± 1.7
10	65.3 ± 7.9	2.9 ± 0.5	46.6 ± 3.2	16.2 ± 0.8	1.18 ± 0.10	28.4 ± 3.7

[a], Values are mean ± SE ($n = 5$). GSH and GSSG levels were expressed in nmol GSx mg^{-1} prot. GSH/GSSG levels were expressed in number of molecules but not moles.
*, $p < 0.05$ according to the Dunnett's test.

Table 2. Effect of exposure to 0.17 mg l^{-1} dichlorvos for 48 h and recovery in NAC solutions for 48 h on glutathione levels in the liver and muscle of the European eel[a].

[NAC] (mM)	Liver			Muscle		
	GSH	GSSG	GSH/GSSG	GSH	GSSG	GSH/GSSG
0	49.9 ± 6.5	3.4 ± 0.3	30.7 ± 5.8	8.1 ± 1.5	1.06 ± 0.24	16.7 ± 2.7
0.1	54.0 ± 7.0	2.3 ± 0.5	50.5 ± 4.7	12.0 ± 1.8	1.32 ± 0.10	19.0 ± 3.8
0.5	54.1 ± 7.9	2.2 ± 0.4	56.2 ± 8.4*	14.3 ± 1.4*	1.57 ± 0.51	25.1 ± 6.1
1	53.9 ± 7.9	2.4 ± 0.4	47.2 ± 5.6	14.8 ± 1.1*	1.33 ± 0.22	25.4 ± 5.4

[a], Values are mean ± SE ($n = 5$). GSH and GSSG levels were expressed in nmol GSx mg^{-1} prot. GSH/GSSG levels were expressed in number of molecules but not moles.
*, $p < 0.05$ according to the Dunnett's test.

Table 3. *F* statistics and corresponding *p*-values (in parenthesis) of the two-factor ANOVAs to test the effect of time and exposure to 0.17 mg l^{-1} dichlorvos and/or 0.5 mM NAC on several biochemical parameters of the European eel.

		Muscle			Brain	
Contrast	Effect	GSH	GSSG	GSH/GSSG	AChE	GR
NAC vs. Control	Time	2.2 (0.07)	0.55 (0.74)	1.66 (0.16)	2.2 (0.07)	1.50 (0.21)
	NAC	8.1 (0.006)	0.21 (0.65)	4.0 (0.05)	0.37 (0.55)	6.7 (0.012)
	Time × NAC	1.12 (0.36)	0.21 (0.96)	0.36 (0.87)	0.93 (0.47)	1.41 (0.24)
Dichlorvos vs. Control	Time	0.81 (0.55)	1.85 (0.12)	6.4 (<0.0001)	2.8 (0.024)	3.8 (0.005)
	Dichlorvos	41 (<0.0001)	17.4 (0.00011)	136 (<0.0001)	194 (<0.0001)	35 (<0.0001)
	Time × Dichlorvos	1.52 (0.20)	1.79 (0.13)	8.0 (<0.0001)	8.1 (<0.0001)	0.32 (0.90)
D+Water vs. Control	Time	0.56 (0.73)	1.14 (0.35)	1.10 (0.37)	5.3 (0.0006)	1.12 (0.36)
	D+Water	55 (<0.0001)	12 (0.0012)	81 (<0.0001)	445 (<0.0001)	42 (<0.0001)
	Time × D+Water	1.22 (0.32)	1.27 (0.29)	0.19 (0.96)	2.4 (0.05)	1.39 (0.25)
D+NAC vs. Control	Time	2.6 (0.038)	0.62 (0.68)	1.79 (0.13)	8.6 (<0.0001)	0.80 (0.56)
	D+NAC	14.1 (0.0005)	14.7 (0.0004)	49 (<0.0001)	269 (<0.0001)	18.4 (<0.0001)
	Time × D+NAC	3.5 (0.008)	1.25 (0.30)	0.81 (0.55)	3.5 (0.009)	1.16 (0.34)
D+Water vs. D+NAC	Time	6.1 (0.00019)	1.95 (0.10)	4.8 (0.0013)	44 (<0.0001)	1.7 (0.15)
	NAC	11.7 (0.0013)	0.34 (0.56)	6.11 (0.017)	48 (<0.0001)	6.3 (0.016)
	Time × NAC	2.3 (0.06)	1.01 (0.42)	1.09 (0.38)	3.7 (0.007)	1.5 (0.21)

Effect of dichlorvos exposure

Exposure of European eels to a sublethal concentration of the OP pesticide resulted in a significant decrease of muscular GSH content ($p < 0.0001$, Table 3) but an increase of GSSG ($p = 0.00011$) that oxidised dramatically the glutathione redox status ($p < 0.0001$), which is indicative of the cellular redox status. In addition, the GSH/GSSG ratio decreased over time ($p < 0.0001$) and a significant interacting effect was found between time and dichlorvos exposure for glutathione redox status ($p < 0.0001$). AChE activity in brain was severely inhibited by the effect of the insecticide ($p < 0.0001$) and was time dependent ($p = 0.024$), with a significant time interaction ($p < 0.0001$). GR activity was also time-dependently ($p = 0.005$) inhibited as a consequence of the exposure to dichlorvos ($p < 0.0001$).

Table 4. Glutathione levels in the muscle of the European eel during exposure to 0.17 mg l^{-1} dichlorvos for 96 h and/or recovery in clean water or a 0.5 mM NAC solution for 96 h[a].

	t (h)	Control	NAC	Dichlorvos	D + Water	D + NAC
GSH	3	17.0 ± 2.2	16 ± 2.2	14.3 ± 1.1	7.5 ± 1.1•••	9.0 ± 1.9••
	6	17.6 ± 0.7	18.2 ± 2.1	13.6 ± 1.3	11.2 ± 1.6••	8.7 ± 1.1•••
	12	16.5 ± 1.6	19.4 ± 1.4	12.8 ± 1.4	13.1 ± 1.4	16.9 ± 1.3
	24	16.4 ± 1.4	20.5 ± 1.3	11.2 ± 1.1•	11.5 ± 1.9•	19.5 ± 1.4§§§
	48	18.7 ± 1.9	24.7 ± 1.6••	10.4 ± 1.4•••	9.2 ± 1.0•••	14.1 ± 2.2§
	96	17.8 ± 2.2	22.3 ± 1.4•	8.7 ± 1.2•••	9.4 ± 1.7•••	12.9 ± 2.2
GSSG	3	1.44 ± 0.13	1.39 ± 0.18	1.42 ± 0.06	2.04 ± 0.23•	1.69 ± 0.26
	6	1.32 ± 0.13	1.28 ± 0.22	1.42 ± 0.12	1.67 ± 0.23	1.61 ± 0.21
	12	1.27 ± 0.20	1.26 ± 0.11	1.69 ± 0.21	1.76 ± 0.06	1.64 ± 0.11
	24	1.23 ± 0.08	1.36 ± 0.07	1.83 ± 0.24•	1.81 ± 0.27•	2.21 ± 0.15•••
	48	1.38 ± 0.11	1.59 ± 0.22	1.92 ± 0.11•	1.53 ± 0.1	1.83 ± 0.2
	96	1.39 ± 0.21	1.40 ± 0.16	2.28 ± 0.29••	1.29 ± 0.17	1.54 ± 0.32
GSH/GSSG	3	23.9 ± 3.2	23.0 ± 1	20.3 ± 2.0	8.1 ± 1.9•••	10.8 ± 1.9•••
	6	27.5 ± 2.3	30.2 ± 2.9	19.2 ± 1.2••	13.8 ± 1.5•••	11.1 ± 1.2•••
	12	28.2 ± 4.1	31.7 ± 3	15.5 ± 1.2•••	14.9 ± 1.6•••	20.9 ± 2§
	24	27.0 ± 2.2	30.2 ± 1.4	12.7 ± 0.8•••	13.3 ± 2.0•••	17.9 ± 1.6•
	48	28.1 ± 4.2	32.8 ± 3.2	10.7 ± 1.0•••	12.3 ± 1.5•••	15.6 ± 2.1••
	96	26.7 ± 3.0	33.1 ± 2.4•	7.6 ± 0.5•••	15.3 ± 2.8••	18.6 ± 3.2•

[a], Values are mean ± SE (n = 5-6). GSH and GSSG levels were expressed in nmol GSx mg^{-1} prot. GSH/GSSG levels were expressed in number of molecules but not moles. The groups D + Water and D + NAC were previously exposed to 0.17 mg l^{-1} dichlorvos for 96 h.

•, ••, ••• $p < 0.05$, 0.01, and 0.001, respectively, compared to the Control group.

§, §§, §§§ $p < 0.05$, 0.01, and 0.001, respectively, compared to the D + Water group.

Table 5. AChE and GR activities in the brain of the European eel during the exposure to 0.17 mg l^{-1} dichlorvos for 96 h and/or recovery in clean water or a 0.5 mM NAC solution for 96 h[a].

	t (h)	Control	NAC	Dichlorvos	D + Water	D + NAC
AChE	3	234 ± 12	279 ± 16	151 ± 9•••	51 ± 8•••	57 ± 6•••
	6	207 ± 22	215 ± 19	158 ± 16•	61 ± 3•••	77 ± 11•••
	12	226 ± 15	214 ± 13	149 ± 19•••	82 ± 5•••	99 ± 8•••
	24	239 ± 12	255 ± 15	95 ± 12•••	73 ± 6•••	131 ± 11•••,§§§
	48	250 ± 20	244 ± 24	69 ± 14•••	81 ± 7•••	132 ± 7•••,§§§
	96	244 ± 14	228 ± 12	48 ± 12•••	140 ± 9•••	173 ± 5•••,§§
GR	3	36.2 ± 3.2	37.3 ± 1.6	31.1 ± 1.6	22.8 ± 2.3•••	24.8 ± 2.2••
	6	32.9 ± 3.5	34.3 ± 2.7	24.4 ± 1.6••	23.9 ± 2.5••	22.9 ± 2.8••
	12	31.1 ± 2.5	29.5 ± 1.5	20.5 ± 2.9••	25.9 ± 1.0	27.2 ± 2.4
	24	32.4 ± 2.0	37.1 ± 1.7	23.2 ± 1.1••	19.8 ± 0.8•••	28.5 ± 2.2§§
	48	29.6 ± 2.3	37.4 ± 2.5•	18.6 ± 3.0••	19.9 ± 1.6••	23.7 ± 0.6
	96	28.6 ± 3.4	37.5 ± 2.2•	19.5 ± 3.3••	25.2 ± 1.4	26.9 ± 1.0

[a], Values are mean ± SE (n = 5-6). Activities were expressed as mU mg^{-1} protein. The groups D + Water and D + NAC were previously exposed to 0.17 mg l^{-1} dichlorvos for 96 h.

•, ••, ••• $p < 0.05$, 0.01, and 0.001, respectively, compared to the Control group.

§, §§, §§§ $p < 0.05$, 0.01, and 0.001, respectively, compared to the D + Water group.

When dichlorvos-exposed fish were compared to controls (Tables 4 and 5), GSH and GSSG levels in muscle were significantly different from 24 to 96 h of exposure, whereas the GSH/GSSG ratio and brain GR activity decreased from 6 h. However, AChE activity in the brain was inhibited from the beginning.

Recovery

Fish that were exposed to a sublethal concentration of dichlorvos for 96 h and were then transferred to clean water, continued with GSH depletion ($p < 0.0001$, Table 3), increased GSSG ($p = 0.0012$), and low GSH/GSSG ratio ($p < 0.0001$) in the muscle and AChE and GR inhibition in the brain ($p < 0.0001$). The recovery of brain AChE was time-dependent ($p = 0.0006$), with an interaction effect of time with treatment ($p = 0.05$).

When fish were allowed to recover in a 0.5 mM NAC solution instead of only water, muscular GSH increased time-dependently ($p = 0.038$) with an interaction effect of time with the treatment ($p = 0.008$). Then, NAC treatment improved muscular GSH content ($p = 0.0013$), which was increased by 69% at 24 h ($p < 0.001$) and 54% at 48 h ($p < 0.05$) compared to fish recovered in water. Although this GSH augment was concomitant to a GSSG increase at 24 h ($p < 0.001$), glutathione redox status was less oxidised than water-recovered fish ($p = 0.017$, Table 3), being 40% higher at 12h ($p < 0.05$). Brain AChE activity increased over time ($p < 0.0001$) and was less inhibited than in water-recovered fish ($p < 0.0001$), with an interaction effect of NAC with time ($p = 0.007$). GR activity was also less inhibited in NAC-treated fish ($p = 0.016$). Then, NAC treatment increased significantly by 47% brain AChE recovery from 12 to 96 h (Table 5 and Fig. 1), being 78% higher at 24 h ($p < 0.001$), whereas GR inhibition was reduced by 43% after 24 h of recovery ($p < 0.01$; Fig. 2), returning to the control levels.

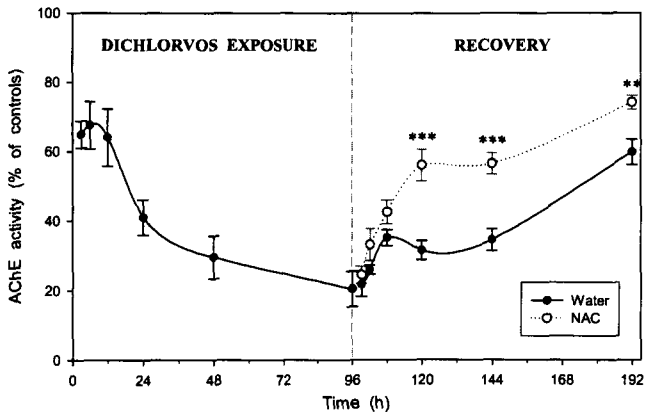

Fig. 1. AChE activity in the brain of the European eel (*A. anguilla*) after 96 h of exposure to 0.17 mg l^{-1} of dichlorvos and recovery for 96 h in clean water (solid line) or in 0.5 mM NAC (dotted line).

*, **, *** $p < 0.05$, 0.01, and 0.001, respectively, at each specific time.

DISCUSSION

The present work demonstrates that the antioxidant and glutathione pro-drug NAC improves the recovery from dichlorvos poisoning in the European eel. A sublethal concentration of this OP pesticide (0.17 mg l^{-1}, 1/5 of the 96-h LC_{50}) decreased and oxidised muscular GSH levels, declining the GSH/GSSG ratio, which is indicative of a process of oxidative stress. In addition, AChE and GR activities were inhibited in the brain.

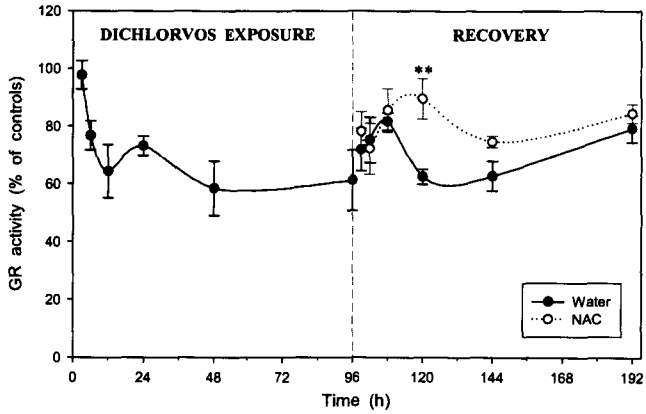

Fig. 2. GR activity in the brain of the European eel (*A. anguilla*) after 96 h of exposure to 0.17 mg l^{-1} of dichlorvos and recovery for 96 h in clean water (solid line) or in 0.5 mM NAC (dotted line).

**, $p < 0.01$ at each specific time.

These results are consistent with the data of Hai et al. (1997), who have shown GSH decrease in the liver and muscle of the common carp (*Cyprinus carpio*), and inhibition of the brain AChE activity after being exposed to 1 and 5 mg l^{-1} of dichlorvos for 24 h.

Dichlorvos is metabolised mainly in the liver *via* two enzymatic pathways: one, producing desmethyldichlorvos, is glutathione dependent, while the other, resulting in dimethyl-phosphate and dichloroacetaldehyde, is glutathione independent (Dicowsky & Morello 1971). Hence, GSH availability can result in a limiting factor for dichlorvos elimination. Although *de novo* synthesis of glutathione by GCL is regulated by feedback inhibition of GSH (Richman & Meister 1975), NAC baths increased muscular GSH content. Furthermore, NAC treatment ameliorated muscular GSH depletion.

The relationship between AChE inhibition and mortality is species- and age-specific, but normally a brain AChE inhibition higher than 70% is associated with mortality (Fulton & Key 2001). In the present study, brain AChE inhibition in dichlorvos-exposed fish increased over time and reached 80% of the control activity after 96 h exposure, but no mortality was experienced. Sancho et al. (1997) found a 64% inhibition of brain AChE in European eels exposed for 96 h to a sublethal (1/5 of the corresponding 96-h LC_{50}) concentration of the OP insecticide fenitrothion, which is a cholinesterase inhibitor less potent than dichlorvos because it requires activation to the oxygen analogue fenitrooxon.

The recovery of brain AChE activity is a function of the degree of the initial inhibition (Morgan et al. 1990). As the recovery of the enzyme activity is basically a result of the *de novo* synthesis of enzyme protein, the greater the degree of inhibition, the more protein synthesis is required (Fulton & Key 2001). Sancho et al. (1997) found that the AChE inhibition of fenitrothion-intoxicated fish decreased from 64% to 49% and 37% after 96 and 192 h, respectively, of recovery in clean water. In the current study, brain AChE was still inhibited by 40% after 96 h of recovery in clean water, but only by 26% in the 0.5 mM NAC solution. These results indicate that bath treatments with NAC enhance the recovery of AChE activity, which to our knowledge have not previously reported without the use of an oxime to reactivate the inhibited AChE.

In a recent study (Peña-Llopis et al. submitted), NAC treatment extended the survival of European eels exposed to a lethal concentration of dichlorvos. This survival was especially associated to the maintenance of the hepatic glutathione redox status, muscular AChE and GR activities, muscular GSH levels, and hepatic GCL activity.

The process of OP toxicity and the involvement of glutathione metabolism are summarised in Fig. 3. The inhibition of brain AChE activity by OP pesticides blocks the hydrolysis of the neurotransmitter acetylcholine (ACh), which can accumulate and activate muscarinic receptors. The overstimulation of cholinergic neurones initiates a process of hyperexcitation and convulsive activity that progresses rapidly to status epilecticus, which leads to profound structural brain damage or the death of the organism if the muscarinic ACh receptor antagonist atropine is not swiftly administered. These OP-induced seizures allow the release of excitatory amino acids, such as glutamate and aspartate, prolonging the convulsive activity, and making atropine treatment ineffective (Shih & McDonough 1997). High concentrations of these excitatory amino acids can activate the *N*-methyl-D-aspartate (NMDA) receptor, leading to intracellular influx of Ca^{2+}, which triggers the activation of proteolytic enzymes and free radical generation (Beal 1995). These effects can be attenuated or reversed using NMDA receptor antagonists (Solberg & Belkin 1997). Free radicals may, in addition, inhibit the activity of AChE probably by oxidation of an amino acid critical for enzyme function (Den Hartog et al. 2002) and prevent the recovery of the enzyme function.

Cytosolic free radicals are either removed non-enzymatically or by antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (GPx), which oxidises GSH to GSSG (Fig. 3). GSSG is then reduced back to GSH by glutathione reductase (GR) through oxidation of NADPH to $NADP^+$, which is recycled by the pentose phosphate pathway. Raising the GSH/GSSG ratio by reducing GSSG to two molecules of GSH is energetically less costly than synthesising GSH *de novo*, which is regulated by the enzyme glutamate-cysteine ligase (GCL). Furthermore, glutathione *S*-transferases (GST) catalyse the conjugation of GSH to the OP pesticide leading to its detoxification and

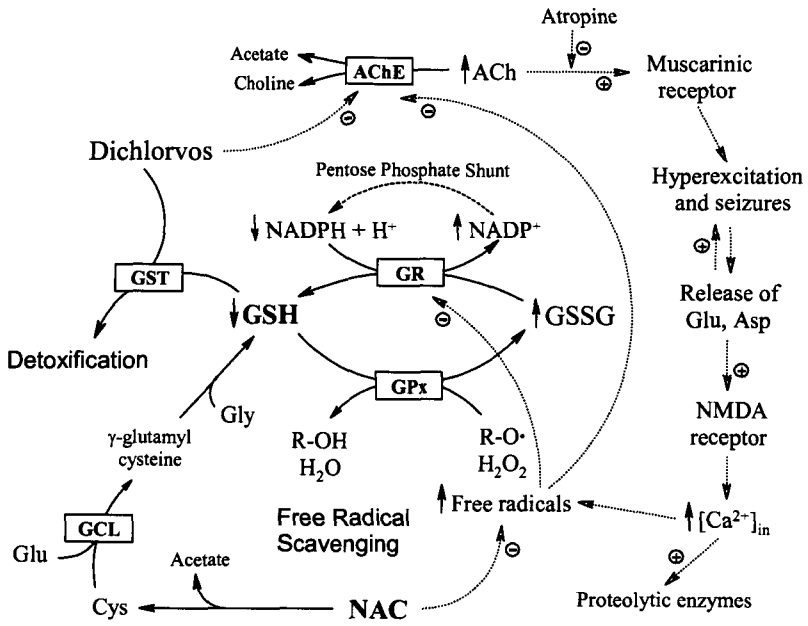

Fig. 3. Scheme of the proposed involvement of the glutathione metabolism in the toxicity of dichlorvos. The OP inhibits the brain AChE activity, leading to excessive accumulation of ACh and overstimulation of muscarinic receptors. This process produces neuronal hyperexcitation and seizures that allow the release of excitatory amino acids, such as Glu and Asp, which activate the NMDA receptor. Excessive activation of NMDA receptors triggers the influx of large amounts of extracellular Ca^{2+}, which activates proteolytic enzymes and leads to formation of free radicals. Then, GSH oxidises to GSSG to remove the reactive oxygen species, catalysed by GPx or non-enzymatically. GSSG is subsequently reduced to GSH by GR at expense of oxidising NADPH to $NADP^+$, which is recycled by the pentose phosphate pathway. In addition, GSH can be conjugated to dichlorvos by GST allowing the detoxification of the pesticide. The antioxidant NAC acts as a reductant and free radical scavenger that can also be deacetylated to Cys, the limiting amino acid for glutathione synthesis, which is regulated by GCL.

elimination. Bath treatments with NAC were a source of the limiting amino acid Cys to synthesise glutathione when it is depleted, enhancing the detoxification of the OP. NAC acts also as a reductant and free radical scavenger ameliorating the enzyme inhibition caused by the free radicals generated by dichlorvos.

Despite the widespread applications and extensive use of dichlorvos since its commercial introduction in 1961, the US Environmental Protection Agency (USEPA) had made public in 1995 its intention to cancel several dichlorvos registrations, because it was classified as a possible carcinogen for man (USEPA 1995). Detailed examination of the animal experimental data by independent experts eventually concluded that no measurable cancer hazard can legitimately be associated with exposure to dichlorvos (Mennear 1998) and downgraded its classification toward *non-classifiable with regard to cancer in man* (Van Maele-Fabry et al. 2000). However, its use is banned in the UK for bath treatments of fish against sea lice infestations (Costello et al. 2001), and as presented reduced efficacy caused by parasite resistance (Jones et al. 1992), it has been replaced by other OPs like azamethiphos (Roth et al. 1996). This insecticide was also found useful to treat parasite infestations in the European eel (Pretti et al. 2002).

Although the standard treatment against OP poisoning consists of reactivation of the inhibited AChE with an oxime and reversal of the biochemical effects of acetylcholine with atropine (Kwong 2002), the present study demonstrates that NAC may be a complementary antidote for OP poisoning, acting at different target sites: increasing the detoxification of the OP and the removal of free radicals. In addition, NAC characteristics, such as its high solubility and low price make it suitable for fish treatment by baths after antiparasitic actions. This is especially important in the case of treatment of parasitic infestations in warm waters because temperature increases the toxicity of pesticides, representing a risk of the fish health. This is the case of the culture of the European eel in recirculated freshwater or marine farms of sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) in the Mediterranean, which are infested by cymothoid isopods (Horton & Okamura 2001), and the use of pesticides is encouraged (Papapanagiotou & Trilles 2001).

CONCLUSIONS

NAC improves fish recovery from a sublethal concentration of dichlorvos through enhancing the glutathione metabolism and decreasing the glutathione loss and oxidation, and enzyme inactivation caused by the OP pesticide. Therefore, NAC increases the tolerance to dichlorvos-induced oxidative stress, being a potential antidote for OP poisoning that can be administered by baths. However, NAC concentrations should be controlled because very high doses may be harmful for fish health.

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APPENDIX

Abbreviations: AChE, acetylcholinesterase; γ-GT, γ-glutamyl transferase; GCL, glutamate- cysteine ligase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidised glutathione or glutathione disulfide; GST, glutathione *S*-transferase; NAC, *N*-acetyl-L-cysteine; NMDA, *N*-methyl-D-aspartate; OP, organophosphate.

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