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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
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ANA MARIA MONTEIRO PAIVA COIMBRA

EFEITO DE POLUENTES ORGANOCLORADOS NO COMPORTAMENTO REPRODUTIVO E NO DESENVOLVIMENTO LARVAR DA TILÁPIA NILÓTICA, *OREOCHROMIS NILOTICUS*

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Dissertação de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Orientadora – Professora Doutora Maria Armanda Reis Henriques
Professora Catedrática do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto
Co-orientadora – Professora Doutora Maria do Rosário Rodrigues de Almeida Martins
Professora Associada do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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RESUMO

O desenvolvimento e a reprodução dos vertebrados são controlados pelo sistema endócrino, no qual estão incluídos os eixos hipotálamo-hipófise-tiróide, hipotálamo--hipófise-inter-renal (equivalente ao eixo hipotálamo-hipófise-supra-renal dos mamíferos) e hipotálamo-hipófise-gónadas. O equilíbrio ou homeostasia destes sistemas pode ser perturbado por efeitos resultantes de contaminantes presentes no meio ambiente, os quais actuam sobre estes eixos, alterando o seu funcionamento e interferindo com os mecanismos que estão sob o seu controlo. Entre os contaminantes ambientais destacam--se compostos possuidores de átomos de cloro, que incluem os bifenis policlorados (PCBs) e os insecticidas organoclorados. Estes compostos, devido às suas propriedades físico-químicas, permanecem no meio ambiente e nos tecidos dos organismos onde exercem os seus efeitos durante longos períodos de tempo.

Este trabalho pretende estudar os efeitos de dois contaminantes organoclorados numa espécie de ciclídeos, mais precisamente tilápia do Nilo ou *Oreochromis niloticus*. Os compostos seleccionados foram o Endosulfan, um insecticida organoclorado, e o Aroclor 1254, uma mistura de congéneres de PCBs. Os compostos foram incorporados na ração, sendo, por isso, a exposição feita através da alimentação.

Os resultados mostraram que o Endosulfan actuou ao nível do eixo hipotálamo--hipófise-inter-renal originando diminuição da concentração plasmática de cortisol e, consequentemente, das actividades das ATPase Na^+,K^+ do rim e da brânquia, o que indica uma menor capacidade de osmorregulação em tilápias expostas.

Este insecticida exerceu uma acção no eixo hipotálamo-hipófise-tiróide, de forma dependente da dose utilizada, dando origem à diminuição da concentração plasmática de T4 e actuou sobre o metabolismo periférico das hormonas da tiróide, interferindo com a actividade das desiodases em alguns tecidos. Apesar das alterações observadas na concentração de T4 e na actividade das desiodases, os níveis circulantes de T3 não foram significativamente alterados pela exposição a este contaminante. Verificou-se ainda que o Endosulfan não interferiu com o transporte das hormonas da tiróide pelas proteínas plasmáticas de transporte em tilápia, a albumina e uma apolipoproteína de 14 kDa. Estes resultados indicam que a acção do Endosulfan no eixo da tiróide ocorre ao nível central, diminuindo a síntese e/ou secreção de T4, e ao nível dos tecidos, podendo alterar a disponibilidade local destas hormonas.

Para além dos efeitos nos dois eixos estudados, observou-se que a exposição ao Endosulfan induziu destruição do tecido hepático, podendo, desta forma, interferir com múltiplas vias metabólicas.

Estudos similares foram realizados com o outro poluente seleccionado, o Aroclor 1254. Neste caso, avaliaram-se os efeitos ao nível do eixo hipotálamo-hipófise-tiróide e do desenvolvimento das gónadas e reprodução, em tilápias adultas expostas durante as fases larvar e adulta.

Os resultados mostraram que, embora a exposição ao Aroclor 1254 tivesse interferido com o metabolismo periférico das hormonas da tiróide, as concentrações circulantes de T4 e T3 não apresentaram variações significativas. Por outro lado, constatou-se que este composto não interferiu com o transporte destas hormonas pelas proteínas plasmáticas de transporte em tilápia.

Os estudos dos efeitos do Aroclor 1254 no eixo hipotálamo-hipófise-gónadas nas tilápias expostas durante a fase adulta mostraram uma diminuição das concentrações plasmáticas de testosterona e progesterona e lesões dos ovários e dos testículos. Quando os animais foram expostos a este composto durante a fase larvar, apresentaram, em adultos, lesões das gónadas semelhantes às observadas quando expostas na fase adulta; como são lesões irreversíveis, levam a concluir que conduzem à diminuição da capacidade reprodutiva, o que tem, obviamente, consequências particularmente graves quando a exposição ocorre na fase larvar.

Em conclusão, os resultados obtidos nestes estudos mostraram que a exposição a compostos organoclorados, por via alimentar, origina alterações em mecanismos de regulação endócrina responsáveis pelo controlo de funções fisiológicas, tais como os eixos hipotálamo-hipófise-tiróide, hipotálamo-hipófise-inter-renal e hipotálamo-hipófise--gónadas, e alterações morfológicas, como as lesões observadas no tecido hepático e nas gónadas. Devido ao número elevado de órgãos em que estes contaminantes podem actuar, a sua presença no meio ambiente origina alterações fisiológicas e morfológicas nos animais expostos, algumas das quais podem pôr em causa a sobrevivência dos indivíduos ou da própria espécie.

SUMMARY

Vertebrate development and reproduction are controlled by the endocrine system, which includes the hypothalamus-pituitary-thyroid, the hypothalamus-pituitary-inter-renal (the equivalent to mammalians hypothalamus-pituitary-adrenal axis) and the hypothalamus-pituitary-gonads axes. The homeostasis of these systems can be disturbed by effects resulting from contaminants present in the environment, that act over these axes, altering their function and interfering with mechanisms under their control. Among environmental contaminants, compounds with chlorine atoms, like polychlorinated biphenyls (PCBs) and chlorinated insecticides are of major importance. Due to their physical-chemical properties, these compounds remain in the environment and in the organism tissues where they originate effects for long periods of time.

In this work it was studied the effects over endocrine systems of two chlorinated compounds in a cichlid specie, more precisely the Nile tilapia, *Oreochromis niloticus*. The selected compounds Endosulfan, a chlorinated insecticide, and Aroclor 1254, a PCBs congeners' mixture, were incorporated in diets.

The results showed that Endosulfan acted on the hypothalamus-pituitary-inter-renal axis creating a decrease in cortisol plasmatic concentration, and consequently of gills and kidney Na^+,K^+ ATPase activities, which indicates a lower osmoregulation capability in exposed tilapias.

This insecticide also had an action in the hypothalamus-pituitary-thyroid axis, dose dependent, creating a decline in T4 plasma concentration. Similarly the thyroid hormones peripheral metabolism was disturbed through the interference with some tissues deiodinases activity. Although the changes observed in T4 concentration and in deiodinases activity, T3 circulating levels were not significantly altered by the exposure to this contaminant. Also, it was observed that Endosulfan does not interfere with thyroid hormones transport through the binding competition to tilapia plasma transport proteins, albumin and 14 kDa apolipoprotein. These results indicate that Endosulfan action in the thyroid axis must occur at central level, decreasing T4 synthesis and/or secretion, and at tissues level, by changing local thyroid hormones availability.

Besides the effects studied in those two axes, it was also observed that exposure to Endosulfan causes hepatic tissue destruction, thus promoting changes in metabolic routes.

Similar studies were performed with the other selected pollutant, Aroclor 1254. In this case, effects were evaluated in the hypothalamus-pituitary-thyroid axis, in gonad development and reproduction, in adult tilapias exposed as adult and as larvae.

Results showed that, although Aroclor 1254 exposure had interfered with thyroid hormones peripheral metabolism, T4 and T3 plasma concentrations did not present significant variations. It was also observed that this compound did not interfere with these hormones transport through the binding competition to tilapia plasma proteins.

Studies on Aroclor 1254 effects in the hypothalamus-pituitary-gonads axis in tilapias exposed as adults showed a decrease in testosterone and progesterone plasma concentrations and the presence of ovarian and testicular lesions. When the exposure was performed in tilapia larvae, these presented, while adults, similar gonad lesions. Since, the damage was irreversible; they lead to the conclusion that there is a decrease in the reproductive capability, which is, obviously, particularly serious when exposure occurs during the larvae phase.

In conclusion, this study shows that the exposure to chlorinated compounds, through alimentary route, induces alterations in endocrine regulatory mechanisms responsible for physiological functions, as the hypothalamus-pituitary-thyroid, the hypothalamus-pituitary--inter-renal, and the hypothalamus-pituitary-gonads axis, and morphological changes, as the ones seen in liver and gonads. Due to the high number of organs where these contaminants action may take place, their presence in the environment creates physiological and morphological changes, some of which may reduce the survival and fitness of individuals or even of species.

RÉSUMÉ

Le développement et la reproduction des vertébrés sont contrôlés par le système endocrinien, qui inclus les axes hypothalamus–hypophyse–thyroïde, hypothalamus--hypophyse–rein (équivalent à l'axe hypothalamus–hypophyse–surrénal chez les mammifères) et hypothalamus–hypophyse–gonades.

L'équilibre ou homéostasie de ces systèmes peut être perturbé sous l'effet de contaminants présents dans l'environnement, qui agissent sur ces axes, modifiant leur fonctionnement et perturbant les mécanismes sous leur contrôle.

Parmi ces contaminants environnementaux, les plus importants sont ceux qui présentent des atomes de chlorue, tels que les biphényls polychlorinés (PCBs) et les insecticides organochlorinés. En raison de leurs propriétés physico-chimiques, ces composés persistent dans l'environnement et dans les tissus organiques pendant longues périodes.

Les effets de deux contaminants organochlorinés sur une espèce de cichlides, plus précisément le Tilapia du Nil (*Oreochromis niloticus*) ont été étudiés.

Les composés utilisés ont été l'Endosulfan, insecticide chloré, et l'Aroclor 1254, mélange d'analogues de PCBs, tous deux incorporés au régime alimentaire.

Les résultats ont montré que l'Endosulfan agit au niveau de l'axe hypothalamus--hypophyse–inter-rénal, provoquant une diminution de la concentration plasmatique de cortisol et, par conséquent, des activités ATPases Na^+,K^+ des branchies et des reins. Ceci indique une capacité réduite de l'osmorégulation chez les tilapias exposés.

Cet insecticide a également un effet sur l'axe hypothalamus–hypophyse–thyroïde, dépendant de la dose utilisée, provoquant une diminution de la concentration plasmatique de T4, de même qu'une perturbation du métabolisme périphérique des hormones de la thyroïde, au travers d'interactions avec l'activité deiodinase de certains tissus. Malgré les changements dans la concentration de T4 et de l'activité deiodinase, les niveaux de T3 en circulation n'ont pas été significativement modifiés par l'exposition à ce contaminant. Nous avons également observé que l'Endosulfan ne perturbait pas le transport des hormones de la thyroïde par les protéines de transport plasmatiques de Tilapia, l'albumine et une apolipoprotéine de 14 kDa. Ces résultats indiquent que l'action de l'Endosulfan sur l'axe de la thyroïde se situe au niveau central, diminuant ainsi la synthèse et/ou la sécrétion de T4, et au niveau des tissus, en modifiant la disponibilité locale de ces hormones.

Au-delà des effets sur les axes étudiés, nous avons de la même façon observée que l'exposition à l'Endosulfan provoquait une destruction des tissus hépatiques, entraînant ainsi des changements dans les voies métaboliques.

Des études similaires ont été réalisées avec l'autre polluant sélectionné, l'Aroclor 1254. Ceci a permis d'évalué les effets sur l'axe hypothalamus–hypophyse–thyroïde, le développement des gonades et la reproduction, sur des Tilapias adultes exposés au stade larvaire et adulte.

Les résultats ont montré que bien que l'Aroclor 1254 ait un effet sur le métabolisme périphérique des hormones de la thyroïde, les concentrations de T4 et T3 plasmatiques ne présentent pas de variation significative.

Nous avons aussi observé que ce composé ne perturbe pas le transport de ces hormones par les protéines plasmatiques de transport chez Tilapia.

Les études des effets de l'Aroclor 1254 sur l'axe hypothalamus–hypophyse–gonades dans les tilapias exposées durant la phase adulte ont montré une diminution des concentrations plasmatiques de testostérone et progestérone, ainsi que des lésions des testicules et des ovaires.

Si l'exposition à ces composés a lieu lors de la phase larvaire, les lésions observées sont similaires à celles observées si l'exposition a eu lieu à l'âge adulte. Les lésions étant irréversibles, permettent de conclure qu'elles provoquent une diminution de la capacité reproductrice, ce qu'induisent des conséquences particulièrement sérieuses, si l'exposition se produit au stade larvaire.

En conclusion, les résultats obtenus lors de cette étude montrent que l'exposition à des composés organochlorés par voie alimentaire provoque des altérations des mécanismes de régulation endocrinienne, eux-mêmes responsables de certaines fonctions physiologiques, tels que les axes hypothalamus–hypophyse–thyroïde, hypothalamus--hypophyse–inter-rénal et hypothalamus–hypophyse–gonades, mais également des altérations morphologiques, comme celles observées sur le foie et les gonades, chez tilapia.

En raison du grand nombre d'organes où ces composés peuvent agir, leur présence dans l'environnement entraîne des dégradations physiologiques et morphologiques sur les animaux exposés, certaines pouvant remettre en cause l'intégrité et la préservation des individus ou même de l'espèce.

ABREVIATURAS

12D:12N	12 horas dia:12 horas noite
14D:10N	14 horas dia:12 horas noite
A	Aroclor 1254
ACTH	Hormona adrenocorticotrópica
Alb	Albumina
An	Proteína anódica de tilápia
Apo A-I/-II	Apolipoproteínas
C	Controlo
cDNA	Ácido desoxi-ribonucleico clonado
CRF	Factor de libertação da corticotropina
Ct	Proteína catódica de tilápia
CYP1A	Família 1A do citocromo P450
D1	Desiodase tipo I
D2	Desiodase tipo II
D3	Desiodase tipo III
DDT	Diclorodifeniltricloretano
DNA	Ácido desoxi-ribonucleico
DTT	Ditiotreitol
E	Endosulfan
EROD	Etoxiresorufina-o-deetilase
FSH	Hormona folículo estimulante
GABA	Ácido γ-aminobutírico
GH	Hormona de crescimento
GnRH	Hormona libertadora das GTH
GTH I	Gonadotropina I (FSH)
GTH II	Gonadotropina II (LH)
GTH	Gonadotropinas
HCB	Hexaclorobenzeno
HT	Hormonas da tiróide
IRD	Desiodinação do anel interno
L	Lisosoma
LH	Hormona luteinizante
Km	Constante de Michaelis
mRNA	Ácido ribonucleico mensageiro
N	Neurohipófise
NAD^+	Nicotinamida adenina dinucleotido
NADH	Hidrogenonicotinamida adenina dinucleotideo
ORD	Desiodinação do anel externo
PCBs	Bifenilos policlorados
PCBs-OH	PCBs hidroxilados
PCR	"Polymerase Chain Reaction"
PI	*Pars Intermedia*
POPs	Compostos orgânicos persistentes
PPD	*Proximal Pars Distalis*
PRL	Prolactina
PTi	Plasma de tilápia
PTU	Propiltiouracilo
RPD	*Rostral Pars Distalis*
rT3	3,3',5'-triiodotironina (T3 reversa)
RT-PCR	"Reverse Transcriptase-PCR"
SB	Dourada ("sea bream")
SDS-PAGE	Electroforese em gel de poliacrilamida
T2	3,3'-diiodotironina
T3	3,3',5-triiodotironina
T4	Tiroxina (3,3',5,5'-tetraiodotironina)
TBG	Globulina de ligação à T4
TBT	Trifenilestanho
TCDD	2,3,7,8-tetraclorodibenzo-p-dioxina
Tg	Tiroglobulina
Tg-T4	Tiroglobulina-tetraiodotironina
TR	Receptor das hormonas da tiróide
TSH	Hormona estimuladora da tiróide
TTR	Transtirretina

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ICBAS

INTRODUÇÃO GERAL



TILÁPIA DO NILO OU TILÁPIA NILÓTICA (*OREOCHROMIS NILOTICUS*, L.)

Taxonomia

A tilápia pertence à família dos Ciclídeos (Fig. 1) e foi classificada em três géneros (*Tilapia*, *Sarotherodon* e *Oreochromis*) com base no seu comportamento reprodutivo, nomeadamente pela forma como os ovos são incubados. No género *Tilapia*, os ovos são incubados num ninho vigiado por ambos os progenitores; no género *Sarotherodon*, ambos os progenitores incubam os ovos na boca e no género *Oreochromis* só as fêmeas realizam incubação bocal (Trewavas, 1983).

Reino	Animalia	Animal	
Filo	Chordata	Cordado	
Sub-Filo	Vertebrata	Vertebrado	
Super-Classe	Osteichthyes	Peixe ósseo	
Classe	Actinopterygii	Peixe com barbatanas espinhosas	
Sub-Classe	Neopterygii	Neopterígios	
Infra-Classe	Teleostei	Teleósteos	
Super-Ordem	Acanthopterygii		
Ordem	Perciformes	Peixe semelhante à perca	
Sub-Ordem	Labroidei		
Familia	**Cichlidae**	Ciclídeos	
Género	*Oreochromis*		
Espécie	*Oreochromis niloticus* (Linnaeus, 1758)		Tilápia do Nilo ou nilótica

Fig. 1: Taxonomia da tilápia do Nilo.

Características gerais

A tilápia é tolerante a um vasto número de parâmetros ambientais e tem a capacidade de se adaptar a situações ambientais extremas (Philippart e Ruwett, 1982).

A reprodução ocorre a temperaturas superiores a 22 °C, o valor ideal encontra-se entre 25 °C e 29 °C, e em períodos de fotoperíodo 12 D:12 N ou 14 D:10 N (Rothbard e Pruginin, 1975). As tilápias do género *Oreochromis* apresentam dicromatismo e dimorfismo sexual (Palada De Vera e Eknath, 1995).

Nas espécies com incubação bocal, a incubação dos ovos dura entre 6 e 10 dias e os alevins permanecem no interior da cavidade bocal durante mais alguns dias (Macintosh e Little, 1995). Durante este período, as fêmeas não se alimentam.

No meio natural, a tilápia tem uma alimentação muito variada que inclui plâncton, macrófitas aquáticas, invertebrados plantónicos e bentónicos, larvas de peixe, detritos e matéria orgânica em decomposição (Fontaínhas Fernandes, 1998).

Produção

A tilápia do Nilo (*Oreochromis niloticus*) (Fig. 2) foi uma das primeiras espécies a ser produzida em aquacultura, sendo o seu cultivo referenciado há mais de 3000 anos em hieróglifos egípcios.

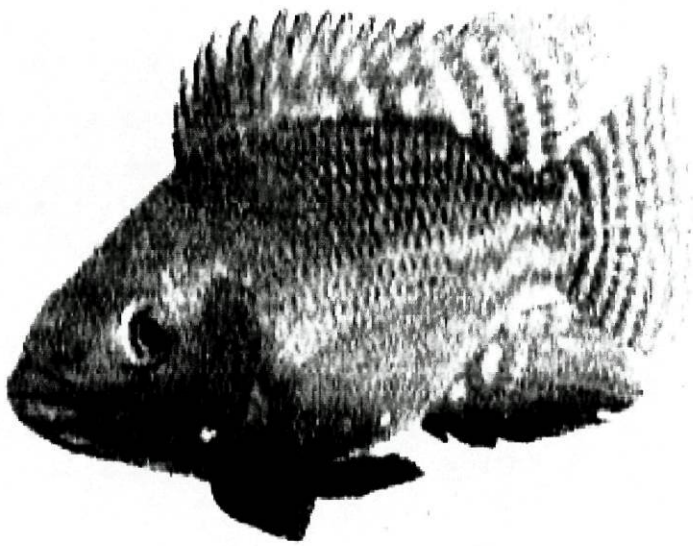


Fig. 2: Espécime de tilápia do Nilo, *Oreochromis niloticus*, adulto.

Esta espécie é actualmente cultivada em três continentes (Fig. 3 e 4): Ásia, África e América (FAO-FIGIS, 2003).

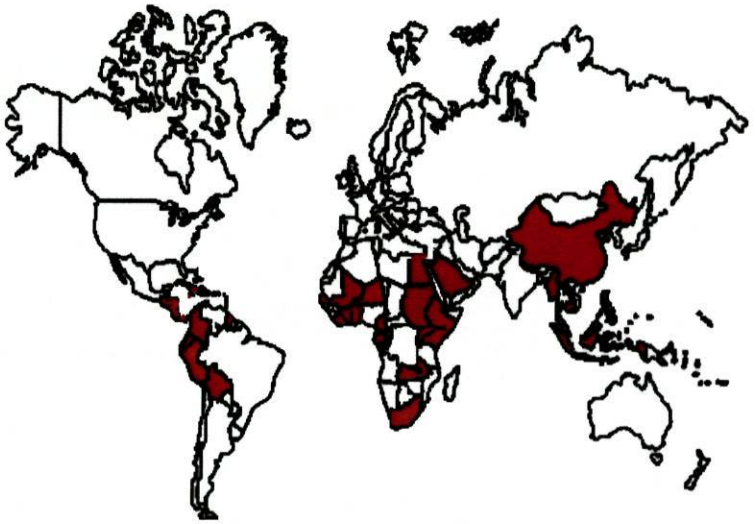


Fig. 3: Países produtores de tilápia do Nilo.

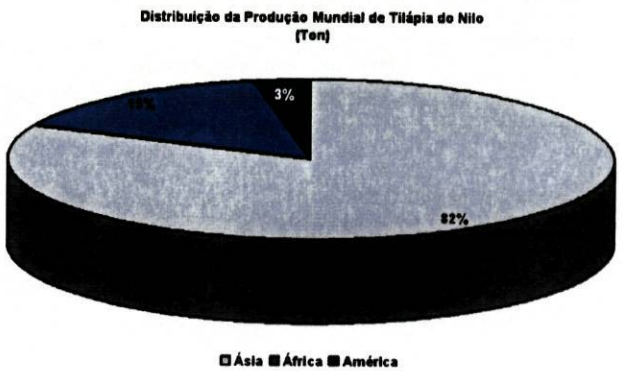


Fig. 4: Distribuição da produção mundial de tilápia do Nilo (FAO-FIGIS, 2003).

Para além do custo de produção desta espécie ser baixo, a sua aceitação pelos consumidores tem vindo a aumentar (Roderick, 2003), o que faz da tilápia uma das principais espécies produzidas em aquacultura e, consequentemente, uma importante fonte proteica em países menos desenvolvidos (Edwards, 2003).

Actualmente, a tilápia ocupa o segundo lugar no cultivo de peixes de água doce a nível mundial e das diferentes espécies de ciclídeos produzidas, 82 % são de tilápia do Nilo. Em 2003, a produção de tilápia do Nilo correspondeu a 5 % (1,4 milhões de toneladas) da produção mundial de peixes de cultura (Fig. 5) (FAO-FIGIS, 2003).

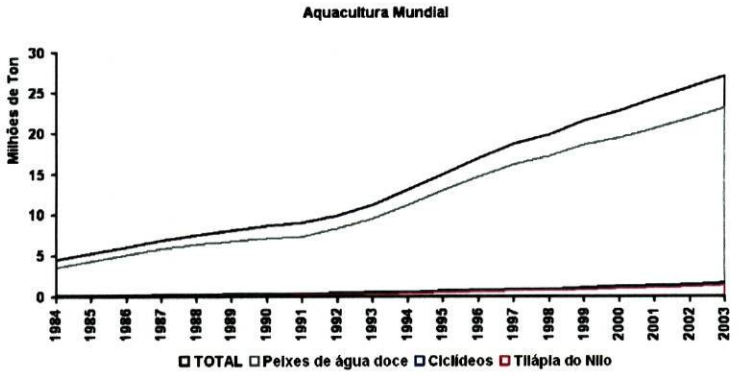


Fig. 5: Evolução (1984-2003) da aquacultura de peixes a nível mundial (FAO-FIGIS, 2003).

A tilápia do Nilo foi escolhida como modelo animal para este trabalho devido ao seu fácil manuseamento e capacidade reprodutiva em cativeiro, assim como à sua importância a nível comercial e actual dispersão geográfica. Outros factores que determinaram a escolha desta espécie foram o uso frequente de poluentes policlorados nos países onde é endémica, assim como nos países produtores, e a possibilidade dos poluentes exercerem a sua acção tóxica, quer nas populações naturais, quer nas populações mantidas em cativeiro.

A exposição de animais a compostos policlorados pode originar, entre outras, alterações no desenvolvimento e na reprodução dos animais, interferindo na regulação dos eixos hipotálamo-hipófise-rim, hipotálamo-hipófise-tiróide e hipotálamo--hipófise-gónada.

Eixo Hipotálamo-Hipófise-Rim

O eixo hipotálamo-hipófise-rim (Fig. 6) é fundamental para a interacção dos organismos com o ambiente, pois é responsável pelas constantes adaptações fisiológicas de resposta aos inúmeros e variáveis estímulos ambientais.

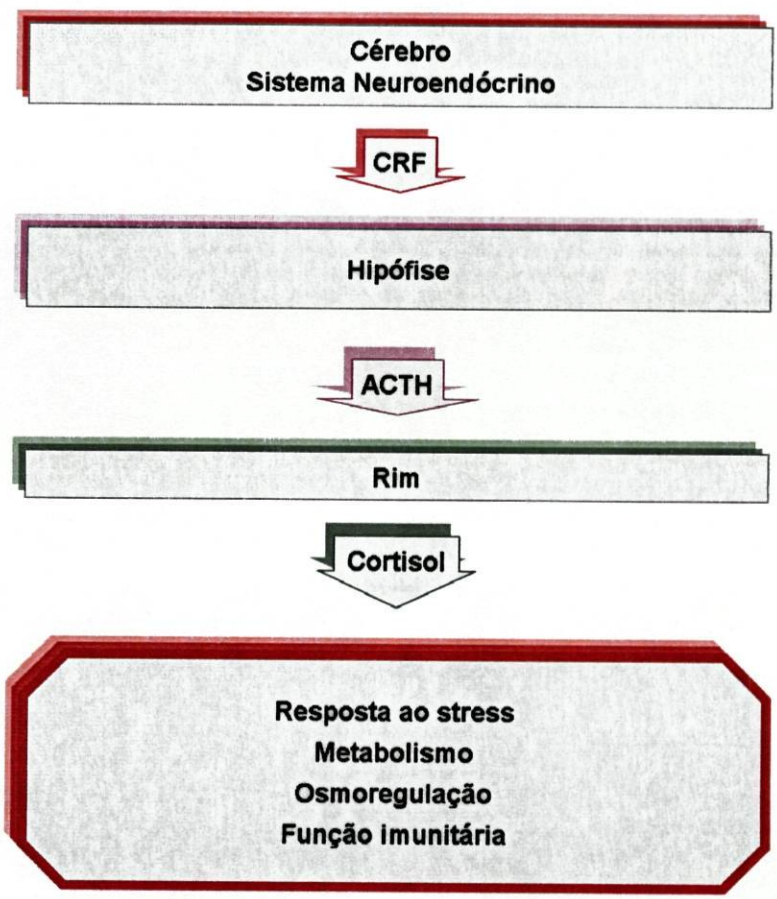


Fig. 6: Eixo hipotálamo-hipófise-rim.

Resumidamente, o hipotálamo produz o factor de libertação da corticotropina (CRF-"corticotropin releasing factor") que é libertado directamente na hipófise, onde induz

a síntese e libertação da hormona adrenocorticotrópica (ACTH). Esta hormona actua sobre as células da glândula inter-renal presentes no rim anterior ou cefálico, onde induz a produção de cortisol e catecolaminas (Flik et al., 2006).

O cortisol tem como principal função o controlo da resposta às situações de stress. Desta forma, interfere com o metabolismo intermediário, nomeadamente com a regulação dos níveis de glucose, com a capacidade de osmoregulação e com a resposta imunitária (Mommsen et al., 1999).

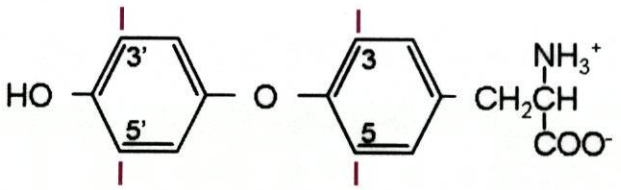
O cortisol é uma hormona hipo-osmoregulatória presente desde as fases iniciais de vida dos peixes e está relacionada com a actividade da ATPase Na^+,K^+, aumentando a actividade desta enzima em situações de transferência para água salgada (Lin et al., 1999, Fontainhas-Fernandes et al., 2003, Pepels e Balm, 2004).

EIXO HIPOTÁLAMO-HIPÓFISE-TIRÓIDE

As principais acções das hormonas da tiróide (HT) em todos os vertebrados envolvem a ligação da hormona bioactiva, 3,3',5-triiodotironina (T3), a receptores nucleares. O complexo T3-receptor actua como factor de transcrição sobre regiões específicas do DNA, os elementos de resposta às HT, regulando a expressão de genes específicos e, consequentemente, a síntese das respectivas proteínas, embora em peixes esta sequência de eventos ainda não esteja completamente esclarecida (Brown et al., 2004a).

Nos mamíferos, as HT (Fig. 7) são responsáveis pela estimulação do metabolismo basal. Nos peixes não existem evidências de que as HT aumentem o consumo de oxigénio, mas conhece-se o seu envolvimento na regulação do crescimento, no desenvolvimento durante as primeiras fases de vida, na metamorfose e em alguns aspectos da reprodução (Brown et al., 2004a).

T4



T3

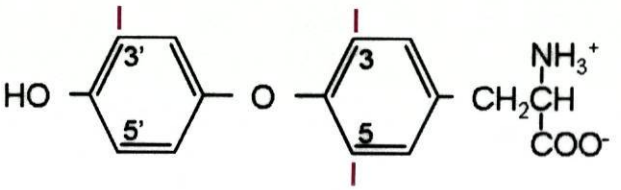


Fig. 7: Hormonas da tiróide: tiroxina (T4) e 3,3',5-triiodotironina (T3).

O sistema da tiróide é exclusivo dos vertebrados e engloba o eixo hipotálamo-hipófise-tiróide e o metabolismo periférico das HT, pelas desiodases. Estas desiodases, por determinarem a disponibilidade intracelular local das HT activas e inactivas, regulam as acções específicas destas hormonas (Bianco et al., 2002, Valverde-R et al., 2004).

Modelo de funcionamento do eixo da tiróide

O modelo proposto para o eixo da tiróide nos peixes envolve dois componentes: o primeiro inclui a síntese, secreção e metabolismo da tiroxina (T4) pela tiróide e encontra-se sob o controlo central do eixo hipotálamo-hipófise-tiróide; o segundo, que engloba a conversão de T4 em T3, o metabolismo da T3 e a acção da T3 mediada pelos receptores nucleares, parece estar sob controlo periférico (tecidos extra-tiróide). Este modelo assume que todas as HT são de origem endógena, no entanto, os peixes podem obter HT por via exógena, através da dieta (Eales, 1997).

O eixo da tiróide pode ser avaliado em três níveis (Fig. 8):

a) no eixo central, que controla a síntese e secreção de T4 (homeostasia da T4), uma pré-hormona relativamente inactiva;

b) no controlo periférico responsável pela disponibilidade da hormona biológicamente activa, a T3 (homeostasia da T3);

c) nos efeitos mediados pela acção da T3 nos receptores nucleares das células alvo (Eales et al., 1999).

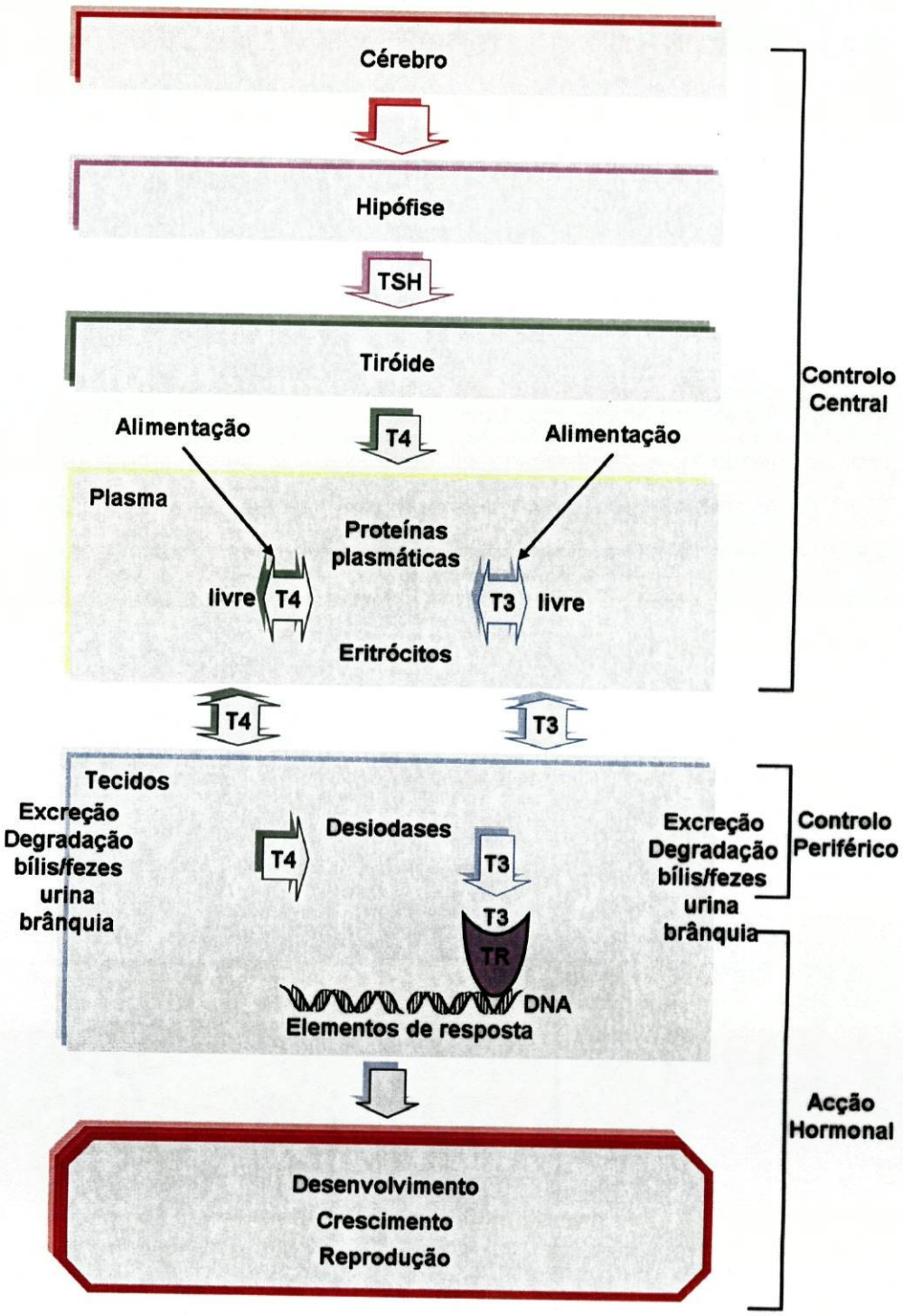


Fig. 8: Componentes e níveis de avaliação do eixo hipotálamo-hipófise-tiróide dos peixes (adaptado de Eales et al., 1999).

Síntese, secreção e metabolismo da T4

O iodo, potencial factor limitante da síntese das HT, encontra-se normalmente sob a forma de ião iodeto (I^-). Os peixes não só obtêm iodo através da dieta, via tracto gastrointestinal, tal como os vertebrados terrestres, mas também a partir da água, via brânquias (Brown et al., 2004a). O iodo pode ser acumulado no ovário e na pele, mas apenas a tiróide possui capacidade para sintetizar T4 (Eales et al., 1999).

Na maioria dos peixes a tiróide é um tecido difuso localizado na região basobranquial. Tal como nos outros vertebrados, este tecido consiste de folículos com uma única camada de células epiteliais a rodear o lúmen cheio de um colóide proteíco, a tiroglobulina (Tg). Em cada folículo (Fig. 9), o iodo plasmático é transportado activamente para o lúmen. A iodoperoxidase, da membrana apical das células do folículo, oxida alguns resíduos de tirosil-tiroglobulina, substituindo o hidrogénio nas posições 3 e 5 por iodo e ligando dois resíduos de diiodotirosil para formar tiroglobulina-tetraiodotironina (Tg-T4). A reserva de Tg-T4 entra, por endocitose, para as células do folículo. As vesículas de endocitose combinam-se com lisossomas, onde as catepsinas digerem o complexo tiroglobulina-T4, libertando a T4 que é secretada para o plasma (Brown et al., 2004a).

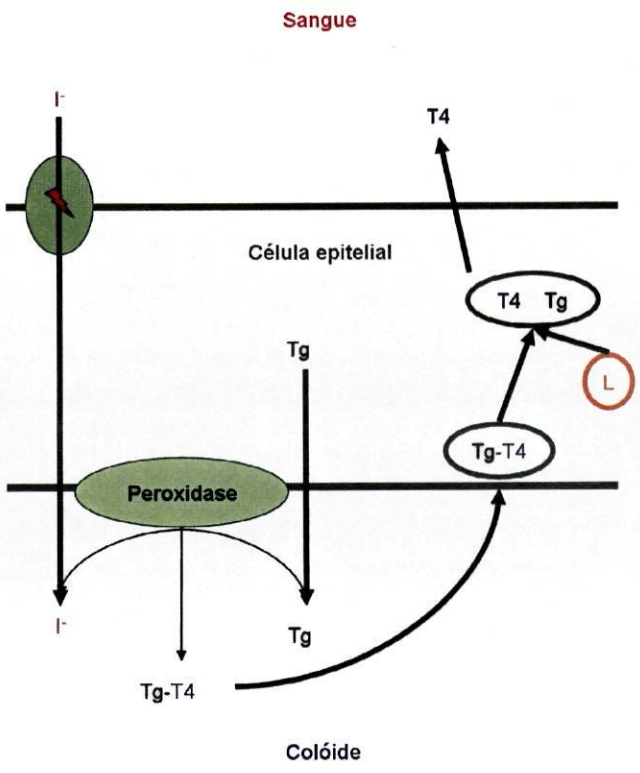


Fig. 9: Produção de T4 nos folículos da tiróide. Tg-tiroglobulina, L-lisossoma.

Nos mamíferos, a T3 é também libertada em quantidades significativas pela tiróide (Chanoine et al., 1993), mas tudo indica que nos peixes a T4 é provavelmente a única das HT secretada (Grau et al., 1986, Byamungu et al., 1990, Eales e Brown, 1993).

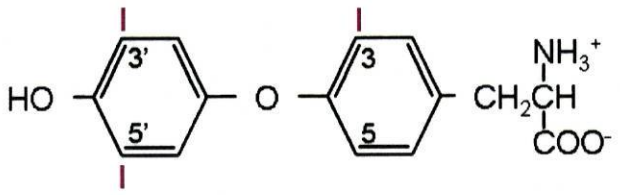
A secreção de T4 é controlada pelo eixo hipotálamo-hipófise-tiróide. A tiróide é estimulada pela hormona estimuladora da tiróide (TSH), uma glicoproteína secretada pelas células tirotrópicas da hipófise. O sistema portal hipofisário nos peixes é inexistente e os tirotrópos são inervados directamente por neurónios provenientes do hipotalámo. O controlo do hipotálamo sobre as células produtoras de TSH é principalmente inibitório, possivelmente devido à secreção de somatostatina, mas outros factores hipotalámicos poderão ter um efeito estimulatório (Brown et al., 2004a).

Da T4 plasmática total menos de 1 % está livre, encontrando-se 99 % ligada reversivelmente a proteínas plasmáticas. A concentração de T4 livre é mantida constante por um mecanismo de "feedback" negativo, ao nível do hipotálamo e da hipófise. No entanto, o aumento dos níveis fisiológicos de T3 plasmática não diminui os níveis de T4 e não exerce uma acção de "feedback" negativo. O eixo hipotálamo-hipófise-tiróide dos peixes parece regular exclusivamente os níveis de T4 livre e o controlo, por "feedback", da secreção de TSH nos peixes parece ocorrer simplesmente em resposta aos níveis de T4 livre (Eales et al., 1999, Brown et al., 2004a).

A entrada de T4 nas células pode fazer-se por difusão simples, mas, em geral, é mediada por proteínas de transporte. A T4 intracelular liga-se de forma reversível a proteínas citoplasmáticas e pode ser metabolizada enzimaticamente por vias de desiodinação ou de conjugação (Brown et al., 2004a).

A desiodinação ocorre fundamentalmente no retículo endoplasmático do fígado e de outros tecidos. A remoção de iodo do anel externo da T4 origina T3, a hormona activa, enquanto a remoção de iodo do anel interno da T4 origina T3 reversa (rT3), uma forma inactiva da hormona. A rT3 é por sua vez desiodinada no anel externo formando diiodotironina (T2) (Fig. 10).

rT3



T2

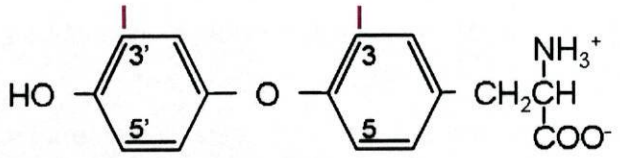


Fig. 10: Hormonas da tiróide: 3,3',5'-triiodotironina (rT3) e 3,3'-diiodotironina (T2).

A conjugação da T4 ocorre principalmente no fígado e os produtos de conjugação são excretados juntamente com a bílis. A T4 e os seus conjugados também podem ser excretados através do rim e a T4 pode ainda ser excretada através do epitélio das brânquias.

Síntese e metabolismo da T3

Existem várias fontes potenciais endógenas de T3 como: a) a secreção directa pela tiróide; b) a desiodinação intra-tiróide da T4; c) a desiodinação extra-tiróide da T4. Os estudos realizados em peixes sugerem que quase toda a T3 é produzida por desiodinação extra-tiróide da T4. O controlo principal dos níveis de T3 ocorre nos órgãos e tecidos periféricos e pode estar limitado a alguns tipos celulares específicos.

No fígado e nos outros tecidos periféricos, a T3 é formada por desiodinação do anel externo da T4 por acção de uma ou mais desiodases. A T3 pode então passar para o plasma, tornando-se numa fonte sistémica de T3 para outros tecidos ou pode ser utilizada pelo tecido onde é formada, satisfazendo as necessidades locais (Brown et al., 2004a).

Por sua vez, a T3 é degradada por remoção de iodo do anel interno para formar T2. Esta actividade de desiodinação da T3 é particularmente elevada no cérebro e na retina.

O balanço entre as actividades de desiodinação do anel externo e interno da T4 e do anel interno da T3 regulam a quantidade de T3 plasmática ou local em tecidos específicos (Brown et al., 2004a).

Tal como a T4, a T3 liga-se a proteínas do plasma, mas com uma maior afinidade do que a T4, o que resulta em menores concentrações de T3 livre (Eales e Shostak, 1985, Eales, 1987).

Parte da T3 livre entra nas células por difusão passiva ou por transporte activo, mas a maior parte entra por difusão facilitada, envolvendo um sistema de transporte distinto do que transporta a T4.

Transporte plasmático das hormonas da tiróide

Nos vertebrados superiores, as HT circulam no plasma ligadas a três proteínas de transporte: a albumina (Alb), a transtirretina (TTR) e a globulina de ligação à T4 (TBG) (Larsson et al., 1985, Schreiber e Richardson, 1997). No plasma de peixe, as lipoproteínas possuem o papel principal no transporte das HT, sendo a maior fracção correspondente a apolipoproteínas: apo A-I (25 kDa) e apo A-II (13 kDa) (Babin, 1992).

No Homem, a TBG circula em concentrações mais baixas do que as outras proteínas de transporte de HT, mas possui maior afinidade de ligação; cerca de 75% da T4 circula ligada a esta proteína. A TTR possui uma afinidade intermédia e a Alb é aquela que tem menor afinidade (Robbins e Edelhoch, 1986). Destas proteínas apenas a Alb está presente no plasma de todos os vertebrados estudados (Richardson et al., 2005).

Estudos em salmonídeos sobre a ligação das HT às proteínas plasmáticas de transporte, realizados nos anos 70 (Falkner e Eales, 1973) e 80 (Eales, 1987, Fingler et al., 1992), determinaram que a ligação da T4 é mais fraca nestes do que nos mamíferos e que a T3 possui maior afinidade que a T4 para algumas proteínas. Trabalhos posteriores mostraram que a TBG está ausente no plasma de salmonídeos e identificaram uma proteína análoga à TTR, mas que ao contrário da TTR de mamíferos, possui afinidade reduzida para a proteína de ligação ao retinol (Larsson et al., 1985, Folli et al., 2003). Recentemente, Yamauchi et al. (1999) isolaram TTR de juvenis de *Onchorhynchus masou* e verificaram que esta proteína possui uma afinidade para a T3 três vezes superior à afinidade para a T4. O gene da TTR foi identificado em peixes, nomeadamente em *Sparus aurata* (Funkenstein et al., 1999, Santos e Power, 1999) e sendo conhecida a sua sequência em *Danio rerio*, *Takifugu rubripes*, *Tetraodon nigroviridis* e *Oncorhynchus mykiss*.

A expressão da TTR e o seu papel durante o ciclo de vida dos peixes encontra-se actualmente em estudo. Os trabalhos publicados sugerem que esta proteína é expressa

apenas durante fases específicas, em que existem níveis mais elevados de HT circulantes ("smoltificação" e desenvolvimento larvar).

Desiodinação

A desiodinação das HT é um processo sucessivo de perda de iodo do anel interno ou externo. Este processo activa ou inactiva as HT e permite também a reciclagem de iodo antes das HT serem excretadas (Fig. 11).

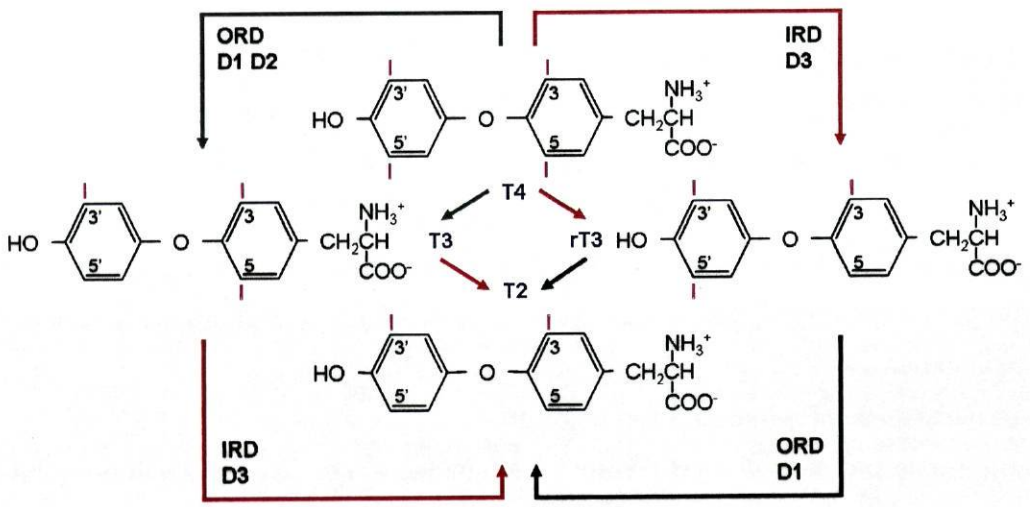


Fig. 11: Desiodinação das hormonas da tiróide e desiodases (D1, D2 e D3) responsáveis pela desiodinação dos anéis externo (ORD) e/ou interno (IRD).

Nos mamíferos, a desiodinação é desempenhada por uma família de proteínas, as desiodases (tipo I-D1, II-D2 e III-D3). As desiodases têm a particularidade de possuírem no local activo da enzima uma selenocisteína, que se encontra na face citosólica do retículo endoplasmático (microssomas). As desiodases dos peixes são também proteínas microssomais com selenocisteínas, mas possuem propriedades diferentes das congéneres dos mamíferos (Mol et al., 1993, Mol et al., 1997, Orozco et al., 1997, Orozco e Valverde-R, 2005).

A actividade das desiodases nos peixes responde a numerosos estímulos fisiológicos, de desenvolvimento e ambientais, sugerindo que são fundamentais para a regulação e manutenção dos níveis de HT.

Desiodases

A desiodinação é o primeiro passo no mecanismo de acção das HT (Orozco e Valverde-R, 2005). A especificidade e a remoção sequencial dos átomos de iodo da T4 originam os isómeros activo (T3) e inactivo (T2). Esta biotransformação das HT ocorre em praticamente todos os tecidos do organismo e, por isso, é designada iodotironina--desiodinação periférica e é catalisada por uma família de halogenases redutoras, denominadas iodotironina-desiodases. Os peixes, à semelhança dos outros vertebrados e com excepção dos anfíbios, expressam os três isótipos de desiodases, responsáveis pelo controlo periférico da bioactividade das hormonas da tiróide.

A distribuição destas enzimas pelos tecidos dos peixes é diferente da sua distribuição nos outros vertebrados, mas é característica desta classe. As desiodases dos peixes apresentam diferenças notáveis na sua resposta à disponibilidade do substrato e aos sinais regulatórios (Orozco e Valverde-R, 2005).

As desiodases possuem propriedades catalíticas diferentes e são expressas de forma específica durante o desenvolvimento e nos vários tecidos. As desiodases D1 e D2 estão envolvidas na via activadora ou de desiodinação do anel externo ("outer ring deiodination"-ORD) e convertem a T4 em T3. A via inactivadora ou de desiodinação do anel interno ("inner ring deiodination"-IRD) é catalizada principalmente pela D3, que converte a T4 em rT3, o metabolito inactivo. Por estas razões, a desiodinação periférica das HT regula, de forma organo-específica, tanto os níveis circulantes, como a concentração intracelular das HT (Bianco et al., 2002).

A actividade ORD hepática tem sido a mais estudada das vias de desiodinação em todas as classes de vertebrados. Nos vertebrados endotérmicos, o fígado é o órgão que apresenta os valores mais elevados da actividade ORD, que é exclusivamente catalisada pela D1, pelo menos nos adultos. A expressão das desiodases nos vertebrados ectotérmicos é diferente, tanto quantitativa como qualitativamente; por exemplo, a actividade da D1 hepática nos répteis encontra-se entre as mais elevadas descritas em vertebrados (Fenton e Valverde, 2000, Shepherdley et al., 2002); em anfíbios, pelo contrário, o fígado não expressa D1 em qualquer estado de desenvolvimento (Becker et al., 1997, Bianco et al., 2002).

No caso dos peixes, a identificação de desiodases idênticas às D1, D2 e D3 dos mamíferos continua a não estar completamente esclarecida, pois embora as desiodases dos peixes apresentem semelhanças moleculares parciais com as desiodases dos mamíferos, a sua equivalência funcional é ainda discutível (Frith e Eales, 1996, Finnson et al., 1999). No entanto, e apesar de não estarem identificadas as proteínas, o uso de diferentes concentrações de T4, T3 e rT3 como substrato e a análise dos produtos de reacção permitiram identificar várias actividades de desiodinação (T4-ORD, T4-IRD,

T3-ORD, T3-IRD e rT3-ORD) em diferentes tecidos de várias espécies de peixe (Sweeting e Eales, 1992, Johnston e Eales, 1995, Frith e Eales, 1996, Adams et al., 2000, Vanputte et al., 2001, Plohman et al., 2002).

Iodotironina Desiodase 1

A presença de D1 no fígado dos peixes tem sido um assunto controverso. Inicialmente, os estudos efectuados em várias espécies, nas quais a actividade ORD hepática foi caracterizada, mostraram apenas a expressão de D2 sob condições basais, demonstrada pelo Km da enzima, pela necessidade de ditiotreitol (DTT), pela preferência de substrato e pela sensibilidade ao propiltiouracilo (PTU) (Mol et al., 1997, Orozco et al., 1997, Mol et al., 1998, Orozco et al., 2000). No entanto, o uso de rT3 como substrato na determinação de actividade das desiodases *in vitro* permitiu identificar uma desiodase semelhante à D1 dos mamíferos (Mol et al., 1993, Mol et al., 1997, Orozco et al., 1997, Finnson et al., 1999, Orozco et al., 2000). Embora, a D1 hepática dos peixes seja cineticamente semelhante às D1 dos mamíferos, apresenta algumas diferenças funcionais, tais como constante de Michaelis-Menten baixa e insensibilidade parcial à inibição por PTU, uma característica única das D1 dos peixes.

Nos mamíferos, as HT são os reguladores fisiológicos mais potentes da actividade das desiodases, aumentando a actividade e a transcrição da D1 numa situação de hipertiroidismo, enquanto que, numa situação de hipotiroidismo ocorre o oposto. Pelo contrário, nos peixes, as HT (T4, T3 e T2) não regulam a actividade da D1 no fígado (Finnson e Eales, 1999, Orozco et al., 2002b, Garcia-G et al., 2004) e o hipertiroidismo não afecta a actividade da D1 renal (Mol et al., 1999). Apesar da falta de efeito na actividade da D1 hepática, após a indução de hipotiroidismo, os níveis de mRNA da D1 hepática diminuem (Garcia-G et al., 2004). Uma interpretação possível é que como a D1 dos peixes não remove iodo do anel interno, o excesso de T4 regula a transcrição da D1 (Orozco e Valverde-R, 2005). Esta hipótese é suportada pelo facto da D1 renal de tilápia possuir uma actividade residual de IRD da T3 (Sanders et al., 1997).

Iodotironina Desiodase 2

A D2 controla a concentração intracelular de T3. As células que expressam D2 produzem localmente T3, que está mais rapidamente disponível para agir sobre os receptores nucleares, do que a T3 plasmática (Bianco et al., 2002). Os estudos efectuados sobre a D2 dos peixes mostram que esta enzima possui uma preferência de

substrato, perfis de inibição pelas HT e valores de Km semelhantes às D2 dos mamíferos (Mol et al., 1993, Orozco et al., 1997, Mol et al., 1998, Orozco et al., 2000). Nos peixes, a D2 tem uma actividade particularmente elevada no fígado (Mol et al., 1993, Orozco et al., 2000).

A concentração das HT é o principal factor de regulação pré e pós-transcripcional da actividade da D2, tal como no caso da D1. No hipertiroidismo a actividade da D2 e a expressão do seu mRNA são suprimidas, enquanto no hipotiroidismo são ambas induzidas. O tempo de semi-vida da D2 (menos de 1 hora) é reduzido nas células expostas aos seus substratos T4 e rT3 ou concentrações elevadas de T3. Os resultados experimentais sugerem que a interacção enzima-substrato induz a proteólise do complexo.

Nos peixes, a D2 é também regulada pelas concentrações dos substratos. A exposição a T4 e T3 diminui a actividade da D2 hepática e a expressão do seu mRNA (Finnson e Eales, 1999, Mol et al., 1999, Garcia-G et al., 2004); a indução de hipotiroidismo origina um aumento da actividade e da expressão do mRNA da D2 hepática (Mol et al., 1999, Van Der Geyten et al., 2001). O mecanismo de regulação da D2 é ainda desconhecido, mas os resultados obtidos até agora sugerem que a regulação desta enzima seja feita a nível pré-transcripcional, pois a diminuição da actividade da D2 é sempre precedida por uma diminuição dos níveis do seu mRNA (Garcia-G et al., 2004).

Iodotironina Desiodase 3

A D3 possui quase a exclusividade da actividade IRD e catalisa a conversão de T4 em rT3 e de T3 em T2. A função principal desta enzima é contribuir para a homeostasia das HT, protegendo os tecidos do excesso de HT activas. Nos peixes, a D3 é expressa abundantemente no cérebro, brânquias (Mol et al., 1998) e pele (Fenton et al., 1997).

Alterações na actividade da D3 controlam os níveis de HT circulantes e dos tecidos, por aumento ou redução da inactivação da T3. A actividade da D3 apresenta um paralelo com os níveis de TH, aumentando em casos de hipertiroidismo e diminuindo em casos de hipotiroidismo (Bianco et al., 2002). Na tilápia, o hipertiroidismo induzido por T3 estimula a D3 hepática, mas não a D3 cerebral e das brânquias (Mol et al., 1999), o mesmo acontece na truta (Eales e Finnson, 1991, Maclatchy e Eales, 1992, Sweeting e Eales, 1992). Isto sugere a existência de mecanismos de resposta da D3 às HT específicos de cada tecido. Nas situações de hipotiroidismo, a actividade da D3 diminui tanto no fígado como no cérebro (Mol et al., 1999, Van Der Geyten et al., 2001), o que sugere um papel

protector contra as concentrações muito baixas de T3, como acontece nos outros vertebrados.

Nos peixes, a D2 hepática e a D3 das brânquias são as principais enzimas responsáveis pela regulação das concentrações de HT e as principais responsáveis pelos valores circulantes de T3 (Orozco e Valverde-R, 2005).

Conjugação

As HT podem ser conjugadas com ácido glucurónico ou com ácido sulfúrico. Os conjugados são biologicamente inactivos e têm uma alta solubilidade em água, o que permite a sua excreção na urina ou na bílis.

Existe também a possibilidade das HT poderem ser excretadas através das brânquias, que são permeáveis à T4 e à T3, mas a principal via de eliminação é a via intestinal, através da bílis e das fezes.

No intestino, as HT podem ser novamente reabsorvidas, especialmente a T3, o que mostra que nos peixes as HT presentes na dieta contribuem também para a quantidade de HT circulantes.

Comparação do sistema da tiróide dos peixes e dos mamíferos

Nos mamíferos, a secreção de T4 e T3 pela tiróide é regulada fundamentalmente pelo eixo hipotálamo-hipófise-tiróide, sendo os níveis de T4 e T3 regulados por um mecanismo de "feedback" negativo de ambas as hormonas. O controlo periférico parece estar restrito ao ajuste dos níveis de T3 nos tecidos (Eales et al., 1999).

Nos peixes, o sistema da tiróide não parece estar sob controlo do eixo hipotálamo--hipófise-tiróide. A principal função do eixo é regular a secreção de T4 pela tiróide, que tudo indica, ser a única hormona segregada. De acordo com este facto, a T4 exerce uma acção muito forte de "feedback" negativo sobre o eixo hipotálamo-hipófise-tiróide, enquanto a T3 apresenta uma acção ineficaz ou inconsistente (Eales e Brown, 1993). O controlo dos níveis de T3 é feito nos tecidos periféricos por acção das desiodases.

Em resumo, o principal papel do eixo hipotálamo-hipófise-tiróide é manter a homeostasia da T4, de forma a que exista uma reserva de T4 suficiente para satisfazer as necessidades periféricas de T3. Assim, a actividade das desiodases indica o potencial dos tecidos para produzir ou degradar T3. A determinação da actividade das desiodases juntamente com a determinação dos níveis plasmáticos de T4 e T3 devem ser capazes de detectar os efeitos da acção de xenobióticos no sistema periférico.

Esta diferença entre os mamíferos e os peixes tem implicações importantes na determinação de alterações do sistema da tiróide (Eales et al., 1999).

As HT actuam, nos peixes, ao nível do desenvolvimento, crescimento e reprodução (Eales e Brown, 1993, Leatherland, 1994) e desta forma a disrupção do sistema da tiróide, por exemplo por xenobióticos, pode comprometer a saúde e a sobrevivência dos indivíduos. Estas alterações podem ocorrer ao nível da síntese, regulação, metabolismo ou acção das HT (Brown et al., 2004a).

O sistema da tiróide pode ser influenciado por inúmeros xenobióticos, sendo, por isso, importante a detecção dos efeitos dos xenobióticos nas funções destas hormonas, a fim de avaliar o risco para os indivíduos e populações de peixes expostos a estes compostos. Para além disso, não existe um único biomarcador capaz de avaliar todos os níveis do sistema da tiróide dos peixes, pelo que desta forma os efeitos dos xenobióticos devem ser determinados nos três níveis de acção: controlo central da secreção de T4 para o plasma; controlo periférico da conversão de T4 em T3; e controlo dos efeitos pós-receptores de T3 (Eales et al., 1999).

Assim, a interpretação dos efeitos dos xenobióticos no sistema da tiróide dos peixes é extremamente complexa. Pelo menos cinco categorias de factores têm de ser considerados: a) variáveis do teste ou espécie; b) variáveis dos xenobióticos; c) determinação de pontos chave da cascata da tiróide; d) acções directas ou indirectas e e) a distinção entre efeitos de exposição e de compensação (Fig. 12) (Brown et al., 2004a).

As variáveis da espécie incluem a espécie usada, o seu estado fisiológico e o seu ambiente, passado e presente. Estudos com a mesma espécie podem diferir devido ao estado geral, reprodutivo ou idade dos espécimes usados. Para além desses factores, o estado fisiológico dos peixes e a sua susceptibilidade aos xenobióticos podem ser influenciados por factores ambientais, que são definidos à partida para cada estudo, mas que diferem entre os vários estudos (Brown et al., 2004a).

As variáveis relativas aos xenobióticos incluem o tipo de composto, o seu modo de administração, a dose e a duração dos tratamentos. Os principais modos de administração são a injecção intra-peritonial, a administração por via oral (juntamente com a comida ou por alimentação forçada) ou a exposição através da água. Os dois últimos modos de administração representam as vias naturais de contacto dos peixes com os xenobióticos (Brown et al., 2004a).

As HT regulam numerosas funções que incluem o crescimento, as respostas à entrada de nutrientes, a reprodução e o desenvolvimento. Desta forma, o sistema da tiróide é sensível e responde a alterações destes estados fisiológicos, pelo que, um composto que

altere uma destas funções interfere, secundariamente, com o sistema da tiróide (Brown et al., 2004a).

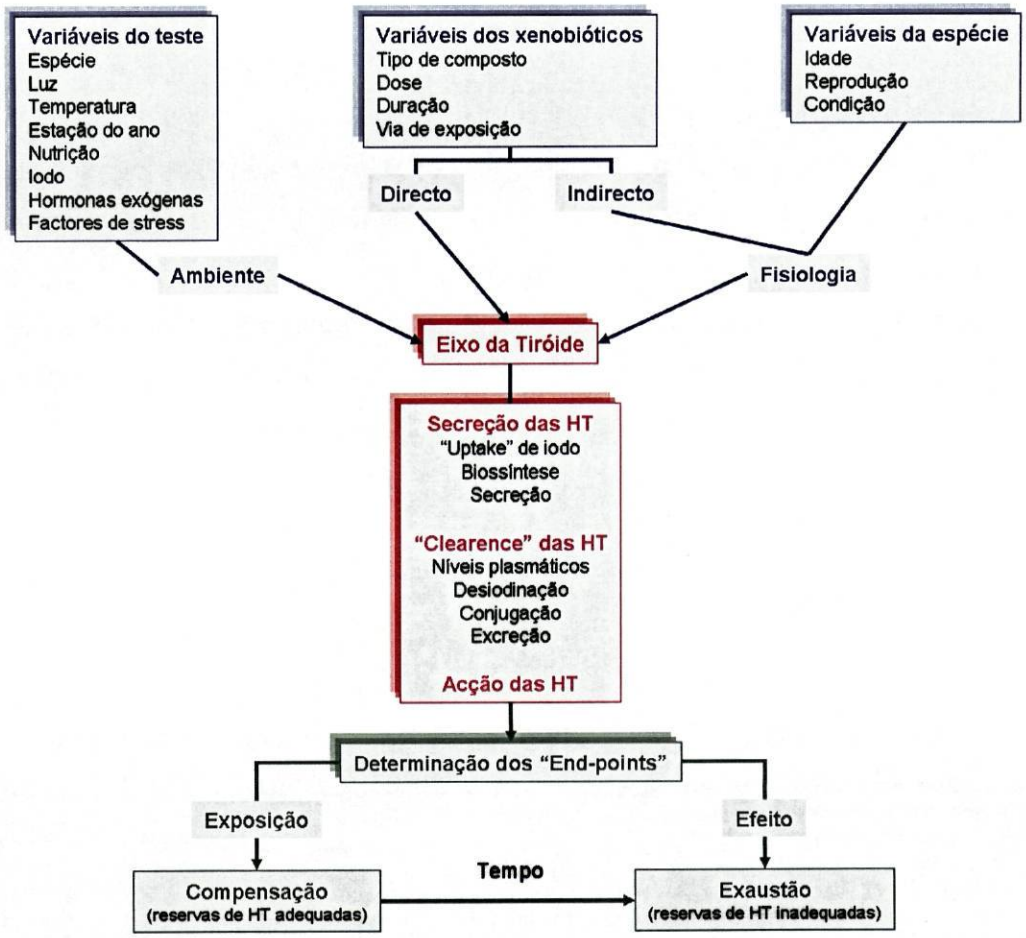


Fig. 12: Factores envolvidos na interpretação da resposta do sistema da tiróide dos peixes à exposição de xenobióticos (Brown et al., 2004a).

A actividade de desiodinação é altamente sensível a factores ambientais e a sua determinação, juntamente com os níveis plasmáticos de T4 e T3, permite avaliar o metabolismo periférico das HT. A tiróide tem a particularidade de possuir uma capacidade de compensação considerável aos efeitos de disrupção. Esta regulação ocorre a nível central e/ou periférico (Brown et al., 2004a), como já foi referido anteriormente.

EIXO HIPOTÁLAMO-HIPÓFISE-GÓNADA

A reprodução é o processo biológico que assegura a continuidade de todas as espécies.

Nos peixes, como nos outros vertebrados, a reprodução, os comportamentos associados e as alterações fisiológicas são reguladas pelo eixo hipotálamo-hipófise--gónada, um sistema de controlo onde cada componente actua directa ou indirectamente sobre os outros (Fig. 13).

Assim, o desenvolvimento das gónadas é sincronizado por séries de factores endógenos e estímulos exógenos (temperatura, fotoperíodo, disponibilidade de alimento, qualidade da água e factores sociais). Os factores exógenos são captados pelo cérebro e traduzidos em impulsos nervosos que estimulam as vias endócrinas do eixo hipotálamo--hipófise-gónada.

As hormonas chave envolvidas neste processo são: as hormonas libertadoras das gonadotropinas (GnRH), as gonadotropinas (GTH) da hipófise (a hormona folículo estimulante-FSH e a hormona luteinizante-LH), os esteróides sexuais (androgénios e estrogénios) e as prostaglandinas.

É importante referir que inicialmente as GTH dos peixes foram denominadas de GTH I e GTH II, contudo, com base na similaridade entre as sequências das subunidades β da GTH I e da FSH e da GTH II e da LH (Querat, 1994, Querat, 1995), em 1999 no "Sixth International Symposium on the Reproductive Physiology of Fish", em Bergen, a comunidade científica adoptou o uso dos termos FSH para a GTH I e LH para a GTH II (Yaron et al., 2003).

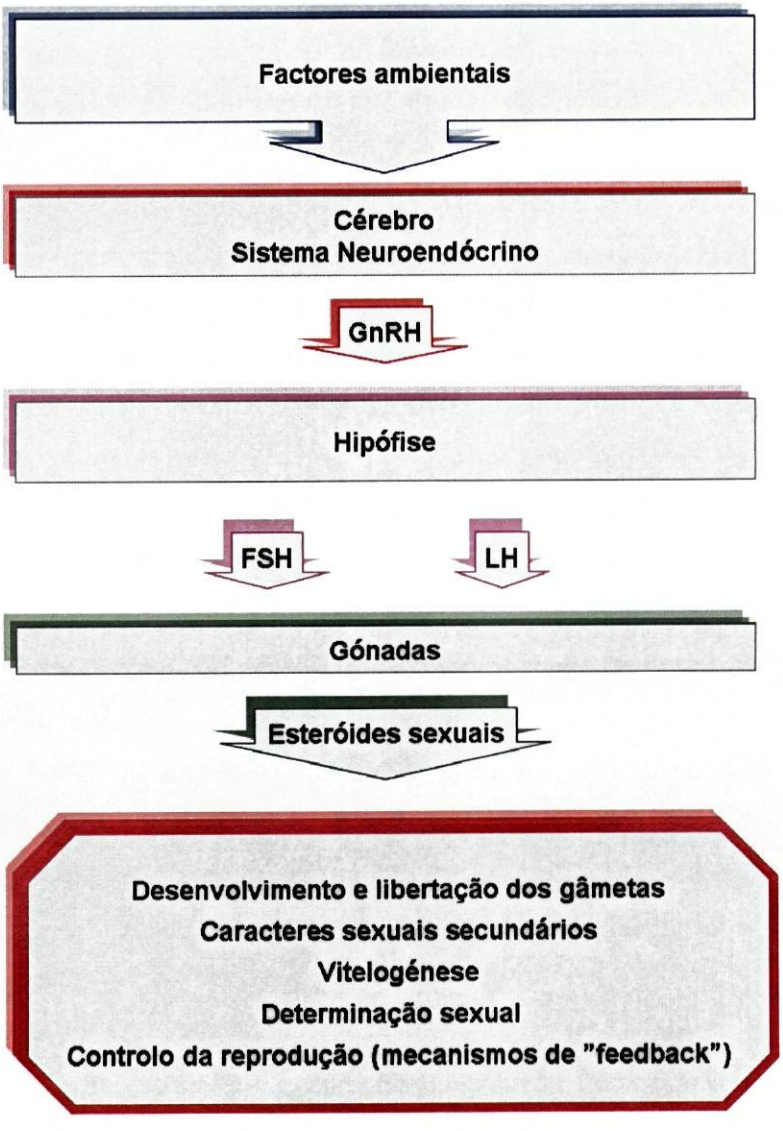


Fig. 13: Eixo hipotálamo-hipófise-gónada: FSH-hormona folículo estimulante; GnRH--hormona libertadora das gonadotropinas; LH-hormona luteinizante.

Cérebro

O cérebro nos peixes, tal como nos outros vertebrados, encontra-se envolvido na regulação da reprodução, através da síntese de substâncias que regulam a produção e secreção das GTH.

Nos mamíferos, o cérebro produz substâncias que chegam à adenohipófise através dos vasos sanguíneos do sistema portal hipofisário. Nos peixes, este sistema está ausente e os estímulos chegam à hipófise, directa ou indirectamente, por via neuronal (Kah et al., 1993, Trudeau, 1997).

Neurotransmissores envolvidos na regulação da reprodução

Para além da GnRH, vários neurotransmissores estão envolvidos no controlo da produção das GTH. Estes são: a dopamina; o ácido γ-aminobutirico (GABA); o glutamato; o neuropéptido Y; a serotonina e a noradrenalina (Trudeau, 1997).

Hormona libertadora das gonadotropinas (GnRH)

A função da GnRH é induzir a síntese e libertação das GTH, FSH e LH pela hipófise (Peter e Yu, 1997, Trudeau, 1997). Durante o desenvolvimento dos gâmetas a GnRH estimula a libertação da FSH, enquanto na fase de maturação predomina a indução da produção da LH (Swanson et al., 1987, Nozaki et al., 1990).

O cérebro dos peixes contém duas ou três formas desta hormona (Lethimonier et al., 2004). A forma da hormona que regula a libertação das GTH é específica da espécie e é designada de GnRH I; esta proteína pode ser encontrada na zona pré-óptica do hipotálamo e na hipófise. A GnRH II ("chicken" GnRH II-cGnRH II) é a forma mais conservada da hormona (Millar, 2003) e está presente no tegumento do cérebro médio. A terceira forma GnRH III ("salmon" GnRH-sGnRH) encontra-se, geralmente, no bolbo olfactivo, no nervo terminal e no cérebro anterior dos peixes (Gothilf et al., 1996, White e Fernald, 1998). Os estudos realizados indicam que as GnRH I são as responsáveis pelo controlo da secreção das GTH e que a GnRH II está envolvida na determinação do comportamento sexual (Millar, 2003).

Na tilápia do Nilo foram identificadas (Parhar et al., 2000) e clonadas (Kitahashi et al., 2005) três formas de GnRH: GnRH I-sbGnRH ("sea bream" GnRH); GnRH II-cGnRH e GnRH III-sGnRH.

Para além da indução da síntese e libertação das GTH, a GnRH também influencia a produção e libertação da hormona de crescimento (GH) (Bosma et al., 1997) e da prolactina (PRL) (Weber et al., 1997).

Dopamina

A dopamina exerce uma função inibidora da secreção das GTH (Fig. 14) e foi o único neurotransmissor identificado com esta acção (Peter e Yu, 1997, Trudeau, 1997). A dopamina actua nos neurónios produtores de GnRH I, inibindo a libertação desta hormona (Yu e Peter, 1991, Yu et al., 1991, Trudeau, 1997). Na hipófise, a dopamina reduz a ligação da GnRH aos seus receptores, por diminuição do número destes receptores (Peter et al., 1986, De Leeuw et al., 1988, Habibi et al., 1989, Sloley et al., 1991, Sloley et al., 1992a, Sloley et al., 1992b, Levavi-Sivan et al., 2004).

Na tilápia, a dopamina inibe a libertação das GTH pelas células produtoras das GTH (Levavi-Sivan et al., 1995), mas, apesar da forte acção inibitória na libertação das GTH, a dopamina não interfere com a estimulação da expressão do gene da LHβ pela GnRH (Melamed et al., 1996).

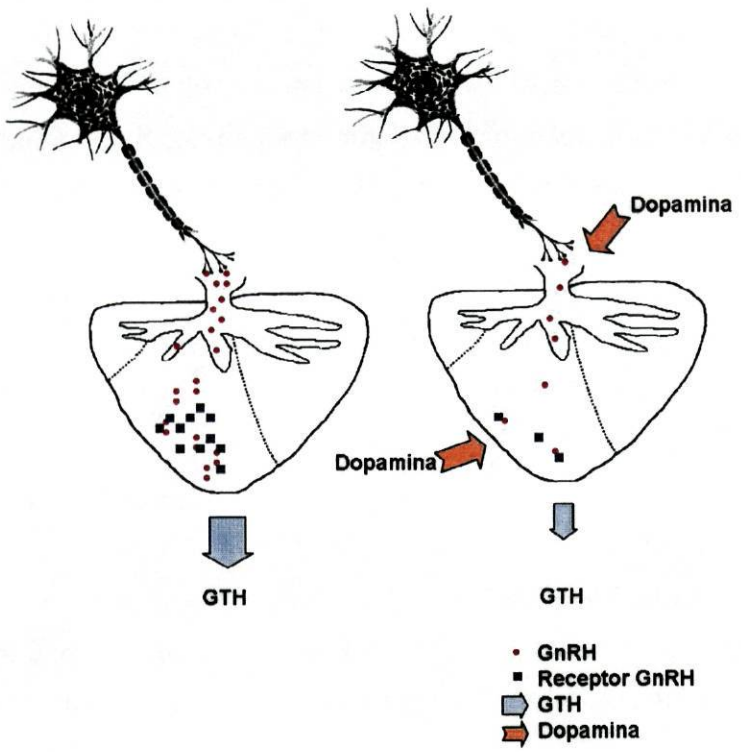


Fig. 14: Acção da dopamina nos neurónios produtores de GnRH e na hipófise.

ICRAS

Ácido γ-aminobutírico (GABA)

Contrariamente à acção inibidora que possui nos mamíferos, durante as fases iniciais do desenvolvimento das gónadas do peixe-dourado (*Carassius auratus*), o GABA, um aminoácido, estimula a libertação de LH. No entanto, em peixes sexualmente maduros, esta estimulação não ocorre (Kah et al., 1992, Trudeau, 1997).

A acção do GABA (Fig. 15) consiste principalmente no aumento da libertação de GnRH e, secundariamente, na redução da actividade dopaminérgica (Trudeau et al., 1993). Foi sugerido que o principal efeito do GABA é estimular a libertação de GnRH nas terminações nervosas e estimular a libertação das GTH, atingindo indirectamente as células produtoras das GTH ou elevando directamente os níveis intercelulares de Ca^{2+} nestas células (Kah et al., 1992, Kah et al., 1993).

Estudos, *in vitro* e *in vivo*, na truta arco-íris (*Onchorynchus mykiss*) mostraram que o GABA, para além de exercer um efeito estimulador da secreção de FSH e LH, dependente do sexo e do estado reprodutivo, também facilita a acção da GnRH (Mananos et al., 1999). Na tilápia, alterações na quantidade de GABA presente no cérebro foram relacionadas com o estado de desenvolvimento do ovário, tendo as concentrações mais elevadas sido encontradas durante o período de pré-desova (Tsai e Wang, 2000).

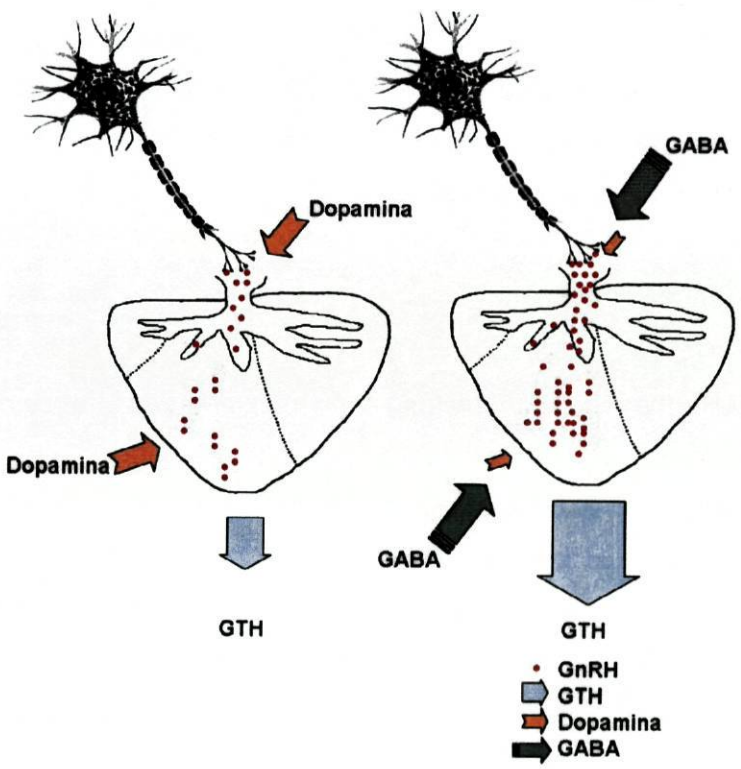


Fig. 15: Acção do GABA nos neurónios GnRH e na hipófise.

Glutamato

O glutamato é outro aminoácido associado à regulação da reprodução; nos mamíferos está associado a uma indução da libertação de GnRH e de LH (Brann e Mahesh, 1994), mas nos peixes a sua função ainda não se encontra esclarecida (Trudeau, 1997).

A acção deste neurotransmissor pode ser directa ou indirecta, como molécula precursora do GABA (Trudeau et al., 2000).

Na tilápia, as concentrações mais elevadas de glutamato, tal como as de GABA, ocorrem durante o período de pré-desova e estão relacionadas com o estado de desenvolvimento do ovário (Tsai e Wang, 2000).

Neuropéptido Y

No peixe dourado, o neuropéptido Y estimula a libertação de LH, por acção directa, sobre um receptor "y1-like" presente nos gonadotrófos e por acção indirecta, através de um receptor "y2-like", promove a libertação de GnRH (Peng et al., 1990, Peng et al., 1993a, Peng et al., 1993b, Cerda-Reverter e Larhammar, 2000).

Na truta, o efeito estimulador do neuropéptido Y na libertação das GTH é dependente da fase do ciclo reprodutivo (Breton et al., 1989, Breton et al., 1991) e a sua acção parece envolver os neurónios produtores de GnRH (Danger et al., 1991, Chiba et al., 1996).

Na tilápia, a exposição de células da hipófise ao neuropéptido Y resultou num aumento da expressão da subunidade α das GTH. Este neurotransmissor aumentou a expressão desta subunidade por estimulação da GnRH (Gur et al., 2001a, Gur et al., 2001b). Estudos posteriores mostraram que o neuropéptido Y também estimula a expressão de LHβ, mas não a de FSHβ (Gur et al., 2002).

Serotonina

Na corvina (*Micropogonias undulatus*) e no peixe-dourado, a serotonina activa os neurónios produtores de GnRH e estimula a libertação de LH (Trudeau, 1997).

A serotonina funciona como um sinal diferencial no desenvolvimento neuronal e induz a formação de uma diferenciação sexual cerebral (Lauder e Krebs, 1976).

Na tilápia, os níveis de serotonina presentes no cérebro são diferentes entre machos e fêmeas. Durante o período de diferenciação sexual da tilápia, a quantidade e o metabolismo de serotonina estão dependentes de esteróides exógenos (Tsai e Wang, 1997).

Noradrenalina

A noradrenalina estimula a actividade dos neurónios produtores de GnRH (Peter et al., 1990, Peter et al., 1991), promovendo ligeiramente a libertação de LH. Na tilápia pouco se conhece sobre a acção deste neurotransmissor; no entanto, sabe-se que a quantidade de noradrenalina presente no cérebro durante o desenvolvimento do sistema de neurotransmissores centrais depende da temperatura (Wang e Tsai, 2000), do fotoperíodo (Huang et al., 2004) e dos níveis de estradiol (Tsai et al., 2001a, Tsai et al., 2001b).

Hipófise

A hipófise (Fig. 16) é uma glândula apenas presente nos vertebrados, formada pela neurohipófise e pela adenohipófise. Com base na morfologia, a adenohipófise dos teleósteos pode ser dividida na *pars distalis* (zona anterior) e na *pars intermedia* (zona posterior). Por sua vez, a *pars distalis* é formada pela *rostral pars distalis* e pela *proximal pars distalis*. Como já foi referido anteriormente, a hipófise dos peixes não apresenta um sistema hipotálamo-hipófise portal para o transporte dos reguladores neurohormonais. Em vez disso, existe um transporte axonal directo do hipotálamo para a hipófise através do pedúnculo hipofisário e da neurohipófise.

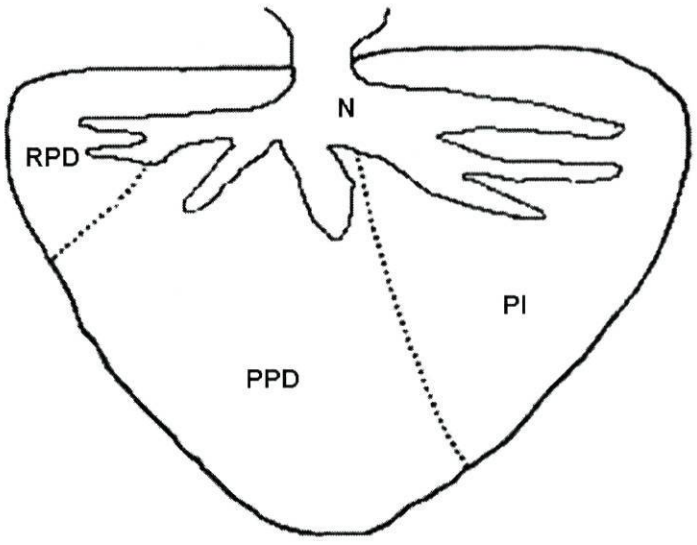


Fig. 16: Hipófise dos teleósteos: N-Neurohipófise, PI-*Pars Intermedia*, PPD-*Proximal Pars Distalis*, RPD-*Rostral Pars Distalis* (adaptado de Weltzien et al., 2004).

Gonadotropinas

As GTH são produzidas na adenohipófise, na *pars distalis*. Tal como nos mamíferos, ambas as GTH são heterodímeros glicoproteicos que partilham uma subunidade idêntica (α) e uma subunidade distinta (β), que lhes confere a especificidade de acção (Yaron et al., 2003).

No salmão, as GTH são igualmente potentes na estimulação de estradiol pelo ovário durante a vitelogénese; no entanto, a LH possui uma maior capacidade de estimulação da produção dos esteróides maturativos (Suzuki et al., 1988, Swanson et al., 1991).

A FSH está envolvida no desenvolvimento das gónadas, na síntese de estradiol, na vitelogénese, na oogénese e na espermatogénese. A LH tem uma acção predominante na fase final do ciclo reprodutivo, induzindo a maturação das gónadas através da estimulação da síntese dos esteróides maturativos.

Os níveis das GTH durante os ciclos reprodutivos variam com o tipo de reprodução da espécie.

Na tilápia, os níveis de expressão da FHSβ mais elevados foram encontrados em peixes com níveis baixos de esteróides sexuais, entre 1 e 10 $ng.ml^{-1}$ de estradiol ou testosterona, enquanto que, os níveis mais elevados de LHβ foram encontrados em peixes com níveis mais elevados de esteróides sexuais (Melamed et al., 2000).

Gónadas

As gónadas têm duas funções principais: a produção de gâmetas e a produção de esteróides sexuais, que regulam a reprodução quer através da sua acção local (parácrina ou autócrina), quer através de mecanismos de "feedback" positivo e negativo no hipotálamo e hipófise.

Peixes com reprodução assíncrona, como é o caso da tilápia, apresentam durante o período reprodutivo gónadas com gâmetas em todas as fases de desenvolvimento.

Esteróides sexuais

A síntese dos esteróides sexuais ocorre principalmente nas gónadas. No testículo, os androgénios são produzidos pelas células de Leydig. No ovário, os androgénios são produzidos pelas células da teca e posteriormente aromatizados, na camada de células da granulosa, a estrogénios (Yoshikuni e Nagahama, 1991).

Os esteróides sexuais (estrogénios, androgénios e progestagénios) actuam: a) localmente, nas gónadas, onde induzem a proliferação de células germinativas e o

desenvolvimento e libertação dos gâmetas; b) perifericamente, onde induzem o aparecimento dos caracteres sexuais secundários e do comportamento reprodutivo; c) ao nível do hipotálamo e da hipófise, onde controlam a sua própria síntese por mecanismos de "feedback"; d) no fígado das fêmeas onde induzem a síntese de vitelogenina, uma proteína que posteriormente é incorporada nos oócitos (Yoshikuni e Nagahama, 1991, Peter e Yu, 1997, Trudeau, 1997).

Resumo da síntese dos principais esteróides sexuais

Os esteróides sexuais (Fig. 17) têm a sua origem numa molécula precursora, o colesterol. O colesterol é inicialmente hidroxilado nos carbonos 20 e 22 pelas hidrolases, originando-se 20,22-dihidroxicolesterol. Posteriormente, os mesmos átomos de carbono são oxidados pela desmolase e a molécula perde seis átomos de carbono formando-se a pregnenolona.

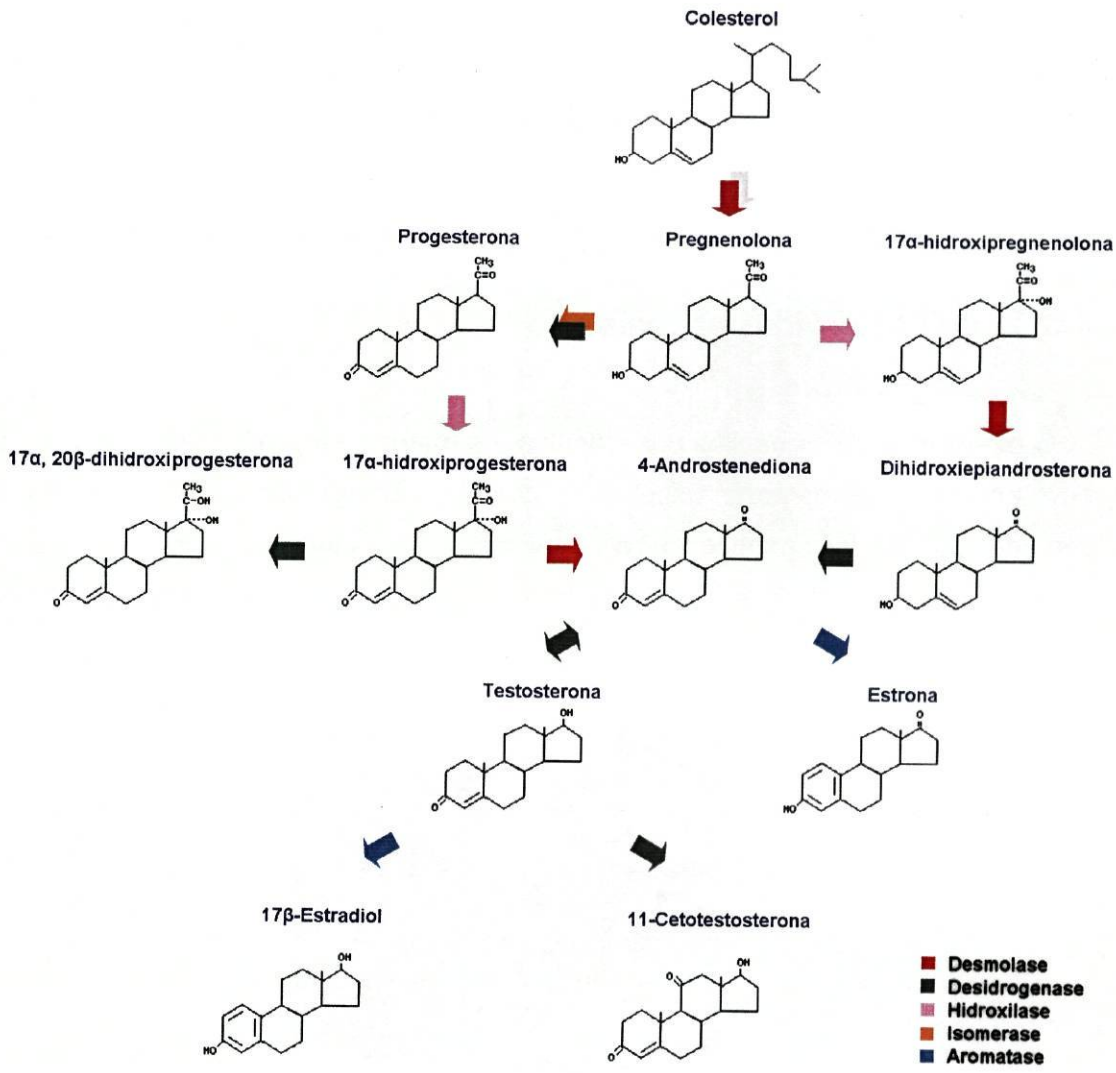


Fig. 17: Resumo da síntese dos esteróides sexuais.

A pregnenolona origina a 4-androstenediona, o precursor da testosterona, por duas vias distintas onde actuam as mesmas enzimas, mas por ordem diferente.

Na via predominante a pregnenolona é convertida por oxidação em progesterona; esta reacção envolve a 3β-hidroxiesteróide-desidrogenase e uma isomerase. A progesterona, por sua vez, sofre uma hidroxilação do carbono 17 por acção da 17α-hidroxilase e forma-se a 17α-hidroxiprogesterona. Esta molécula sofre uma oxidação pela 17,20-desmolase e origina a 4-androstenediona.

Na segunda via, a pregnenolona é hidroxilada pela 17α-hidroxilase em 17α-hidroxipregnenolona; esta molécula é oxidada no carbono 17 pela 17,20-desmolase e convertida em dihidroxiepiandrosterona, que por sua vez é transformada pela 3β-hidroxiesteróide-desidrogenase em 4-androstenediona.

A 17β-hidroxiesteróide-desidrogenase reduz de forma reversível a 4-androstenediona a testosterona, com a conversão de NAD^+ a NADH.

Por aromatização, a testosterona é convertida em estradiol e a 4-androstenediona em estrona.

A aromatase e todas as hidroxilases envolvidas na síntese dos esteróides sexuais são enzimas cuja actividade está dependente do citocromo P450, presente no sítio activo da enzima (Vance, 1993).

Acção dos esteróides sexuais

Nas gónadas

O ovário contém os oócitos nos folículos e a matriz ovárica (Fig.18). Cada oócito está envolvido por uma membrana acelular, o córion ou *zona radiata*, limitada por uma única camada de células granulosas e pela teca. As células da granulosa e células especializadas da teca são as responsáveis pela síntese de esteróides sexuais e maturativos.



Fig. 18: Ovários de tilápia.

Durante a oogénese a FSH estimula o aumento dos níveis de estrogénios e androgénios até estes atingirem um pico no início da ovulação (Peter e Yu, 1997). Nos salmonídeos, a FSH estimula a síntese de testosterona nas células da teca, que é depois aromatizada a estradiol, por uma enzima do complexo citocromo P450, a aromatase, na camada de células da granulosa. No *Fundulus heterclitus* e na *Oryzias latipes* este processo ocorre, na totalidade, na camada de células da granulosa (Onitake e Iwamatsu, 1986, Nagahama, 1987, Petrino et al., 1989).

Na fase final de maturação e na ovulação, um pico de LH induz, na camada de células da granulosa, a produção do esteróide maturativo a partir da 17α-hidroxiprogesterona produzida nas células da teca. No caso da tilápia este é a 17,20β-dihidroxi-4-pregnen-3--ona (Yoshikuni e Nagahama, 1991, Rocha e Reis-Henriques, 1996).

O testículo (Fig.19) da maioria dos teleósteos encontra-se organizado em túbulos ramificados ou com anastomoses contendo espermatocistos, onde ocorre a espermatogénese. Cada espermatocisto possui células de Sertoli e espermatogónias. Na tilápia existe um número limitado de células de Sertoli por espermatocisto (Matta et al., 2002, Vilela et al., 2003). No espaço intersticial existente entre os túbulos encontram-se as células de Leydig, a principal fonte de androgénios (Yaron, 1966, Yoshikuni e Nagahama, 1991).



Fig. 19: Testículos de tilápia.

A espermatogénese é estimulada pela FSH por indução da síntese de 11--cetotestosterona nas células de Leydig, que, por sua vez, activa a produção de vários factores necessários à proliferação das espermatogónias e à formação de espermatozóides, pelas células de Sertoli.

Durante a espermiação, as células de Leydig, sob acção da LH, produzem esteróides maturativos, 17α, 20β-dihidroxi-4-pregnen-3-ona (Ueda et al., 1984, Rocha e Reis--Henriques, 1996) ou 17α, 20β-trihidroxi-4-pregnen-3-ona (Trant e Thomas, 1989).

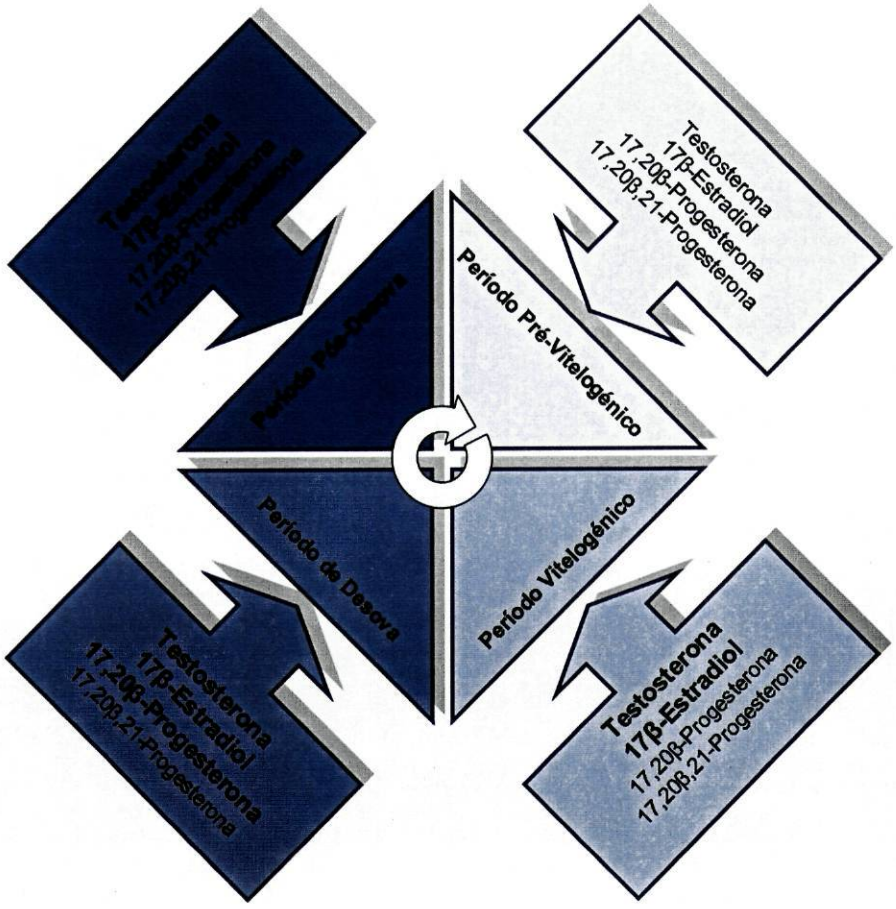


Fig. 20: Principais esteróides sexuais durante o ciclo reprodutivo da tilápia (adaptado de Rocha, 1996).

Nos tecidos periféricos

Os esteróides sexuais, para além das acções exercidas nas gónadas, actuam em alguns tecidos periféricos, induzindo respostas do mesmo modo fundamentais ao desenvolvimento da reprodução.

Nestas respostas incluem-se o aparecimento dos caracteres sexuais secundários e a vitelogénese. A vitelogénese consiste no sequestro e acumulação de vitelogenina produzida no fígado pelos oócitos em desenvolvimento (Yoshikuni e Nagahama, 1991). A síntese desta proteína é estimulada pelo estradiol, assim como o seu sequestro (Hyllner et al., 1991). Na truta, esta proteína regula a sua própria síntese, por inibição da síntese de androstenediona e estradiol quando atinge níveis elevados dentro dos oócitos (Reis--Henriques et al., 2000).

No cérebro e na hipófise

Para além das acções nas gónadas e nos tecidos periféricos, anteriormente descritas, os esteróides sexuais controlam a sua própria acção por mecanismos de "feedback" a nível do cérebro e da hipófise.

Durante as primeiras fases de vida, embriogénese e larvar, estas hormonas são fundamentais para a determinação sexual a nível do cérebro, originando diferenças estruturais e funcionais entre os cérebros masculino e feminino (Callard, 1983).

Estudos de exposição a esteróides exógenos em tilápia revelaram que, durante a fase de determinação sexual, a modulação da proliferação de células cerebrais é feita pelo estradiol (Tsai et al., 2001a, Tsai et al., 2001b), que também determina os níveis de neurotransmissores presentes no cérebro (Tsai e Wang, 1998, Tsai e Wang, 1999, Tsai et al., 2000). Assim, a exposição ao estradiol determinou a feminização cerebral, enquanto que, a exposição à metiltestosterona, um androgénio sintético, determinou a masculinização cerebral (Tsai et al., 2000).

Na fase reprodutiva, as flutuações dos esteróides sexuais que ocorrem são determinadas por acções directas destas hormonas no cérebro e/ou na hipófise ou indirectas sobre a síntese dos neurotransmissores (Peter e Yu, 1997, Trudeau, 1997). Nos peixes imaturos predomina o "feedback" positivo (Goos et al., 1987). A testosterona actua directamente ou após aromatização promovendo a libertação de GnRH e aumentando a resposta dos gonotrofos à GnRH (Trudeau, 1997). No peixe-dourado, a testosterona também aumenta a síntese de GABA e a sua acção estimulatória (Trudeau, 1997).

Na truta durante a vitelogénese, os esteróides sexuais, principalmente o estradiol, aumentam o "turnover" da dopamina, aumentando a sua acção inibidora sobre a libertação de LH (Saligaut et al., 1992, Linard et al., 1995). Na tilápia, a acção de "feedback" negativo exercida pelo estradiol é feita através de um aumento do número de receptores de dopamina na hipófise (Levavi-Sivan et al., 2003).

O estradiol e a testosterona apresentam no peixe dourado um pico antes da fase final da reprodução, originando o aparecimento do pico de LH (Kobayashi et al., 1989), acompanhado de uma estimulação da síntese do neuropéptido Y, que nesta espécie estimula a libertação de GnRH e de LH (Peng et al., 1993a, Peng et al., 1993b).

Na tilápia, como já foi referido, vários estudos mostraram que em peixes com concentrações plasmáticas de estradiol (fêmeas) ou testosterona (machos) relativamente baixas, a expressão da FSHβ é elevada e que quando os níveis destas hormonas aumentam predomina a expressão de LHβ (Melamed et al., 2000). Para determinarem se a acção dos esteróides sexuais na expressão das GTH é directa, células de hipófises de tilápia em vários estados de desenvolvimento foram incubadas na presença de

testosterona. Estes estudos revelaram que nos animais imaturos a testosterona (10 nM) promove a expressão de FSHβ e que nos animais maduros só doses de testosterona entre 10 e 100 pM aumentam a expressão de FSHβ (Melamed et al., 1997). No fim da época reprodutiva a expressão de FSHβ diminui após a exposição a concentrações elevadas de testosterona (10 e 100 nM) e de estradiol (1 nM-100 nM); nesse mesmo estudo a adição de testosterona ou estradiol (10 pM-100 nM) não alterou a expressão de LHβ (Melamed et al., 2000). Assim, a expressão de FSHβ responde aos níveis de esteróides sexuais dependendo da fase do ciclo reprodutivo, sendo mais sensível durante a fase de desenvolvimento dos gâmetas e menos sensível na fase final de maturação; em contrapartida a expressão de LHβ é independente dos níveis de esteróides sexuais.

A exposição das células da hipófise a 11-cetotestosterona não teve qualquer efeito na expressão das duas GTH (Melamed et al., 1998) e a expressão da subunidade α das GTH só é afectada pela presença de testosterona nas células provenientes de animais maduros (Gur et al., 2000). Os resultados mostram que os efeitos da testosterona na expressão das unidades das GTH são semelhantes aos efeitos do estradiol e que o androgénio não aromatizável não produz esses efeitos. Isto indica que a acção da testosterona ao nível da hipófise da tilápia é feita após aromatização a estradiol (Yaron et al., 2003).

COMPOSTOS POLICLORADOS

Actualmente, existe uma considerável preocupação pela presença no ambiente de disruptores endócrinos químicos, isto é, substâncias naturais ou produzidas pelo Homem que interferem com os sistemas endócrinos dos vertebrados (Kavlock et al., 1996, Ankley et al., 1998, Tyler et al., 1998, Vos et al., 2000). Estes compostos têm a capacidade de modular ou interferir com a síntese, secreção, transporte, ligação ou eliminação das hormonas endógenas afectando a homeostasia e, consequentemente, o desenvolvimento, a reprodução e o comportamento dos organismos. Estudos de campo, particularmente no meio aquático, sugerem uma relação entre a exposição a hormonas ou a compostos presentes no ambiente e alterações no desenvolvimento e na reprodução dos organismos expostos (Guillette et al., 1996, Janssen et al., 1997, Jobling et al., 1998, Oberdorster e Cheek, 2001).

Os compostos orgânicos persistentes ("**P**ersistent **O**rganic **P**ollutants"-POPs) podem dissiminar-se por milhares de quilómetros, acumular-se nas cadeias tróficas e persistirem no ambiente, podendo ser necessário séculos para a sua degradação. Sabe-se, que os POPs contribuem para o aparecimento de anomalias do desenvolvimento, de cancro, de disfunção do sistema imunitário e de problemas reprodutivos em animais selvagens (Smialowicz et al., 1989, Colborn et al., 1993, Vos e Van Loveren, 1995, Toppari et al., 1996) e no Homem (Binelli e Provini, 2004, Hardell et al., 2006).

Os POPs são compostos orgânicos que resistem à degradação fotolítica, biológica e química. A maioria são compostos semi-voláteis, o que permite a sua distribuição através da atmosfera por longas distâncias antes de ocorrer a sua deposição. A sua distribuição pelo globo é realizada por um processo denominado de "grasshopper effect", efeito gafanhoto, que envolve ciclos de libertação para a atmosfera e deposição, permitindo a sua distribuição global (Fingler et al., 1992, Wild e Jones, 1992, Abbassy et al., 1999).

Muitos destes compostos são halogenados, ou seja, possuem um ou mais elementos da família dos halogénios (flúor, cloro, bromo ou iodo) e caracterizam-se por uma solubilidade reduzida na água e elevada nos lípidos. Estas propriedades, em combinação com a sua persistência, permitem a bioconcentração e bioacumulação nos tecidos gordos dos organismos. Ambos os processos levam à biomagnificação, em que a concentração do contaminante nos tecidos aumenta à medida que sobe de nível trófico (Pocar et al., 2003).

O Programa Ambiental das Nações Unidas identificou doze POPs, nove dos quais insecticidas, como ameaças sérias para a saúde humana e animal (Tabela 1).

Tabela 1: Fontes ambientais e alimentares de POPs (adaptado de Abelsohn et al., 2002).

Tipo	Fontes ambientais	Exemplos de fontes alimentares
Dioxinas, Furanos	Produtos secundários da indústria petroquímica e do papel; hospitais e incineradores	Carne, laticínios e peixe
PCBs	Produtos resistentes ao fogo fabricados antes de 1977, equipamento eléctrico antigo, contentores de PCBs em locais de despejo	Peixe (dos Grandes Lagos), mamíferos marinhos do Ártico e leite materno
Aldrin	Pesticida usado contra insectos do solo, protecção de cultivos de milho e batata	Laticínios, carne, peixe, óleos e gorduras, batatas e legumes (cenoura, nabo)
Chlordane	Insecticida de contacto de largo espectro usado em vegetais, cereais, batatas, cana do açúcar, frutos, algodão e juta	Devido às restrições de uso, os alimentos não são uma fonte deste composto
DDT	Pesticida usado durante a 2ª Guerra Mundial para proteger contra doenças transmitidas por insectos	Peixe, laticíneos, gorduras animais, ovos e vegetais
Dieldrin	Insecticida usado contra insectos do solo	Os mesmos do aldrin
Endrin	Insecticida usado nas folhas das plantas, por exemplo, na cultura de algodão	Devido às restrições de uso, os alimentos não são uma fonte deste composto
Heptachlor	Insecticida de contacto usado contra insectos do solo	Devido às restrições de uso, os alimentos não são uma fonte deste composto
HCB	Fungicida aplicado na preservação de sementes	Grãos tratados com HCB; devido às restrições de uso, os alimentos não são uma fonte deste composto
Mirex	Insecticida estomacal usado no controlo de formigas e térmitas	Carne, peixe, caça e mamíferos marinhos
Toxaphene	Insecticida de contacto usado na cultura de algodão, cereais, frutos e vegetais e no controlo de carraças do gado	Devido às restrições de uso, os alimentos não são uma fonte deste composto

Os bifenilos policlorados ("**Poly**C**hl**orinated **B**yphenils"-PCBs) são misturas de hidrocarbonetos clorados que foram extensivamente usados desde 1930 para fins industriais, incluindo transformadores eléctricos, aditivos de tintas e plásticos (Fig. 21).

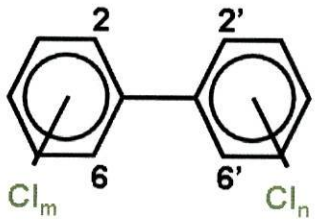


Fig. 21: Estrutura geral dos bifenilos policlorados, compostos formados pela união de dois anéis benzénicos com substituições por átomos de cloro. $_m$ e $_n$ ($1 \leq _m \leq 5$, $1 \leq _n \leq 5$) representam o número de átomos de cloro.

No ambiente, os PCBs podem ser encontrados associados com a matéria orgânica presente no solo, com a matéria orgânica em suspensão na água, nos sedimentos e mesmo nos tecidos dos diversos organismos. A principal fonte de PCBs para o Homem são os alimentos, onde se incluem os peixes.

Dependendo do número e da posição de átomos de cloro, existem 209 congéneres de PCBs, doze dos quais (Tabela 2) possuem as mesmas características das dioxinas ("dioxin-like") e, por isso, uma maior toxicidade e persistência ambiental.

Tabela 2: Congéneres de PCBs semelhantes às dioxinas, nº do congénere (IUPAC), nº e posição dos átomos de cloro.

Congénere	Nº átomos de Cl	Posição dos átomos de Cl
77	4	3 , 3', 4 , 4'
81	4	3 , 3', 4', 5
105	5	2 , 3 , 3', 4 , 4'
114	5	2 , 3 , 4 , 4', 5
118	5	2 , 3', 4 , 4', 5
123	5	2 , 3', 4 , 4', 5'
126	5	3 , 3', 4 , 4', 5
156	6	2 , 3 , 3', 4 , 4', 5
157	6	2 , 3 , 3', 4 , 4', 5'
167	6	2 , 3', 4 , 4', 5 , 5'
169	6	3 , 3', 4 , 4', 5 , 5'
189	7	2 , 3 , 3', 4 , 4', 5 , 5'

Várias misturas de congéneres de PCBs foram comercializadas sob o nome de Aroclor, Clophen ou Kanechlor e usadas em fluídos dieléctricos em transformadores eléctricos, tintas de impressão, adesivos e tintas (Strachan, 1988). Os PCBs são compostos muito estáveis, que persistem no ambiente e que passam e se acumulam nos vários níveis das cadeias tróficas; os valores mais elevados são encontrados nos

predadores, mas nem todos os PCBs são activos, e o seu impacto varia com a sua estrutura e dose (Brucker-Davis, 1998).

Desde meados da década de 70 que a sua produção, importação e uso estão sob um controlo apertado, o que reduziu os níveis presentes no ambiente, mantendo-se actualmente níveis constantes.

O Aroclor 1254 é uma mistura de PCBs que contém aproximadamente: 21% $C_{12}H_6Cl_4$, 48% $C_{12}H_5Cl_5$, 23% $C_{12}H_4Cl_6$, e 6% $C_{12}H_3Cl_7$, com um conteúdo médio de cloro de 54 % (USAF-US, 1989) (Fig. 22).

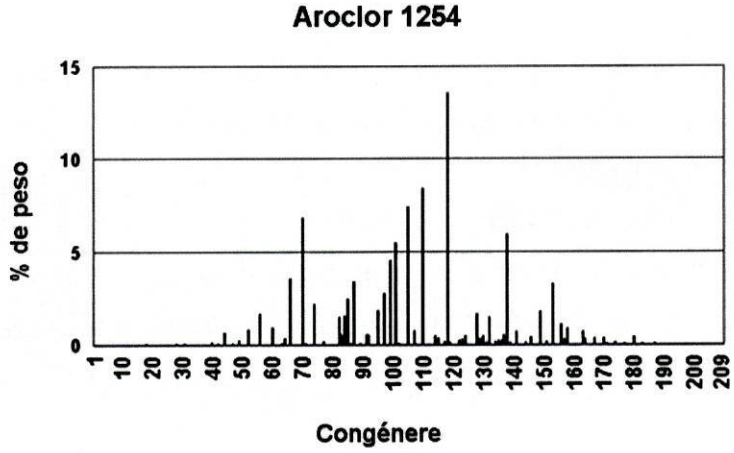


Fig. 22: Perfil da composição do Aroclor 1254 (EPA-"Environmental Protection Agency").

Os PCBs, incluindo o Aroclor 1254, são compostos inertes, termicamente estáveis e com propriedades dieléctricas. No meio ambiente, o comportamento das misturas de PCBs está directamente relacionado com o seu conteúdo em cloro. De uma forma geral, um número maior de átomos de cloro indica uma maior adsorção e um transporte e degradação menores. Apesar de serem compostos químicos resistentes à oxidação e à hidrólise, pode ocorrer a sua biodegradação (Goerke e Weber, 2001, Antunes et al., 2004). Estudos efectuados em tainha (*Mugil cephalus*) mostraram que, após um período de depuração, existe uma concentração mais elevada de congéneres menos clorados (tri e tetraclorados) no músculo do que antes da depuração (Antunes et al., 2004, Antunes et al., in press); entretanto, os mesmos autores mostraram que este aumento é devido à metabolização dos PCBs mais clorados, inicialmente presentes no músculo, em congéneres menos clorados durante o período de depuração (Antunes et al., 2005). Para além disso, os PCBs possuem factores de bioacumulação elevados devido à sua

lipofilicidade, especialmente os congéneres mais clorados, que tendem a acumular-se nos tecidos gordos dos animais (ATSDR, 1995). Estes compostos policlorados são metabolizados pelo sistema de mono-oxigenases microssomais, catalizado pelo citocromo P450 (CYP1A), em metabolitos polares que podem ser posteriormente conjugados (ATSDR, 1995). Os resultados de experiências realizadas em animais sugerem o seguinte metabolismo: a) a hidroxilação é favorecida na posição *para* do anel com menor número de átomos de cloro; b) nos congéneres menos clorados, a posição *para* de ambos os anéis bifenilos e os átomos de carbono que se encontram em posição *para* em relação ao carbono com ligação ao cloro são rapidamente hidroxilados; c) a ocorrência de dois átomos de carbono adjacentes facilita o metabolismo de oxidação do PCB; d) à medida que aumenta o número de átomos de cloro ligados a ambos os anéis a capacidade de serem metabolizados diminui; e) o metabolismo dos diferentes congéneres resulta em inúmeras combinações de metabolitos (Safe, 1980).

Para além dos bifenilos policlorados existem outros compostos organoclorados, alguns dos quais usados como insecticidas, que são objecto de preocupação, uma vez que podem atingir outros organismos que não os organismos alvo. Neste grupo encontra-se o Endosulfan, 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzo--o-dioxathiepin 3-oxide (Fig. 23).

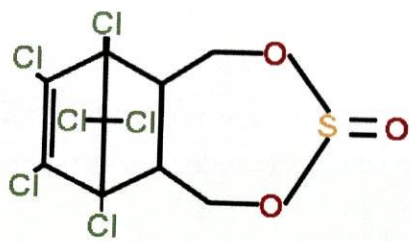


Fig. 23: Estrutura química do Endosulfan.

O Endosulfan é um ciclodieno, usado como um conservante de madeiras e como insecticida/acaricida em diversas colheitas. Este pesticida actua como antagonista dos canais de cloro dependentes do ácido γ-aminobutirico (GABA). A sua toxicidade para os peixes é elevada, sendo o valor de LC50 ao fim de 96h próximo de 1,5 $\mu g.l^{-1}$ para algumas espécies (Johnson e Finley, 1980). No entanto, de acordo com a "World Health Organization"-WHO, o Endosulfan não é considerado um POP, pois não possui propriedades de bioacumulação e persistência nos tecidos (WHO, 1984).

De acordo com o Ministério da Agricultura, do Desenvolvimento Rural e das Pescas (Vieira, 2005), o Endosulfan foi o insecticida organoclorado mais vendido em Portugal no ano de 2003 (cerca de 41 toneladas). Este insecticida foi já detectado (0,18 $\mu g.l^{-1}$) em águas superficiais portuguesas (Cerejeira et al., 2003) e pode ser encontrado nas águas de todos os continentes, como representado na Tabela 3.

Tabela 3: Alguns exemplos da concentração de Endosulfan encontrada na água.

País	Concentração	Referência
Espanha	0,1 $ng.l^{-1}$	(Vilanova et al., 2001)
Áustria	0,07 $ng.l^{-1}$	(Vilanova et al., 2001)
Egipto	3,4 – 290,2 $\mu g.l^{-1}$	(El-Kabbany et al., 2000)
Gana	62,3 $ng.l^{-1}$	(Ntow, 2001)
África do Sul	nd - 26,3 $\mu g.l^{-1}$	(Dalvie et al., 2003)
China	445,9 $ng.l^{-1}$	(Zhang et al., 2004)
Austrália	13-911 $\mu g.l^{-1}$	(Leonard et al., 2000)
E.U.A.	0,01 - 0,85 $\mu g.l^{-1}$	(Scott et al., 1999)

Em suma, as principais características que tornam os POPs potencialmente perigosos são: a) a persistência, os tempos de semi-vida ambientais muito longos, o que resulta num incremento global contínuo das substâncias presentes no meio ambiente; b) a dispersão, podendo ser encontrados a grandes distâncias dos locais onde foram usados ou libertados; c) a bioacumulação, originada pela baixa solubilidade em água e pela elevada solubilidade em lípidos, o que leva à acumulação destes compostos nos tecidos gordos; d) a capacidade de disrupção endócrina, por possuírem actividades semelhantes às das hormonas naturais.

Existem três tipos principais de mecanismos de disrupção endócrina (Pocar et al., 2003): o composto imita as hormonas naturais, iniciando uma reacção da mesma forma que uma hormona natural; o composto bloqueia os receptores hormonais, impedindo as hormonas naturais de se ligarem e executarem as suas funções; o composto actua por vias semelhantes às usadas pelas hormonas, mas induz uma resposta diferente.

Um factor importante relacionado com os efeitos de exposição a tóxicos que interferem com a reprodução é o estado de desenvolvimento reprodutivo na altura da exposição. Os embriões e fetos (ou larvas) parecem ser mais sensíveis à disrupção endócrina do que os animais adultos e alterações do comportamento reprodutivo podem ser induzidas nestas fases por doses muito inferiores às necessárias para induzir toxicidade nos adultos.

ACÇÃO DO ENDOSULFAN NO EIXO HIPOTÁLAMO-HIPÓFISE-RIM

Os compostos organoclorados podem actuar sobre o rim interferindo com a síntese de esteróides, alterando o funcionamento do eixo hipotálamo-hipófise-suprarenal e a síntese da ACTH, a hormona mediadora da resposta ao stress (Hontela et al., 1995). Estudos realizados com peixes capturados em locais contaminados com compostos organoclorados mostraram a ocorrência de uma diminuição da libertação de cortisol e, consequentemente, uma resposta menor ao stress (Hontela et al., 1995). Esta disfunção endócrina resulta em alterações a nível do metabolismo, da reprodução, da osmoregulação e da função imunológica, o que compromete a competência fisiológica e a saúde dos animais.

Estudos *in vitro* mostraram que células do rim cefálico de truta expostas ao Endosulfan apresentam uma menor capacidade de síntese de cortisol (Leblond et al., 2001, Bisson e Hontela, 2002) e que esta deve-se à acção de enzimas antioxidantes (Dorval e Hontela, 2003, Dorval et al., 2003).

COMPOSTOS ORGANOCLORADOS E FUNÇÃO DA TIRÓIDE

Os efeitos dos xenobióticos na função da tiróide têm sido avaliados frequentemente pelas alterações que induzem na biossíntese, secreção e níveis plasmáticos de HT, que estão principalmente relacionados com a eficácia da libertação de HT da tiróide para o sangue. Estes índices, no entanto, não detectam necessariamente disrupção a nível do metabolismo ou efeitos a nível dos receptores ou pós-receptores nos tecidos periféricos. O metabolismo periférico das HT nos peixes é o principal factor de controlo dos níveis de HT, apresentando-se as actividades das desiodases nos tecidos periféricos como fundamentais na determinação da disrupção da tiróide (Eales et al., 1999).

Acção de compostos bifenilos policlorados (PCBs)

Leatherland (1993) relatou alterações da histologia da tiróide e dos níveis de HT plasmáticas em salmonídeos pescados nos Grandes Lagos ou expostos a água dos Grandes Lagos. Anteriormente, o mesmo autor (Leatherland e Sonstegard, 1978) tinha exposto, através da alimentação, salmões aos Aroclor 1242 e 1254 (misturas de PCBs com 42 % e 54 % de cloro, respectivamente), de forma a determinar se os PCBs eram responsáveis pelas alterações observadas. Esta exposição ao Aroclor originou uma diminuição dos níveis da T3 mas não dos da T4 e não causou hiperplasia da tiróide. Em trutas alimentadas com Aroclor 1254 (500 $\mu g.g^{-1}$) e depois de dois meses de exposição,

não foram encontradas alterações nem dos níveis de HT, nem da histologia da tiróide. Numa segunda experiência, trutas alimentadas com uma dieta com 500 $\mu g.g^{-1}$de uma mistura 1:4 de Aroclor 1242 e 1254 apresentaram níveis de T4 e T3 diminuídos (Leatherland et al., 1980). Depois destes ensaios os autores concluiram que outros factores, que não os PCBs, eram responsáveis pelas alterações observadas na histologia da tiróide (Leatherland, 1993).

Durante a smoltificação de salmões, uma única injecção de Aroclor 1254 (150 $\mu g.g^{-1}$) aumentou os níveis plasmáticos de T3 (Folmar e Hodgins, 1982). Em solha, injectada intraperitonialmente com 500 $\mu g.g^{-1}$ de Clophen A50, ocorreu um aumento de duas vezes dos níveis de T4 plasmática e em solhas injectadas com 100 $\mu g.g^{-1}$ os níveis de T3 observados dois dias depois do tratamento foram 3 vezes superiores; no entanto, ao fim de dez dias, só os níveis de T4 permaneciam elevados (Besselink et al., 1996).

No conjunto, os estudos efectuados sugerem que as misturas de PCBs podem alterar o funcionamento do eixo da tiróide. Um dos mecanismos de interferência envolve a ligação dos PCBs ou dos seus metabolitos hidroxilados, às proteínas de transporte de HT plasmáticas (Van Den Berg, 1990, Lans et al., 1993, Lans et al., 1994, Cheek et al., 1999, Chauhan et al., 2000) e ao aumento do metabolismo e excreção das HT, com consequente diminuição dos níveis de T4 circulantes (Mcclain, 1989). Independentemente da sua influência no metabolismo das HT, os PCBs podem causar lesões da ultra-estrutura das células foliculares da tiróide (Kasza et al., 1978, Byrne et al., 1987).

Alguns dos congéneres de PCBs são metabolizados no fígado, por hidroxilação aromática, pelas monoxigenases do citocromo P450, formando PCBs hidroxilados (PCBs-OH). Nos roedores, alguns dos PCBs-OH podem competir com a T4 para a ligação à proteína plasmática, transtirretina (TTR) (Brouwer, 1991, Brouwer et al., 1998). Hood e Klaassen (2000) relataram alterações na actividade das desiodases em tecidos específicos de ratos tratados com Aroclor 1254.

Acção do Endosulfan

Tanto exposições curtas como prolongadas a pesticidas organoclorados em doses elevadas podem alterar a actividade da tiróide dos peixes, interferindo na síntese de HT.

Tilápias (*Oreochromis mossambicus*) expostas durante 20 dias a 1 $\mu g.l^{-1}$ de endosulfan apresentaram hipertrofia e hiperplasia dos folículos da tiróide, assim como degeneração geral do tecido da tiróide (Bhattacharya, 1995). No peixe gato (*Clarias batrachus*) exposto a 8 ou a 15 $\mu g.l^{-1}$ durante 4 ou 16 dias ocorreu um aumento dos níveis de T4 e uma diminuição dos níveis de T3. Estes efeitos foram relacionados com

um aumento das enzimas produtoras de T4, uma diminuição da eliminação de T4 (Sinha et al., 1991a, Sinha et al., 1991b) e uma diminuição da conversão de T4 em T3 nos tecidos periféricos (Sinha et al., 1992).

PCBs E REPRODUÇÃO

A exposição a PCBs está relacionada com numerosas alterações da reprodução em peixes e disrupção do sistema endócrino regulador da reprodução. Assim, os PCBs podem alterar os tecidos das gónadas e interferir com a oogénese e espermatogénese reduzindo o sucesso reprodutivo ou podem alterar o desenvolvimento das larvas, alterando a capacidade de sobrevivência dos indivíduos (Orn et al., 1998).

Na corvina, *Micropogonias undulatus*, a exposição através da dieta a Aroclor 1254 (1 $mg.kg^{-1}$ de peso) durante 30 dias interfere com a capacidade reprodutiva desta espécie. Os mesmos estudos mostraram que esta mistura de PCBs é capaz de inibir a produção de serotonina por inibição da actividade da triptofano hidroxilase, inibindo a estimulação da serotonina sobre os neurónios GnRH e consequente diminuição da secreção e libertação da LH. O Aroclor 1254 diminui também a quantidade de receptores da GnRH na hipófise, diminuindo os efeitos desta hormona sob a estimulação da LH e o crescimento das gónadas nesta espécie de peixe (Khan e Thomas, 2000, Khan e Thomas, 2001, Khan et al., 2001). Ensaios *in vitro* mostraram que o Aroclor 1242 e o 1254 alteram o funcionamento das sinapses, modificando o "uptake" de dopamina, glutamato, GABA e serotonina (Mariussen e Fonnum, 2001, Bemis e Seegal, 2004) e, segundo Hany et al. (1999), os PCBs podem também alterar a actividade da aromatase cerebral.

Estudos realizados em ratos (*Rattus norvegus*) adultos mostraram que ao fim de 30 dias de injecções de Aroclor 1254 os níveis plasmáticos de ambas as GTH diminuiram (Muthuvel et al., 2006), assim como os níveis plasmáticos de estradiol e testosterona e dos receptores de LH nas células de Leydig (Murugesan et al., 2005a, Murugesan et al., 2005b). A diminuição das hormonas esteróides deve-se em parte à diminuição da actividade das enzimas esteroidogénicas, nomeadamente das hidroxilases (Murugesan et al., 2005a, Murugesan et al., 2005b). Nas fêmeas, os PCBs originam um aumento ou uma diminuição do peso do útero, alteram a libertação das GTH (Jansen et al., 1993, Khan e Thomas, 1997) e alteram o comportamento sexual (Chung e Clemens, 1999). Nos machos, os PCBs alteram o peso dos testículos, diminuem os níveis plasmáticos de testosterona (Sager, 1983, Hany et al., 1999) e reduzem a capacidade e o sucesso reprodutivos.

Em ratos Sprague Dawley, expostos a Aroclor 1242 durante o período de aleitamento, foi observado, na fase adulta, um aumento do peso dos testículos, atrofia e hiperplasia das células de Leydig e uma diminuição dos níveis de LH e de testosterona (Kim et al., 2001).

Outros estudos mostraram ainda, que a exposição ao Aroclor 1248 em ratos inibe a produção de androgénios pelo testículo (Andric et al., 2000). Para além disso, em mamíferos e aves expostas a Aroclor 1242 (Ahmad et al., 2003) ou 1254 (Alston et al., 2003, Zhang e Qiao, 2004) foram observadas lesões do tecido do testículo. Em primatas expostos a Aroclor 1248 foram observadas alterações dos ciclos menstruais, aumento do número de abortos e de reabsorção de embriões (Barsotti et al., 1976).

OBJECTIVOS



A presença de compostos policlorados, POPs ou não, no meio aquático é um facto que tem gerado uma grande preocupação a nível mundial. Os estudos efectuados centram-se principalmente na acção dos POPs, sendo os compostos policlorados não pertencentes a este grupo afastados para segundo plano. Esta preferência de objecto de estudo deve-se às propriedades, já anteriormente descritas, dos POPs: persistência, dispersão, bioacumulação e capacidade de actuarem como disruptores endócrinos. Os outros compostos policlorados, apesar de não possuírem todas estas características, são contudo também causadores de efeitos deletérios sobre os organismos.

Actualmente, os compostos policlorados encontram-se dispersos pelas águas de todo o planeta e os organismos aquáticos estão expostos a este tipo de contaminação, quer pela água, quer pela alimentação. Assim, estes compostos policlorados podem exercer a sua acção ao nível dos eixos que regulam o desenvolvimento e a reprodução, induzindo nos organismos expostos alterações destes mecanismos fisiológicos.

Como modelo biológico foi escolhida a tilápia do Nilo, *Oreochromis niloticus*, que para além das características que a tornam uma das espécies de peixe preferenciais em estudos laboratoriais, tem, actualmente, uma vasta dispersão geográfica e uma significante e crescente importância como fonte proteíca a nível mundial.

De forma a avaliar o impacto da exposição através da alimentação a compostos policlorados nos eixos hipotálamo-hipófise-rim, hipotálamo-hipófise-tiróide e hipotálamo--hipófise-gónada foram escolhidos dois compostos, o Endosulfan e o Aroclor 1254. O Endosulfan é o insecticida organoclorado mais usado em Portugal e não pertence à lista dos POPs, enquanto o Aroclor 1254 é uma mistura de PCBs, compostos que pertencem à lista dos POPs.

Os efeitos da exposição ao Endosulfan no eixo hipotálamo-hipófise-rim foram um dos objectivos deste trabalho e foram determinados através do estudo dos efeitos na síntese de cortisol e da actividade da ATPase Na^+,K^+ do rim e da brânquia (Capítulo 1).

Outro dos objectivos deste trabalho foi estudar alguns dos efeitos da exposição ao Endosulfan e ao Aroclor 1254 no eixo hipotálamo-hipófise-tiróide da tilápia do Nilo. O estudo dos efeitos destes compostos foi efectuado a vários níveis do eixo: ao nível da síntese de T4; ao nível do transporte pelas proteínas plasmáticas de transporte das hormonas da tiróide e ao nível do metabolismo periférico das HT pelas desiodases (Capítulos 1, 2, 3 e 4).

Os efeitos no desenvolvimento dos descendentes de tilápias expostas ao Aroclor 1254 foi inicialmente um dos objectivos deste trabalho, que teve de ser alterado, uma vez que se verificou que tilápias adultas expostas a este composto tornaram-se incapazes de se reproduzir. Assim, em alternativa, foram avaliados os efeitos da exposição a este

composto no desenvolvimento das gónadas de tilápias expostas durante a fase adulta e durante a fase larvar (Capítulos 5 e 6).

CAPÍTULO 1

COIMBRA AM[a], ALMEIDA M[a]R, SILVA L, REIS-HENRIQUES M[a]A. Some effects of Endosulfan on Nile tilapia (*Oreochromis niloticus*). *Proceedings of the 21[st] Conference of European Comparative Endocrinologists* (Monduzzi Editore):75-79.



Some Effects of Endosulfan on Nile Tilapia (*Oreochromis niloticus*)

A.M[a] Coimbra[1,3], M[a]R. Almeida[2,3], L. Silva[3] and M[a]A. Reis-Henriques[1,3]

[1]*Center for Marine and Environmental Research CIIMAR, Porto, Portugal*
[2]*Institute for Molecular and Cell Biology IBMC, Porto, Portugal*
[3]*Institute of Biomedical Sciences Abel Salazar ICBAS, Porto University, Portugal*

Summary

Endosulfan is a chlorinated cyclodiene insecticide, toxic for aquatic life. In order to evaluate some of its effects tilapias were fed a diet containing endosulfan (1 μg g^{-1}food) during three weeks. Total T3 plasma levels were evaluated and we found that exposed animals (1.99±0.05 ng ml^{-1}) presented lower levels than control (2.25±0.19 ng ml^{-1}). *In vitro* assays of plasma incubated with labelled hormone revealed a different TH binding protein pattern for the exposed animals. Endosulfan exposure leads to a decrease on plasma cortisol, from 235.94±56.98 ng ml^{-1} on control to 44.45±3.56 ng ml^{-1}on treated animals, that may explain the decline found on both gill and kidney Na^+,K^+-ATPase activity. The liver cytochrome P450 activity showed an increase on exposed tilapias.

Introduction

Many chemicals produced by the industry and used in agriculture become available to aquatic species. Polychlorinated compounds (PC) are among these and cause a wide range of effects on physiological mechanisms as development, reproduction, metabolism and stress response.

Thyroid hormones (TH) are essential to a normal development. In fish blood, there are several TH binding proteins, including transthyretin (TTR) that has been recently identified in fish (review in Power *et al*, 2000). It is known that some PC bind to human TTR, inhibiting TH binding and transport (Lans *et al*, 1994).

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Endosulfan interferes with osmoregulation, because it inhibits cortisol secretion by head kidney cells (Leblond, Bisson and Hontela, 2001) and depresses the Na^+,K^+-ATPase activity (Sharma, 1988).

These pollutants also induce the liver cythocrome P450 monooxygenase enzymatic system, responsible for their detoxification metabolism.

In our work we evaluated effects of endosulfan on cortisol and total T3 plasma levels. We analysed TH binding to plasma proteins *in vitro*. Endosulfan effects on gill and kidney Na^+,K^+-ATPase and P450 activities were also studied.

Materials and Methods

A group of tilapias were fed during 3 weeks a diet containing endosulfan (1 $\mu g\ g^{-1}$food). After this time endosulfan fed and control fish were anaesthetised, blood was collected from the caudal vein and plasma was stored at –80 °C. Liver, kidney and gills were removed and stored until use at –80 °C. Total T3 and cortisol plasma levels were measured by radio immuno assay (RIA) according to the kits manufacturer instructions (Kit Count-a-Count, DPC). Gill and kidney Na^+, K^+-ATPase activity was determined as described by Reis-Henriques *et al*, 1996. The P450 EROD activity was measured by the method employed by Fent and Bucheli (1994). TH binding proteins were analysed by polyacrilamide gel electrophoresis (PAGE) after incubation of plasma with the labelled hormones, ^{125}I-T4 (1300 $\mu Ci\ \mu g^{-1}$) or ^{125}I-T3 (1200 $\mu Ci\ \mu g^{-1}$) (Dupont-NEN, Boston, Ma) (Saraiva *et al*, 1988). T4 binding competition assays, based on a gel filtration procedure (Lans *et al*, 1994), were carried out on diluted plasma using T4, T3 and endosulfan as competitors. One-way analysis of variance was used to compare results.

Results

During the time of exposure neither mortality nor feeding behaviour alterations occurred.

In the TH binding assays plasmas incubated with labelled hormone were analysed by PAGE. The results of the gel autoradiography are presented in fig.1.

Concerning T4, a different protein pattern was evident for the plasma of exposed tilapias where a band with a cathodic migration in relation to albumin was detected.

Although, tilapia plasmas incubated with labelled T4 in the presence of endosulfan showed no significant differences, a very faint band migrating in front of albumin seems to be present in male tilapia plasma. A similar but less pronounced result was obtained for ^{125}I-T3 binding (data not presented).

Competition studies with diluted whole human and tilapia plasmas

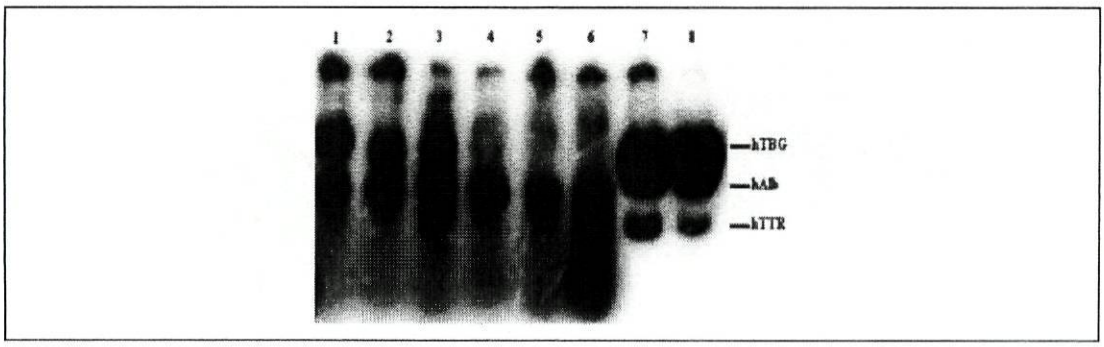


Fig. 1 ^{125}I-T4 incubated plasmas analysed by native PAGE. 1-female tilapia exposed to endosulfan, 2-male tilapia exposed to endosulfan, 3-female tilapia, 4-male tilapia, 5-female tilapia incubated with endosulfan, 6- male tilapia incubated with endosulfan, 7-human incubated with endosulfan, 8-human. hTGB-human thyroxine binding protein, hAlb-human albumin, hTTR-human transthyretin.

indicate that endosulfan does not compete with T4 for the binding to human TTR and presumably to tilapia TTR (fig 2).

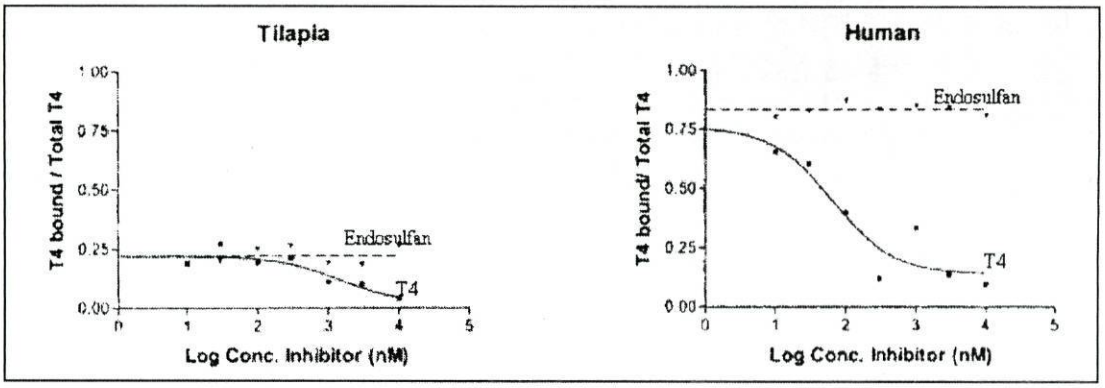


Fig. 2 Competition with T4 for the binding to plasma proteins. Tilapia plasma was diluted 10 times and human plasma 40 times. (-) T4 (--) Endosulfan.

Endosulfan fed tilapias showed a decrease on plasma total T3 and cortisol when compared to control (Table I).

*Table I: Total T3 and cortisol plasma levels. Values presented as Mean±SE. Statically differences: *p<0.05, between control and endosulfan treatment.*

	Control	Endosulfan
Total T3 (ng ml^{-1})	2.25 ± 0.19	1.99 ± 0.05
Cortisol (ng ml^{-1})	235.94 ± 56.98	44.45 ± 3.56 *

Regarding endosulfan effects on enzymatic systems, both gill and kidney ATPase activities decreased, being this decrease 60% on gill and 47% on kidney. On another hand, endosulfan exposed tilapias showed an increase on P450 EROD activity, when compared to control fish (Table II).

*Table II: Kidney and gill ATPase activities and liver P450 EROD activity. Values presented as Mean±SE. Statically differences: *p<0.05, between control and endosulfan treatment.*

	Control	Endosulfan
Kidney ATPase (μmolPi mg protein^{-1} h^{-1})	23.96±2.21	12,62±0.58 *
Gill ATPase (μmolPi mg protein^{-1} h^{-1})	4.55 ± 0.58	1.81 ± 0.18 *
EROD (nmol min^{-1}mg protein^{-1})	35.18 ± 3.79	41.52 ± 1.31

Conclusions

Total T3 plasma levels decreaseed with endosulfan exposure. *In vitro* assays showed that plasma from exposed tilapias presented a different TH binding protein pattern when compared to control. In the same study tilapia plasma incubated with endosulfan showed some T4 displacement from other plasma binding proteins to a protein migrating similar to human TTR. All together these results indicate that endosulfan interacts differently with the diverse binding proteins originating unlike TH distribution among them. More studies are underway to further confirm these preliminary results.

Endosulfan fed tilapias presented a decrease on cortisol plasma levels and a resulting decrease of both gill and kidney Na^+, K^+-ATPase activity, showing that *in vivo*, endosulfan inhibits the secretion of this hormone and interferes with osmoregulation mechanisms.

The increase on the P450 EROD activity on exposed tilapias was expected, since it is widely known that this system is involved in the PC detoxification metabolism.

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References

Fent, K and Bucheli, TD. Inhibition of hepatic microsomal monooxygenase system by organotins in vitro in freshwater fish. Aquat Toxicol, 28, 107-126, 1994.

Lans, MC, Spiertz, C, Brouwer, A and Koeman, JH. Different competition of thyroxine binding to transthyretin and thyroxine-binding globulin by hydroxy-PCBs, PCDDs and PCDFs. Eur J Pharmacol, 270, 129-136, 1994.

Leblond; VS, Bisson, M and Hontela,A. Inhibition of cortisol secretion in dispersed head kidney cells of rainbow trout (Oncorhynchus mykiss) by endosulfan, an organochlorine pesticide. Gen Comp Endocrinol, 121, 48-56, 2001.

Power, DM, Elias, NP, Richardson, SJ, Mendes, J, Soares, CM and Santos, CRA. Evolution of the thyroid hormone-binding protein, tranthyretin. Gen Comp Endocrinol, 119, 241-255, 2000.

Saraiva, MJM, Costa, PP and Goodman DS. Transthyretin (prealbumin) in

familial amyloidotic polineuropathy: genetic and functional aspects. New York. DiDonato, S Manoli, DiMauro S and Rowland LP Eds. 1988.

Reis-Henriques, MA, Silva, L and Coimbra J. Gill Na+-K+ATPase, carbonic anhydrase activities and plasma osmotic and ionic variations during smoltification of Atlantic salmon in the north of Portugal. Aquaculture International, 4, 117-128, 1996.

Sharma, RM. Effect of endosulfan on adenosine triphosphatase (ATPase) activity in liver, kidney and muscle of *Channa gachua*. Bull Environ Contam Toxicol, 41, 317-323, 1988.

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CAPÍTULO 2

COIMBRA AM[a], Figueiredo-Fernandes A, REIS-HENRIQUES M[a]A. Nile tilapia (*Oreochromis niloticus*) thyroid hormones, liver morphology and CYP1A activity after endosulfan dietary exposure. (Submitted to: *Pesticides Biochemistry and Physiology*).



Nile tilapia (*Oreochromis niloticus*), liver morphology, CYP1A activity and thyroid hormones after Endosulfan dietary exposure

Ana Maria Coimbra[1,2], António Figueiredo-Fernandes[3,4], Maria Armanda Reis-Henriques[1,2]

[1]ICBAS-Instituto de Ciências Biomédicas Abel Salazar, Largo do Prof. Abel Salazar nº2, 4099-003, Porto, Portugal

[2]CIIMAR-Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas 289, 4050-123, Porto, Portugal

[3]UTAD-Universidade de Trás-os-Montes e Alto Douro, Apartado 1013, 5000-911 Vila Real, Portugal

[4]CETAV-Centro de Estudos Tecnológicos, do Ambiente e da Vida, Apartado 1013, 5000-911 Vila Real, Portugal

Correspondence to Ana Maria Coimbra

Present address: CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas 289, 4050-123, Porto, Portugal

Tel: +351 22 340 18 00

Fax: +351 22 339 06 08

E-mail: anacoimbra@ciimar.up.pt

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Abstract

Endosulfan is an insecticide used worldwide that can be found in aquatic ecosystems and is known to cause fish mortality.

Most studies regarding Endosulfan choose the water as exposure route for aquatic life, as this pollutant can also be found in aquatic organisms tissues. In this study, Nile tilapia were exposed to Endosulfan through the diet.

Initially, tilapia were exposed to 1 and 0.5 $\mu g.g^{-1}$ of Endosulfan per gram of food for 21 days. Qualitative histological liver analysis showed that Endosulfan exposure induced hepatic damages such as hepatocyte destruction, vessel endothelium rupture and increased melanomacrophages aggregates. Therefore and in order to reduce the hepatic damage, a second exposure was performed using lower Endosulfan doses, 0.1 $\mu g.g^{-1}$ and 0.001 $\mu g.g^{-1}$, though the exposure period was enlarged to 35 days. In this case the hepatic tissue did not present the damages previously observed, although increased hepatocyte vacuolization and increased eosinophil granular cell aggregates were present in Endosulfan exposed tilapia.

Liver CYP1A metabolic activity was evaluated by determining the EROD activity. This enzyme activity was significantly enhanced in tilapia exposed to the lowest concentration of Endosulfan (0.001 $\mu g.g^{-1}$).

Also, thyroid hormones (T4 and T3) plasma concentrations were determined by RIA. Significant Endosulfan induced changes were only observed for T4 plasma concentration on tilapia fed with the 0.1 $\mu g.g^{-1}$ Endosulfan dose for 35 days.

The results showed that the Endosulfan doses used were not lethal for tilapia, though the higher doses had induced liver damages and enabled CYP1A EROD activity.

Introduction

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzo--o-dioxa-thiepin-3-oxide) is a chlorinated cyclodiene insecticide used worldwide in a large variety of crops and as a wood preservative [1].

Agriculture runoff, irrigation waters and wetland applications are the major sources of this contaminant to the aquatic environment and responsible for fish mortality [1, 2]. Endosulfan is highly toxic to fish, presenting a 96h LC50 of 1.5 $\mu g.l^{-1}$ for *Ictalurus punctatus* and *Pimephales promelas* and of 1.2 $\mu g.l^{-1}$ for *Lepomis macrochirus* [3]. Due to its use this insecticide is disseminated through all continents [2, 4-7]. It can also be found in tissues, as for example was observed by Henry and Kishimba [8] in *Oreochromis niloticus*, Nile tilapia, and *Lates niloticus* from the Lake Victoria where total Endosulfan levels ranged between 0.02 and 0.2 $mg.kg^{-1}$ of fresh weight. Thus, the presence of Endosulfan in the water poses a threat to aquatic organisms being fish species highly sensitive to the presence of this insecticide [1].

Agriculture runoff is the major source of aquatic systems contamination by this insecticide. Studies regarding its effects in fish usually choose water as the exposure route, which is known to cause tissue damage, as was shown in *Gambusia affinis,* exposed to 1.0, 2.5 and 5.0 $\mu g.L^{-1}$ of Thiodan (33.7 % Endosulfan) for 7, 14, 21 and 30 days [9]. Observations detected liver hypertrophy, dilatation of sinusoids and hepatocytes vacuolization and gut epithelium degeneration after 7 days of exposure to the lower concentration, being the damages progressively higher; the fish that were exposed to the highest concentration only survived 14 days [9]. Also, *Oncorhynchus mykiss* exposed to two Endosulfan concentrations, 2.6 and 23.7 $\mu g.L^{-1}$, for 96 h presented gill lamellar edema, liver edema and necroses, and spleen and kidney necroses [10].

However, Endosulfan can be found in tissues of aquatic organisms and the diet can be a secondary Endosulfan source, especially for animals at the top of the food web. To our knowledge only one work was performed in fish exposed to Endosulfan using the diet as exposure rout. In that work Endosulfan was capable to alter the liver ultrastructure of *Cyprinus carpio*, which were exposed for 5 weeks to 0.5 µg of Endosulfan per kg of food; these fish presented larger nucleolus, increased number and size of Golgi fields and endoplasmatic reticulum, proliferation of lysosomes and peroxisomes, which indicated a general stimulation of the hepatic metabolism [9, 11].

Ethoxiresorufin-o-deetilase (EROD) activity, an indicator of the induction of cytochrome P4501A (CYP1A) monooxygenases, has been widely used as a biomarker of exposure to halogenated compounds [12].

Thyroid hormones, tiroxine (T4) and 3,3',5-triiodothyronine (T3) are involved in several physiological functions, such as development, reproduction and growth [13-16]. Short or

extended exposure to chlorinated pesticides may alter the thyroid activity, interfering with thyroid hormones synthesis; therefore, disruption of the thyroid axis may result in innumerous deleterious consequences [17]. Previously, we showed that Endosulfan exposure (0.1 and 0.5 $\mu g.g^{-1}$) results in deiodinase activity alterations in tilapia exposed through the diet [18].

The aims of this study were to evaluate in Nile tilapia effects of Endosulfan dietary exposure in liver morphology, in liver metabolism using CYP1A EROD activity as indicator and in circulating thyroid hormones concentration.

Materials and Methods

Adult (119.4±44.68 g of mean body weight and 18.1±2.28 cm of mean body length) Nile tilapia *O. niloticus* used in this study were obtained originally from the Aquaculture Station of the "Universidade de Trás-os-Montes e Alto Douro" and raised for one generation in the "Centro Interdisciplinar de Investigação Marinha e Ambiental".

Endosulfan (technical grade ±95%) was purchase from AGROQUISA (Lisboa, Portugal). Contaminated diets were prepared by immersion of a commercial fish diet in Ethanol-Endosulfan solutions containing the desired Endosulfan concentrations. Ethanol was evaporated under air current, before diet administration. Control tilapia, were fed with the same commercial fish diet, which was only immersed in ethanol.

Both control and Endosulfan exposed tilapia, groups of six fish, were placed in 70 L tanks with individual mechanical and biological filtration. Supplemental aeration was provided to maintain dissolved oxygen near saturation. The water source was the public water supply which was de-chlorinated before entering the tanks; an approximate 3 L renovation occurs per hour in each system. Water temperature (20 °C) and photoperiod (12 hour-light: 12 hour-dark) were maintained constant during the experimental periods.

To begin with, tilapia were fed with diets containing 0.5 or 1 $\mu g.g^{-1}$ food of Endosulfan for 21 days, but as the results observed showed that both doses induced severe hepatic destruction, a second exposure was performed where tilapia were fed with 0.001 or 0.1 $\mu g.g^{-1}$ food of Endosulfan for 35 days. Both exposures included a control group.

At the end of the experiment, both Endosulfan exposed and control tilapia were anesthetized with ethylene glycol monophenyl ether (Merck). The experiments described comply with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

Total weight and length were determined; blood was collected from the caudal vessels into heparinised tubes for further plasma analyses; and liver were collected for morphological and CYP1A EROD activity evaluation. Until being analyzed all samples were kept at -80 °C.

In order to evaluate possible effects of Endosulfan exposure on liver morphology, a liver portion was fixed on Bouin's liquor (Panreac). After tissue dehydration, it was included on paraffin and cut on 5 µm sections. Liver slides were then prepared for optic microscopy and stained with Hemathoxilin-Eosin. Qualitative slide analysis was performed.

Liver microsomes preparation for CYP1A EROD activity determination was performed by the method described by Fent and Bucheli [19] and EROD activity evaluation was done by the method described by Ferreira et al. [20], adapted for tilapia. EROD activity was using 100 µl of microsomal extract and fluorescence was determined every 10 sec for 6 min

(excitation wavelength-530 nm, emission wavelenght-585 nm). A calibration curve was performed using resorufin sodium salt (Sigma) standards.

Protein concentration was determined by the method of Lowry adapted to microplates and using bovine serum albumin (BSA) standards.

Total T3 and total T4 plasma concentrations were determined by radio immuno assay (RIA) using commercially available kits (Kit Count-a-Count, DPC) and according to manufactures instructions. Both kits use antibodies with high specificity (100 % of reactivity) to the correspondent hormone (T3 or T4) and a reduce reactivity to other thyroid hormones and their metabolites. Cross-reactivity of each kit is very low; the T3 kit presents a reactivity of 0.05% with T4, while the T4 kit presents 2% of reactivity with T3. Inter-assay variation was avoided by measuring all samples in the same assay. All samples were analyzed in duplicate.

After testing ANOVA assumptions, statistical analysis was performed by one-way analysis of variance (ANOVA), followed by the multiple comparison LSD test ($p<0.05$) (STATISTICA 6.0).

Results

Neither tilapia mortality nor feeding behavior alterations were observed in Endosulfan exposed groups and in control groups.

The qualitative analysis of the liver slides showed that, at the end of 21 days of exposure to 1 $\mu g.g^{-1}$ of Endosulfan, tilapia presented severe hepatic lesions (Fig 1: C and D). Examples include: hepatocyte damage, in particular near pancreatic areas, blood vessels endothelial rupture and an increase on melanomacrophage aggregates (circled areas), both on the hepatic parenchyma and on pancreatic areas; these alterations were also observed at lower extent in tilapia exposed to 0.5 $\mu g.g^{-1}$ of Endosulfan.

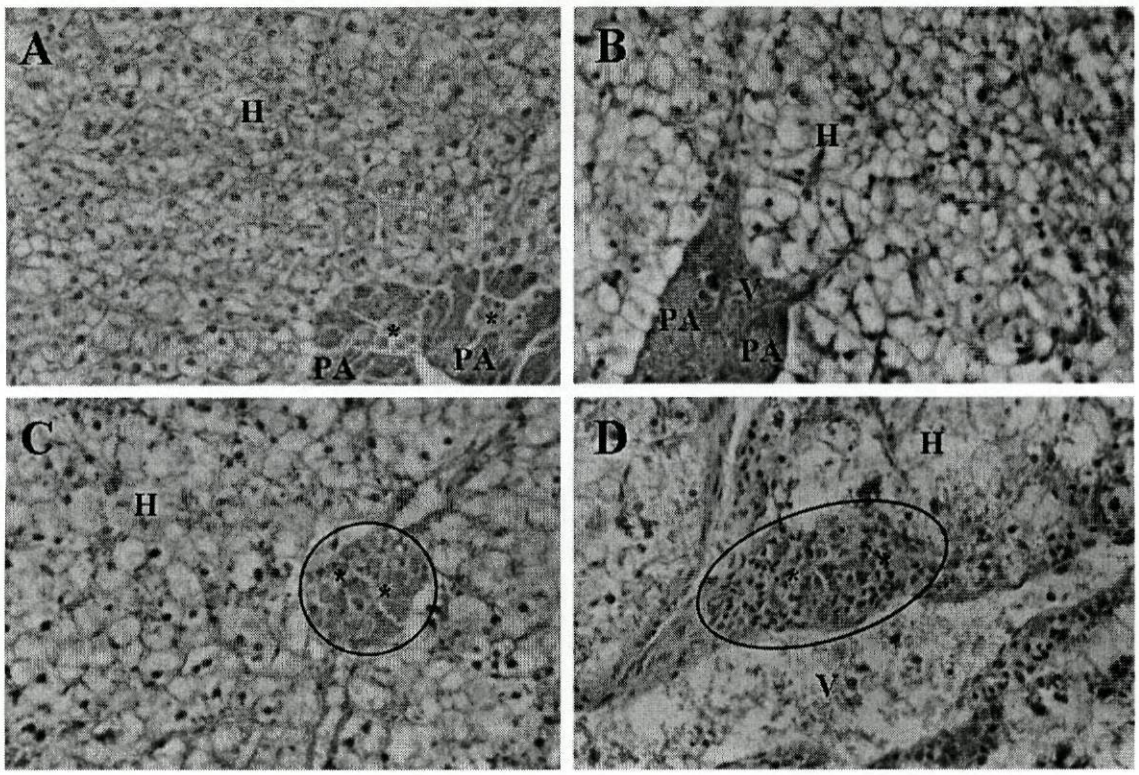


Fig. 1: Histological tilapia liver sections (X 400). A and B-Control tilapia liver sections; C and D-1 $\mu g.g^{-1}$ of Endosulfan exposed tilapia for 21 days. H -Hepatocytes; PA –Pancreatic area; V -vein; * melanomacrophages.

Additionally after 35 days of Endosulfan exposure, liver histological analysis showed that tilapia from the group exposed to 0.1 $\mu g.g^{-1}$ of Endosulfan (Fig 2: A and B) presented an increase on hepatocytes vacuolization and an increment in the number of eosinophil granular cells, both among hepatocytes and near blood vessels. Likewise, tilapia exposed to 0.001 $\mu g.g^{-1}$ (Fig 2: C and D) presented similar alterations.

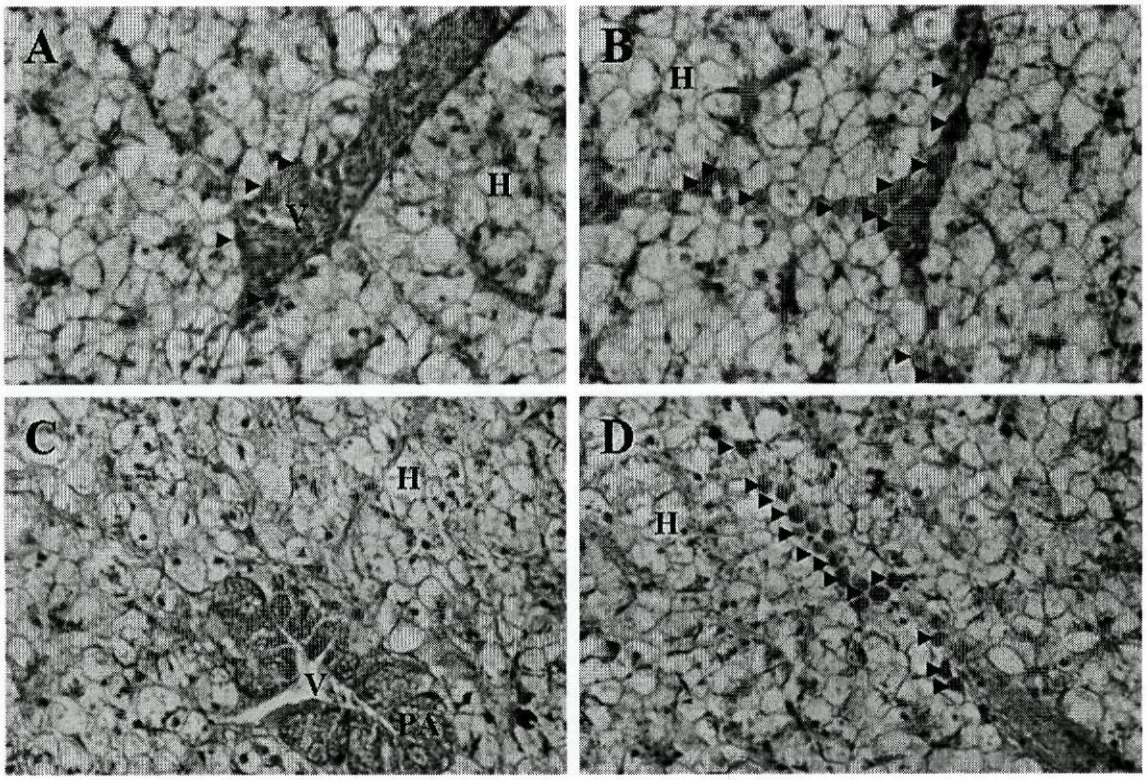


Fig. 2: Histological tilapia liver sections (X 400). A and B-0.1 $\mu g.g^{-1}$ of Endosulfan exposed tilapia for 35 days; C and D-0.001 $\mu g.g^{-1}$ of Endosulfan exposed tilapia for 35 days. H -Hepatocytes; PA -Pancreatic area; V –vein; ►eosinophilic granular cells.

Regarding liver CYP1A EROD activity, at the end of 21 days of exposure, it was found not to be significantly affected by both Endosulfan doses used 1 and 0.5 $\mu g.g^{-1}$; while, liver CYP1A EROD activity, at the end of 35 days, was found to be superior in tilapia exposed to Endosulfan, but only significantly in the group exposed to 0.001 $\mu g.g^{-1}$ ($p<0.05$) (Table 1).

Table 1: Liver CYP1A EROD activity variation percentage in tilapia exposed to Endosulfan. Values presented as mean±SE; n=6.

Time (days)	Dose ($\mu g.g^{-1}$)	Variation (%)
21	0.5	(+) 18 ± 19.6
	1.0	(-) 25 ± 18.0
35	0.001	(+) 102 ± 30.8*
	0.1	(+) 65 ± 46.9

Note: Significant differences: *$p<0.05$; (+) increase in EROD activity; (-) decrease in EROD activity

Concerning thyroid hormone plasmatic concentrations, at the end of 21 days, both Endosulfan doses, 0.5 $\mu g.g^{-1}$ and 1 $\mu g.g^{-1}$, exposed tilapia presented amounts of total T4 and total T3 similar to the ones presented by control tilapia. Despite that fact, at the end of 35 days of exposure, tilapia exposed to 0.1 $\mu g.g^{-1}$ of Endosulfan presented a lower T4 plasma concentration than tilapia control and tilapia exposed to 0.001 $\mu g.g^{-1}$ of Endosulfan. The decrease observed on T4 plasma concentration was not followed by alterations on T3 plasma concentration (Table 2).

Table 2: Total T4 and total T3 plasma concentration (nM) in tilapia control and exposed to Endosulfan. Values presented as mean±SE; n=6.

Time (days)	Dose ($\mu g.g^{-1}$)	T4 (nM)	T3 (nM)
21	0.0	12.3 ± 1.87	2.9 ± 0.34
	0.5	11.1 ± 1.20	3.1 ± 0.87
	1.0	12.8 ± 1.38	2.8 ± 0.60
35	0.0	11.3 ± 2.25	4.5 ± 1.23
	0.001	11.9 ± 1.62	5.5 ± 1.04
	0.1	5.8 ± 0.70*	6.8 ± 1.23

Note: Significant differences: *p<0.05

Discussion

Our results showed that Endosulfan exposure through the diet was not as toxic as described for water exposures [1], but likewise induced severe damages in fish. This may be related with the organs that contact primarily with this insecticide, being in water exposures the gills and in dietary exposures the intestine and the liver, which was the evaluated organ in this study.

This compound is able to destroy fish tissues as shown in this and in previous studies [9-11]. It was observed in an anterior study [21] that exposure to this insecticide induces a decline in tilapia gills and inter-renal Na^+,K^+ ATPase activities, simultaneously with a significant depression of cortisol plasma concentration. Gills Na^+,K^+ ATPase activity depression could also result from gill destruction, mainly of chlorine cells; this was observed by Capkin et al. [10] in a study with *Oncorhynchus mykiss* where specimens exposed to Endosulfan water concentrations of 2.6 and 23.7 $\mu g.l^{-1}$ for 96 hours, presented gills with lamellar edema, separation of the epithelium from lamellae, lamellar fusion and swelling of the epithelium cells; in the same study the liver showed severe edema and necrosis and the authors concluded that mortality was likely related with the disruption of both gill and liver functions.

Tilapia liver destruction, in our study, seemed to be proportional both with the dose and time of Endosulfan exposure. Similar results have been reported for *Gambusia affinis* and *Oncorhynchus mykiss* water exposed to Endosulfan [9, 10]. Mosquitofish exposed to 1.0, 2.5 and 5.0 $\mu g.l^{-1}$ for 7, 14 and 30 days showed liver morphological alterations as cellular hypertrophy, sinusoids dilatation, cell vacuolization, disorganization of areas around blood vessels, hemorrhage, lymphocyte infiltration, cell disconnection and cellular degeneration [9]; and trout exposed to 2.6 for 24 and 96 h showed liver edema and necroses [10]. Also, Braunbeck and Appelbaum [11] showed that Endosulfan dietary exposure induces liver ultrastructural alterations; proliferation of the endoplasmic reticulum and other organelles was observed in *Cyprinus carpio* exposed for 5 weeks to a diet containing (0.5 $\mu g.kg^{-1}$) Endosulfan, which is half of the lowest dose used in our study with tilapia (0.001 $\mu g.g^{-1}$), and the authors state that these morphological changes are related with an increase in the liver metabolic function. In tilapia, a raise of 102 % in CYP1A EROD activity (6.7±1.34 $pmol.mg^{-1}protein.min^{-1}$ in control) was observed in the group exposed to the lowest Endosulfan dose (13.6±2.07 $pmol.mg^{-1}protein.min^{-1}$), which also was the group presenting less hepatic lesions, suggesting that liver metabolic pathways where induced by the presence of Endosulfan. The fact that tilapia exposed to the higher Endosulfan doses did not present CYP1A EROD activity induction, may be related with the hepatic destruction observed; others suggest that it can be related with the destruction of the

intestine walls by this contaminant, thus resulting in a diminished Endosulfan uptake [9, 11].

Liver macrophages and eosinophil granular cells are commonly seen in the major pancreatic venous complexes [22] and are known to be involved in defense mechanisms [23, 24]. In this study, the qualitative analysis of tilapia liver showed (Fig. 1 and 2) an increase in both these cells number and a migration of eosinophil granular cells from areas near blood vessels to the hepatic parenchyma, associated with hepatocytes destruction can be connected with the exposure to Endosulfan and to macrophages and eosinophil granular cells role in defense mechanisms. But while, Figueiredo-Fernandes et al. [25] showed in a study with tilapia exposed to the fungicide Mancozeb (0.5 to 5.0 $mg.L^{-1}$) for four days that this compound did not increase the macrophages and eosinophil granular cells number, Pacheco and Santos [26] observed in eel, *Anguilla anguilla*, an increase macrophage number and severe liver lesions associated with the exposure to benzo(a)pyrene, dehydroabietic acid and bleached kraft pulp mill effluent.

Tilapia exposed to 0.1 $\mu g.g^{-1}$ of Endosulfan was the only group showing a decrease on T4 plasma concentration (Table 2). This group, as all the others of tilapia exposed to Endosulfan did not show significant alterations on T3 plasma concentration. Coimbra et al. [18] showed in a previous study where tilapia were exposed to 0.1 and 0.5 $\mu g.g^{-1}$ of Endosulfan, that this compound interfered with liver deiodinase type 1 and type 3 activity, though kidney type 1 and liver type 2 deiodinase activity, the ones responsible for the maintenance of T3 plasma concentration [27, 28], were not affected. In the present study, the higher doses (1 $\mu g.g^{-1}$ and 0.5 $\mu g.l^{-1}$) did not induce alterations in the concentration of thyroid hormones, which probably result from an Endosulfan decreased uptake due to intestine epithelium destruction as was suggested by Cengiz et al. [9]. Previously, we raised some hypothesis to explain the decrease on T4 plasma concentration without significant alterations on T3 plasma concentration [18]: i) Endosulfan acts at central level inhibiting the synthesis or release of T4 by the thyroid gland; ii) Endosulfan could compete with T4 for the binding to thyroid hormones binding plasma proteins increasing this hormone clearance. Thus, Endosulfan seems to be responsible for a depression in T4 secretion or for an increase in this hormone clearance; therefore further studies regarding thyroid stimulating hormone levels and T4 secretion by the thyroid gland are necessary to identify Endosulfan targets responsible for T4 decrease and competition with thyroid hormones for the binding to tilapia plasma transport proteins should be evaluated.

Although Endosulfan exposure did not result in tilapia mortality, the observations made suggest that if higher concentrations had been used or if longer exposure periods had been assayed, liver alterations would be more severe and possibly would result in metabolic failure and consequently death.

In conclusion, although the Endosulfan doses used were sublethal for tilapia, all of concentrations tested induced liver morphological alterations which rose in severity with the increase of the concentration used. This insecticide induces liver metabolic pathways when used in low doses as shown in this study and by Braunbeck and Appelbraun [11] in carp; nevertheless, due to the liver destruction originated by the higher doses, CYP1A EROD activity is not significantly different from control animals and even a decline, not significant ($p>0.05$), in this enzyme activity occurred when tilapia were exposed to 1 $\mu g.g^{-1}$. Endosulfan dietary exposure apparently is less toxic than water exposure; this is not necessarily truth, since the results presented suggest that Endosulfan may only take longer periods to act when entering via alimentary tract. Also, this seems to be related with the tissues that contact with this compound in each exposure route, since gill destruction by water exposure will induce respiratory failure more rapidly than dietary exposure induces liver metabolic failure, resulting in earlier mortality. In addition, the dose of 0.1 $\mu g.g^{-1}$ was the only to decreased T4 plasma concentration, though T3 plasma concentration was not affected by any dose, suggesting the existence of compensatory mechanisms that maintain the active hormone concentration within physiological normal levels.

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References

[1] S.M. Naqvi, C. Vaishnavi, Bioaccumulative Potential and Toxicity of Endosulfan Insecticide to Nontarget Animals, Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology, 105 (1993) 347-361.

[2] G.I. Scott, M.H. Fulton, D.W. Moore, E.F. Wirth, G.T. Chandler, P.B. Key, J.W. Daugomah, E.D. Strozier, J. Devane, J.R. Clark, M.A. Lewis, D.B. Finley, W. Ellenberg, K.J. Karnaky, Assessment of Risk Reduction Strategies for the Management of Agricultural Nonpoint Source Pesticide Runoff in Estuarine Ecosystems, Toxicology and Industrial Health, 15 (1999) 200-213.

[3] W. Johnson, M. Finley, Handbook of Acute Toxicity of Chemicals to Fish and Aquatic Invertebrates. ed. by United States Departement of the Interior Fish and Wildlife Service. Vol. 137, Resource Publication Washington DC, (1980), pp. 1-98.

[4] M.J. Cerejeira, P. Viana, S. Batista, T. Pereira, E. Silva, M.J. Valerio, A. Silva, M. Ferreira, A.M. Silva-Fernandes, Pesticides in Portuguese Surface and Ground Waters, Water Research, 37 (2003) 1055-1063.

[5] S. El-Kabbany, M.M. Rashed, M.A. Zayed, Monitoring of the Pesticide Levels in Some Water Supplies and Agricultural Land, in El-Haram, Giza (Are), Journal of Hazardous Materials, 72 (2000) 11-21.

[6] A.W. Leonard, R.V. Hyne, R.P. Lim, F. Pablo, P.J. Van Den Brink, Riverine Endosulfan Concentrations in the Namoi River, Australia: Link to Cotton Field Runoff and Macroinvertebrate Population Densities, Environmental Toxicology and Chemistry, 19 (2000) 1540-1551.

[7] Z.L. Zhang, J. Huang, G. Yu, H.S. Hong, Occurrence of Pahs, Pcbs and Organochlorine Pesticides in the Tonghui River of Beijing, China, Environmental Pollution, 130 (2004) 249-261.

[8] L. Henry, M.A. Kishimba, Pesticide Residues in Nile Tilapia (Oreochromis Niloticus) and Nile Perch (Lates Niloticus) from Southern Lake Victoria, Tanzania, Environmental Pollution, 140 (2006) 348-354.

[9] E.I. Cengiz, E. Unlu, K. Balci, The Histopathological Effects of Thiodan (R) on the Liver and Gut of Mosquitofish, Gambusia Affinis, Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes, 36 (2001) 75-85.

[10] E. Capkin, I. Altinok, S. Karahan, Water Quality and Fish Size Affect Toxicity of Endosulfan, an Organochlorine Pesticide, to Rainbow Trout, Chemosphere, 64 (2006) 1793-1800.

[11] T. Braunbeck, S. Appelbaum, Ultrastructural Alterations in the Liver and Intestine of Carp Cyprinus Carpio Induced Orally by Ultra-Low Doses of Endosulfan, Diseases of Aquatic Organisms, 36 (1999) 183-200.

[12] J.J. Whyte, R.E. Jung, C.J. Schmitt, D.E. Tillitt, Ethoxyresorufin-O-Deethylase (Erod) Activity in Fish as a Biomarker of Chemical Exposure, Critical Reviews in Toxicology, 30 (2000) 347-570.

[13] A. Biswas, S. Kundu, S. Roy, J. De, M. Pramanik, A.K. Ray, Thyroid Hormone Profile During Annual Reproductive Cycle of Diploid and Triploid Catfish, Heteropneustes Fossilis (Bloch), General and Comparative Endocrinology, 147 (2006) 126-132.

[14] J.F. Leatherland, Reflections on the Thyroidology of Fishes : From Molecules to Humankind Guelph Ichthyology Reviews (1994) 1-67.

[15] I.S. Parhar, T. Soga, Y. Sakuma, Thyroid Hormone and Estrogen Regulate Brain Region-Specific Messenger Ribonucleic Acids Encoding Three Gonadotropin-Releasing Hormone Genes in Sexually Immature Male Fish, Oreochromis Niloticus, Endocrinology, 141 (2000) 1618-1626.

[16] D.M. Power, L. Llewellyn, M. Faustino, M.A. Nowell, B.T. Bjornsson, I.E. Einarsdottir, A.V.M. Canario, G.E. Sweeney, Thyroid Hormones in Growth and

Development of Fish, Comparative Biochemistry and Physiology C-Toxicology & Pharmacology, 130 (2001) 447-459.

[17] S.B. Brown, B.A. Adams, D.G. Cyr, J.G. Eales, Contaminant Effects on the Teleost Fish Thyroid, Environmental Toxicology and Chemistry, 23 (2004) 1680-1701.

[18] A.M. Coimbra, M.A. Reis-Henriques, V.A. Darras, Circulating Thyroid Hormone Levels and Iodothyronine Deiodinase Activities in Nile Tilapia (Oreochromis Niloticus) Following Dietary Exposure to Endosulfan and Aroclor 1254, Comparative Biochemistry and Physiology C-Toxicology & Pharmacology, 141 (2005) 8-14.

[19] K. Fent, T.D. Bucheli, Inhibition of Hepatic-Microsomal Monooxygenase System by Organotins in-Vitro in Fresh-Water Fish, Aquatic Toxicology, 28 (1994) 107-126.

[20] M. Ferreira, P. Antunes, O. Gil, C. Vale, M.A. Reis-Henriques, Organochlorine Contaminants in Flounder (Platichthys Flesus) and Mullet (Mugil Cephalus) from Douro Estuary, and Their Use as Sentinel Species for Environmental Monitoring, Aquatic Toxicology, 69 (2004) 347-357.

[21] A.M. Coimbra, M.R. Almeida, L. Silva, M.A. Reis-Henriques, 'Some Effects of Endosulfan on Nile Tilapia (Oreochromis Niloticus)', in Proceedings of the 21st Conference of European Comparative Endocrinologists, ed. by R. Keller, H. Dircksen, D. Sedlmeier,H. Vaudry, (2002), pp. 75-79.

[22] A. Figueiredo-Fernandes, A. Fontainhas-Fernandes, R.A.F. Monteiro, M.A. Reis-Henriques, E. Rocha, Spatial Relationships of the Intrahepatic Vascular-Biliary Tracs and Associated Pancreatic Acini of Nile Tilapia, Oreochromis Niloticus (Teleostei, Cichlidae): A Serial Sectioning Analysis Study by Light Microscopy, Annals of Anatomy (In press).

[23] T. Matsuyama, T. Iida, Degranulation of Eosinophilic Granular Cells with Possible Involvement in Neutrophil Migration to Site of Inflammation in Tilapia, Developmental & Comparative Immunology, 23 (1999) 451-457.

[24] E. Rocha, R. Monteiro, 'Histology and Cytology of Fish Liver: A Review', in Icthiology: Recent Research Advances, ed. by D.N. Saksena New Dalhi, India: Oxford & IBH Publishin Co. Pvt. Ldt. , (1999), pp. 321-344.

[25] A. Figueiredo-Fernandes, A. Fontainhas-Fernandes, R. Monteiro, M.A. Reis-Henriques, E. Rocha, Effects of the Fungicide Mancozeb on Liver Structure of Nile Tilapia, Oreochromis Niloticus: Assessment and Quantification of Induced Cytological Changes Using Qualitative Histopathology and the Stereological Point-Sampled Intercept Method, Bulletin of Environmental Contamination and Toxicology, 76 (2006) 249-255.

[26] M. Pacheco, M.A. Santos, Biotransformation, Genotoxic, and Histopathological Effects of Environmental Contaminants in European Eel (Anguilla Anguilla L.), Ecotoxicology and Environmental Safety, 53 (2002) 331-347.

[27] K. Mol, E. Kaptein, V.M. Darras, W.J. Degreef, E.R. Kuhn, T.J. Visser, Different Thyroid Hormone-Deiodinating Enzymes in Tilapia (Oreochromis-Niloticus) Liver and Kidney, Febs Letters, 321 (1993) 140-144.

[28] K.A. Mol, S. Vandergeyten, V.M. Darras, T.J. Visser, E.R. Kuhn, Characterization of Iodothyronine Outer Ring and Inner Ring Deiodinase Activities in the Blue Tilapia, Oreochromis Aureus, Endocrinology, 138 (1997) 1787-1793.

CAPÍTULO 3

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Circulating thyroid hormone levels and iodothyronine deiodinase activities in Nile tilapia (*Oreochromis niloticus*) following dietary exposure to Endosulfan and Aroclor 1254

Ana Maria Coimbra[a,b,*], Maria Armanda Reis-Henriques[a,b], Veerle M. Darras[c]

[a]*ICBAS-Instituto de Ciências Biomédicas de Abel Salazar, Laboratório de Fisiologia Aplicada, Largo do Prof. Abel Salazar 2, 4099-003, Porto, Portugal*
[b]*CIIMAR-Centro Interdisciplinar de Investigação Marinha e Ambiental, Laboratório de Toxicologia Ambiental, Rua dos Bragas 289, 4050-123, Porto, Portugal*
[c]*Laboratory of Comparative Endocrinology, Zoological Institute K.U. Leuven, Naamsestraat 61, B-3000 Leuven, Belgium*

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Abstract

We evaluated the effects of two organochlorinated environmental contaminants, Endosulfan and Aroclor 1254 on peripheral thyroid hormone metabolism and thyroid hormone plasma levels in Nile tilapia (*Oreochromis niloticus*). Tilapia were exposed through diet to 0.1 and 0.5 μg g^{-1} of Endosulfan and 0.5 μg g^{-1} of Aroclor 1254 for 21 and 35 days. Decreased plasma T4 and rT3 levels were observed in tilapia exposed to the lower dose of Endosulfan, while treatment with a higher dose and Aroclor 1254 produced no changes. Plasma T3 levels were not affected by these compounds. Hepatic type I deiodinase (D1) activity was depressed by a lower dose of Endosulfan and hepatic type III (D3) activity was increased following 35 days of exposure to the lower dose of Endosulfan and following 21 and 35 days of exposure to Aroclor 1254; while type II (D2) remained unchanged in liver as well as in all other organs analysed. Apart from hepatic D3 activity, Endosulfan and Aroclor 1254 also increased D3 activity in gill, but not in other tested organs. It is concluded that dietary exposure of tilapia to Endosulfan or Aroclor 1254 can lead to changes in circulating thyroid hormone levels and/or in peripheral thyroid hormone metabolism. The changes in hormone metabolism differ between tissues, eventually reflecting tissue-specific differences in adaptation.
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Keywords: Thyroid hormone; Deiodination; Thyroid hormone metabolism; Endosulfan; Aroclor 1254; Endocrine disruptor; Tilapia; Fish; Plasma; Tissues

1. Introduction

Thyroid hormones are fundamental for fish early development, growth and reproduction. These hormones were found to be present in eggs and embryos of a high variety of fish species (Tagawa et al., 1990) showing their importance even at these early life stages.

The main secretion product of the thyroid gland is thyroxine (T4), but the receptor-active thyroid hormone is 3,5,3′-triiodothyronine (T3). The activation to T3 is carried out almost entirely in peripheral tissues by deiodination of the outer ring (ORD) of T4. Deiodination of the inner ring (IRD) results in the production of the inactive metabolite 3,3′,5′-T3 or reverse T3 (rT3) (Visser, 1996). IRD also inactivates T3 by converting it to 3,3′-diiodothyronine (T2). Deiodination controls plasma and tissue thyroid hormone levels and thyroid hormone biological activity. The enzymes responsible for thyroid hormone deiodination are called iodothyronine-deiodinases (St. Germain and Galton, 1997).

High and low Km deiodinating activities are present in fish. In fact, deiodinases highly similar to the mammalian D1, D2 and D3 have been cloned from tilapia, killifish and the Australian lungfish (Orozco et al., 2002, 2003; Sanders et al., 1997, 1999; Sutija et al., 2003, 2004; Valverde et al.,

* Corresponding author. Laboratório de Toxicologia Ambiental, CIIMAR-Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas 289, 4050-123, Porto, Portugal. Tel.: +351 22 340 18 00; fax: +351 22 339 06 08.
E-mail address: anacoimbra@cimar.org (A.M. Coimbra).

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1997). The tissue distribution of these deiodinases, however, is somewhat different from the pattern typical for mammals. In tilapia, D1 is mainly present in kidney and to a lesser extent in the liver (Mol et al., 1997, 1999). The enzymes responsible for T4 to T3 conversion in tilapia are D2, which is abundantly expressed in liver and also present in several other tissues (Mol et al., 1993, 1997, 1999) and D1 in kidney (Byamungu et al., 1992). The highest levels of the inactivating D3 are found in the brain (Byamungu et al., 1992; Mol et al., 1993, 1997).

The presence of synthetic chemicals in the environment is currently a major problem for both human and animal health. Compounds used in industry and agriculture most of the time end up in the aquatic ecosystems. Due to their chemical properties such as lipophilicity, chemical stability and miscibility with organic compounds, some of them are still present long after their use has been forbidden or finished. Also, these compounds are known to enter the food chain and to bioaccumulate in tissues, increasing their potency to cause health damages (reviewed by Fisher, 1999). Environmental chemicals that act on endocrine systems interfering and altering their function are called endocrine disruptors. Most of the studies on endocrine disruptors focus on the action of these compounds on the reproductive system and many chemicals are already known to act as sex steroid agonists or antagonists (reviewed by Colborn, 2002). A review by Brown et al. (2004a) on the effects of several environmental contaminant classes on the thyroid axis in fish shows that much remains to be investigated to clearly understand how these compounds interfere with thyroid function.

Endosulfan is a polycyclic organochlorine insecticide used on a wide variety of crops and also as a wood preservative. Based on EPA's ecological incident information, Endosulfan poisoning is among the most reported causes of aquatic organism incidents. Although Endosulfan does not readily bioaccumulate in tissues, it has a relatively high acute toxicity in fish (Ferrando et al., 1991) and fish kills are a known consequence of its runoff into waters in agricultural areas (Naqvi and Vaishnavi, 1993). This compound was found to alter thyroid hormone levels in the freshwater catfish (*Clarias batrachus*), increasing T4 levels and decreasing T3 levels (Sinha et al., 1991a,b). Mozambique tilapia (*Oreochromis mossambicus*) exposed for 20 days to 1 μg l^{-1} of Endosulfan presented thyroid follicle hypertrophy and hyperplasia as well as general thyroidal degeneration (Bhattacharya, 1995).

Polychlorinated biphenyls (PCB) are contaminants usually present as complex mixtures of several PCB congeners. Aroclor 1254 is a commercial PCB mixture with a 54 weight percentage of chlorine. Fish thyroidal responses to PCBs vary between studies and/or species (Adams et al., 2000; review by Brown et al., 2004a). For example: Aroclor 1242 and 1254 depressed T3 levels without altering T4 plasma concentrations when fed to coho salmon (*Oncorhynchus kisutch*); while in rainbow trout (*O. mykiss*) thyroid hormone plasma levels remained unaltered when fed with Aroclor 1254, but decreased when fed with a mixture of Aroclor 1242 and 1254. Injections of Aroclor 1254 (150 μg kg^{-1}) in coho salmon led to an increase only in T3 levels, although Clophen A50 (500 mg kg^{-1}), also a PCB mixture, increased both T4 and T3 levels in flounder (*Platichthys flesus*).

In chick embryos injected with PCBs 54, 77 or 80 liver D1 activity did not change. However, when injected with the PCB mixtures Aroclor 1242 or 1254 this enzyme activity decreased or was completely suppressed while no alterations in the circulating thyroid hormone levels were observed (Gould et al., 1999). Also, in male Sprague–Dawley rats exposed to Aroclor 1254 liver D1 activity decreased while free T3 plasma levels remained unaltered and free T4 levels were decreased (Hood and Klaassen, 2000). Liver D3 activity in the American plaice (*Hippoglossoides platessoides*) was found to be increased by the exposure to PCB 126, while PCB 77 caused no alterations in this enzyme activity (Adams et al., 2000).

In the present study we investigated the effects of oral exposure to Endosulfan and Aroclor 1254 on Nile tilapia (*Oreochromis niloticus*) thyroid hormone concentration in plasma and on deiodinase activity in several organs. The results demonstrate that these compounds can interfere with peripheral thyroid hormone metabolism, especially in liver and gill, but have only minor effects on circulating thyroid hormone levels.

2. Material and methods

2.1. Animals

Adult Nile tilapia (*O. niloticus*) were maintained in freshwater tanks of 70 L with water recirculation and aeration at 21 °C, under a 12:12 h light/dark photoperiod. The average mass and length of the fish were 140.51 ± 6.57 g and 18.90 ± 0.29 cm.

Tilapia were fed once a day with a commercial fish diet, to which Endosulfan or Aroclor 1254 were added dissolved in ethanol, followed by drying at room temperature under air current. Tilapia were fed with two different doses of Endosulfan, a low (L) dose of 0.1 μg g^{-1} (μg Endosulfan g^{-1} of food) or a high (H) dose of 0.5 μg g^{-1} and with one dose of Aroclor 1254 (0.5 μg g^{-1}) during a 21- or 35-day period. Table 1 summarizes the different treatments per group. Half of the control tilapia were killed on day 21 and the other half on day 35.

At the end of the exposure period tilapia were anaesthetised with ethylene glycol monophenyl ether (Merck), blood was collected from the caudal vein and plasma was stored until use at −80 °C. All the organs collected were frozen in liquid nitrogen and stored at −80 °C until use.

Table 1
Compound, dose and exposure time description for each tilapia group

Group	Compound	Dose	Exposure time
C21	–	0.0	21
C35	–	0.0	35
EL21	Endosulfan	0.1	21
EL35	Endosulfan	0.1	35
EH21	Endosulfan	0.5	21
EH35	Endosulfan	0.5	35
A21	Aroclor 1254	0.5	21
A35	Aroclor 1254	0.5	35

Dose represented as μg of compound per g of food. Exposure time is expressed in days.

2.2. Chemicals

Endosulfan used was of technical grade (±95%) and supplied by AGROQUISA (Lisboa, Portugal). Aroclor 1254 was purchased from NSI Solutions, Inc. as a neat standard. T4 and T3 antisera and standards were purchased from BykBelga (Brussels, Belgium). Labelled T3, T4 and rT3 were prepared with the chloramine T method using ^{125}I from ICN. The specific activity was comparable to that from commercially available "high specificity" hormone preparations (100–120 MBq μg^{-1}).

2.3. Radio immunoassay (RIA)

Total plasma T4, T3 and rT3 concentrations were measured by RIA. The T4 RIA had a detection limit of 5.03 fmol, an intra-assay variability of 2.8% and an inter-assay variability of 11.0%. The T3 RIA had a detection limit of 2.01 fmol, an intra-assay variability of 2.2% and an inter-assay variability of 9.6%. T4 RIA cross-reactivity with T3 was 3.5%, while T3 RIA cross-reactivity with T4 was 0.1 to 0.5% (Van der Geyten et al., 2001). The detection limit of the rT3 RIA was 1.5 fmol using an antiserum provided by Visser et al. (1977). All samples were analysed in duplicate.

2.4. Deiodinase assays

Microsomal fractions were prepared from liver, kidney, gills and gonads. Since earlier tests had shown that preparation of microsomes from brain tissue resulted in a clear reduction in deiodinase activity, homogenates were prepared from this tissue. Homogenates were also prepared from muscle samples since microsomal protein yield from this tissue is very low. Microsomal fractions and homogenates were prepared according to the following procedures.

Microsomal fractions: 0.5–1 g of tissue was homogenized in 5 mL of buffer A (10 mM HEPES, 1 mM dithiothreitol (DTT), pH 7) for liver and 5 mL buffer A+0.25 M sucrose for the other tissues (Mol et al., 1993). After centrifugation for 20 min at 10,000×g, the supernatant was centrifuged for 1 h at 135,000×g for liver and at 100,000×g for the other tissues. The resulting pellet was resuspended in 1 mL of buffer B (0.2 M phosphate pH 7.2, 4 mM EDTA, 1 mM DTT) by use of a motorized pestle and then snap frozen in aliquots before storage at −80 °C.

Homogenates: the whole brain was directly homogenized in 1 mL buffer B while 0.25 g of muscle were homogenized in 3 mL buffer B and centrifuged at 10,000×g for 20 min. The supernatant was snap frozen and stored at −80 °C until analysis.

All procedures were carried out on ice, while centrifugations were done at 4 °C. Protein concentrations were determined with the BCA protein assay reagent (Bio-Rad) using bovine serum albumin as a standard.

Deiodinase activities were measured as described by Van der Geyten et al. (1998). D1 (rT3 ORD) activity was measured by incubating 1 μM rT3 (plus 50,000 cpm labelled rT3), 15 mM DTT with 1 and 0.5 mg mL^{-1} of liver and kidney microsomes respectively for 120 min at 37 °C. D2 (T4 ORD) activity was determined by incubation of 1 nM T4 (plus 50,000 cpm labelled T4 and 100 nM T3), 30 mM DTT with 1 mg mL^{-1} of microsomal protein from kidney, ovaries and gills, 0.5 from testes and 0.1 from liver, and 1 mg protein mL^{-1} of muscle and brain homogenates. Incubation was done at 37 °C and lasted 120 min for all organs, except for liver (60 min). D3 (T3 IRD) activity was measured by incubating 1 nM T3 (plus 200,000 cpm labelled T3 and 1 μM rT3), 30 mM DTT with 1 mg protein mL^{-1} of liver, kidney, gills and ovaries microsomes and brain and muscle homogenates and 0.5 mg mL^{-1} of testes microsomes for 120 min at 37 °C.

2.5. Statistical analysis

After testing ANOVA assumptions, statistical analysis was performed by one-way analysis of variance (ANOVA), followed by the multiple comparison Student–Newman–Keuls test ($p<0.05$). Statistical differences were evaluated between controls and exposed tilapia (C21 vs. EL21; C35 vs. EL35, C21 vs. EH21, C35 vs. EH35, C21 vs. A21 and C35 vs. A35), between control groups (C21 and C35), between different Endosulfan doses (EL21 vs. EH21 and EL35 vs. EH35) or exposure times (EL21 vs. EL35 and EH21 vs. EH35) and between different Aroclor 1254 exposure times (A21 vs. A35). Correlation coefficients were calculated using the Pearson correlation method.

3. Results

3.1. Plasma thyroid hormone levels

Different effects were found depending on the dose of pollutant used and on the duration of the exposure time (Fig. 1). In tilapia exposed to the lower dose of Endosulfan (EL21) plasma T4 levels were found to be lower than in control fish (C21). In fact, in fish exposed for 21 days (EL21) the T4 levels were one third of the control levels. The decrease in plasma T4 levels was no longer significant after 35 days of treatment (EL35). Also, for the higher dose

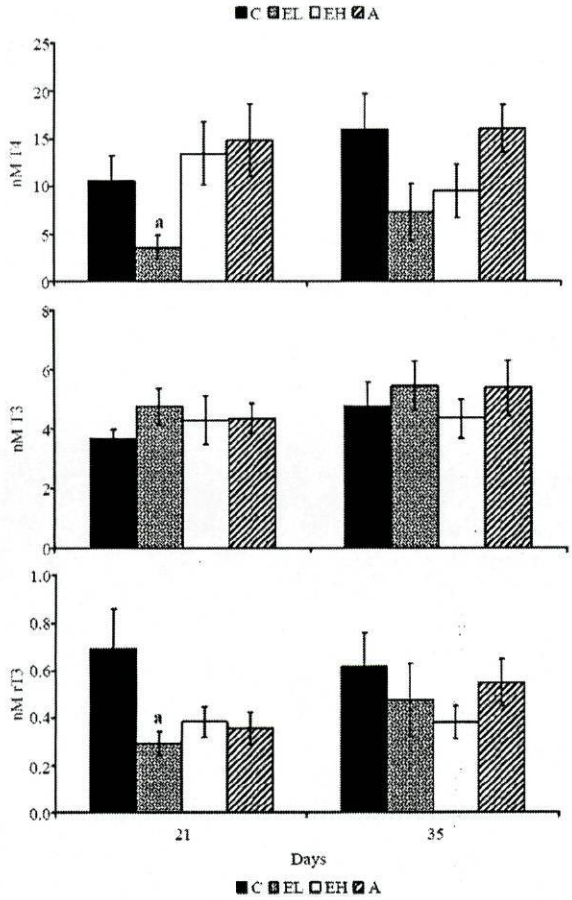


Fig. 1. Plasma total T4 (top), total T3 (middle) and rT3 (bottom) concentrations (nM) in tilapia control and exposed through the diet to two Endosulfan (E) doses, 0.1 μg g^{-1} (L) or 0.5 μg g^{-1} (H), and to one Aroclor 1254 (A) dose, 0.5 μg g^{-1}, during 21 or 35 days. Values are represented as mean ± SEM, $n=6-8$. Significant differences obtained by ANOVA followed by Student–Newman–Keuls test are indicated as follows: between control and treated tilapia with the same exposure time—$^{a}p<0.05$.

(EH21 and EH35), no significant effects were observed. Plasma T4 levels in Aroclor 1254 exposed tilapia (A21 and A35) were not different from those of the control group.

Total plasma T3 levels were not significantly altered by any of the treatments.

Total plasma rT3 levels were only found to be significantly depressed in the EL21 group. The concentration of this inactive hormone presented a very strong positive correlation with plasma T4 ($r=0.6898$, $p=0.782\times10^{-10}$).

3.2. Deiodinase activities

3.2.1. D1 activity

Liver D1 ORD activity (Fig. 2) was significantly decreased by two of the Endosulfan treatments. Significant D1 activity depletion was observed in tilapia exposed to the lower dose of Endosulfan, especially in fish with the longest exposure period (EL35); tilapia from this group also presented a significant liver D1 activity decrease when compared to tilapia exposed for the same time to the higher dose of Endosulfan (EH35). On the other hand, kidney D1 activity (Table 2) in all treatment groups remained unaltered and was found to be positively correlated with T3 plasma levels ($r=0.2670$, $p=0.0375$). These enzymes activities were also positively related with each other ($r=0.2783$, $p=0.0285$).

3.2.2. D2 activity

Liver D2 activity (Fig. 3) was not affected by any of the treatments. Significant differences were only observed between EH21 and EH35 ($p<0.01$). Positive correlations were found between this enzyme activity and plasma T4 ($r=0.2700$, $p=0.0353$) and T3 levels ($r=0.3276$, $p=0.0010$) and liver D1 activity ($r=0.3695$, $p=0.0031$).

Kidney D2 activity (Table 2) in treated tilapia was not significantly altered when compared to control animals, but significant differences were observed between EL21 and EH21 ($p<0.001$) and EH21 and EH35 ($p<0.01$); the EH21 group presented the highest D2 activity levels.

Brain D2 activity (Table 2) was only found to be significantly depressed in tilapia belonging to the A21 treatment.

In testes, D2 activity (Table 2) was found to be significantly enhanced by all treatments compared to the control group at the end of 35 days of exposure.

Ovary, muscle and gill D2 activities (Table 2) were not altered by any of the treatments.

3.2.3. D3 activity

D3 activity was found to be below the detection limit in kidney, muscle and gonads.

Liver D3 activity (Fig. 4) was found to be significantly increased in treatment groups EL35, A21 and A35. This effect seemed to be related with the time of exposure since

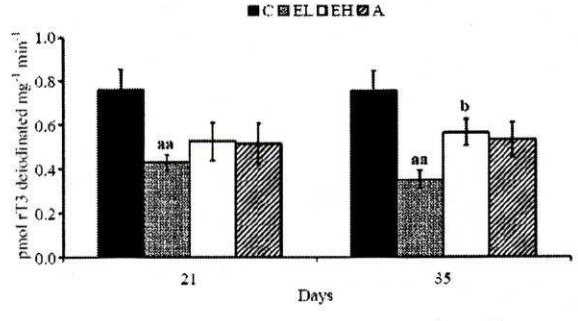


Fig. 2. Liver type I deiodinase activity (rT3 pmol mg^{-1} min^{-1}) in tilapia control (C) and exposed through the diet to two Endosulfan (E) doses, 0.1 μg g^{-1} (L) or 0.5 μg g^{-1} (H), and to one Aroclor 1254 (A) dose, 0.5 μg g^{-1}, during 21 or 35 days. Values are represented as mean ± SEM, $n=7-8$. Significant differences obtained by ANOVA followed by Student–Newman–Keuls test are indicated as follows: between control and treated tilapia with the same exposure time—$^{aa}p<0.01$; between different doses and the same exposure time—$^{b}p<0.05$.

Table 2
Deiodinases activities in different tissues of tilapia control (C) and exposed through the diet to two Endosulfan (E) doses, 0.1 μg g^{-1} (L) or 0.5 μg g^{-1} (H), and to one Aroclor 1254 (A) dose, 0.5 μg g^{-1}, during 21 or 35 days

		C21	C35	EL21	EL35	EH21	EH35	A21	A35
D1	Kidney	1.10±0.11	1.19±0.15	1.12±0.17	1.06±0.17	1.39±0.20	1.23±0.16	1.17±0.16	1.03±0.12
D2	Kidney	0.40±0.08	0.38±0.08	0.29±0.01	0.30±0.05	$0.44\pm0.03^{bbb,c}$	0.36±0.02	0.38±0.04	0.37±0.06
	Brain	0.16±0.02	0.12±0.01	0.13±0.01	0.14±0.01	0.12±0.01	0.14±0.02	0.10 ± 0.01^{aa}	0.12±0.02
	Gills	0.93±0.12	0.63±0.10	0.98±0.11	0.83±0.08	0.97±0.13	0.76±0.07	0.83±0.05	0.75±0.06
	Muscle	0.14±0.02	0.10±0.01	0.11±0.01	0.11±0.02	0.11±0.02	0.10±0.01	0.10±0.02	0.08±0.01
	Testes	2.63±0.55	1.23±0.21	3.03±0.54	3.92 ± 0.44^{aa}	3.00±0.74	2.82 ± 0.35^{a}	2.66±0.53	3.10 ± 0.52^{aaa}
		(4)	(3)	(4)	(3)	(6)	(6)	(3)	(5)
	Ovary	0.23±0.04	0.17±0.05	0.16±0.02	0.26±0.04	0.46	0.14	0.13±0.02	0.09±0.00
		(4)	(4)	(3)	(4)	0.23	0.08	(5)	(3)
D3	Brain	4.26±0.21	4.65±0.27	4.55±0.22	4.86±0.11	4.15±0.31	4.68±0.29	4.33±0.22	4.74±0.34

D1 activity expressed as rT3 pmol mg^{-1} min^{-1}; D2 activity expressed as T4 fmol mg^{-1} min^{-1} and D3 activity expressed as T3 fmol mg^{-1} min^{-1}. Values represented as mean±SEM, $n=7-8$ except when indicated (n). Significant differences were obtained by ANOVA followed by Student–Newman–Keuls test. Significant differences between control and exposed tilapia with the same exposure time—$^{a}p<0.05$, $^{aa}p<0.01$, $^{aaa}p<0.001$, significant differences between different doses and the same exposure time—$^{bbb}p<0.001$, significant differences between different exposure time for the same dose—$^{c}p<0.05$.

the increase in activity was always superior in tilapia exposed during 35 days for both compounds. Also, on day 35, the Endosulfan lower dose (EL35) had a higher effect on liver D3 activity than the higher dose (EH35) ($p<0.05$).

Gill D3 activity (Fig. 5) was significantly increased by all 21 days treatments. The increase was also significant compared to control activity at 35 days for the lower Endosulfan dose. The lower Endosulfan dose exposure for a longer period (EL35) had a significantly ($p<0.05$) stronger effect on gill D3 activity when compared to the shorter exposure period (EL21). This enzyme activity was found to be negatively related with liver D1 activity ($r=-0.2507$, $p=0.0494$).

Brain D3 activity (Table 2) was not affected by the exposure to both pollutants.

4. Discussion

The present study shows that in tilapia fed with Endosulfan, a chlorinated pesticide, or Aroclor 1254, a PCB mixture, a number of changes occur in total plasma T4 and rT3 levels and in tissue deiodinase activities.

The only significant changes in circulating thyroid hormone levels were the decrease in plasma T4 and rT3 following 21 days of exposure to the lower Endosulfan dose. Tilapia Aroclor 1254 exposure through the diet did not alter any of the thyroid hormones levels which is in accordance with the studies performed with rainbow trout (reviewed by Brown et al., 2004a) fed with a 1000 times higher dose than the one we used.

It was interesting to observe that despite the changes in T4 and rT3, total plasma T3 concentration remains unaltered. This is in agreement with a study made by Mol et al. (1999) on experimentally induced hyper- and hypothyroidism in tilapia. The authors concluded that tilapia is able to keep plasma T3 relatively stable despite changes in T4 concentration.

Liver was the organ that was most affected by the exposure to Endosulfan or Aroclor 1254. In our study, tilapia liver D1 activity was found to be depressed by both low

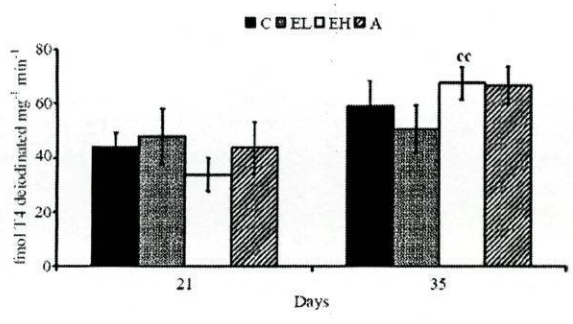


Fig. 3. Liver type II deiodinase activity (T4 fmol mg^{-1} min^{-1}) in tilapia control (C) and exposed through the diet to two Endosulfan (E) doses, 0.1 μg g^{-1} (L) or 0.5 μg g^{-1} (H), and to one Aroclor 1254 (A) dose, 0.5 μg g^{-1}, during 21 or 35 days. Values are represented as mean±SEM, $n=7-8$. Significant differences obtained by ANOVA followed by Student–Newman–Keuls test are indicated as follows: between different exposure times for the same dose—$^{cc}p<0.01$.

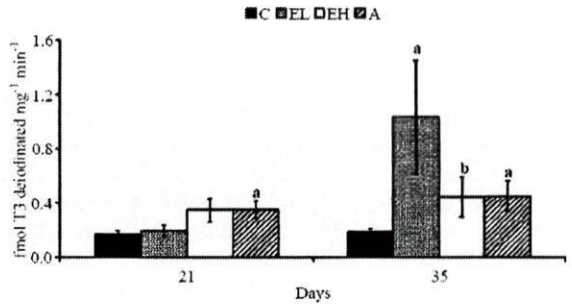


Fig. 4. Liver type III deiodinase activity (T3 fmol mg^{-1} min^{-1}) in tilapia control (C) and exposed through the diet to two Endosulfan (E) doses, 0.1 μg g^{-1} (L) or 0.5 μg g^{-1} (H), and to one Aroclor 1254 (A) dose, 0.5 μg g^{-1}, during 21 or 35 days. Values are represented as mean±SEM, $n=7-8$. Significant differences obtained by ANOVA followed by Student–Newman–Keuls test are indicated as follows: between control and treated tilapia with the same exposure time—$^{a}p<0.05$; between different doses and the same exposure time—$^{b}p<0.05$.

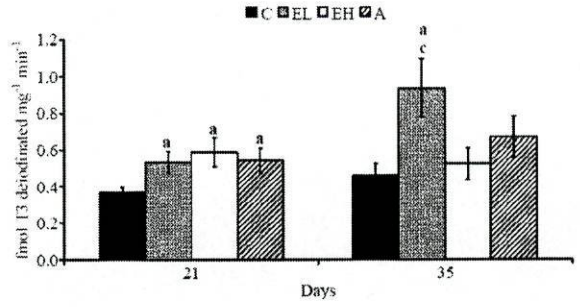


Fig. 5. Gills type III deiodinase activity (T3 fmol mg^{-1} min^{-1}) in tilapia control (C) and exposed through the diet to two Endosulfan (E) doses, 0.1 μg g^{-1} (L) or 0.5 μg g^{-1} (H), and to one Aroclor 1254 (A) dose, 0.5 μg g^{-1}, during 21 or 35 days. Values are represented as mean ± SEM, $n = 7–8$. Significant differences obtained by ANOVA followed by Student–Newman–Keuls test are indicated as follows: between control and treated tilapia with the same exposure time—$^{a}p < 0.05$; between different exposure times for the same dose—$^{c}p < 0.05$.

Endosulfan treatments while liver D3 was found to be enhanced by Endosulfan lower treatment in relation to the duration of the exposure. Taken together, these changes could result in a decreased T3 availability. The fact that plasma T3 remains unaltered, both in our and in other studies, is probably indicative of the prominent role of hepatic D2 activity and renal D1 activity, which both remained stable, in determining circulating T3 levels in fish. Our results also agree with the lack of change in hepatic D2 activity in lake trout (*Salvelinus namaycush*) after gavage exposure to PCB 126 (Brown et al., 2004b) and for American plaice injected with the same PCB congener and with PCB 77 (Adams et al., 2000).

Liver deiodinase activities, alterations may be related with the major metabolic function of this organ and the chemical properties of the used compounds. As liver possesses a high fat content and these pollutants are lipophilic, they tend to concentrate in the hepatic tissue. Also, since Endosulfan and Aroclor 1254 were administrated through the diet, liver is one of the first organs to enter in contact with these compounds. Therefore it is probably submitted to higher doses of the pollutants and eventually of their metabolites than the other organs analysed, as was also suggested by Adams et al. (2000).

While gill D3 activity was increased by the exposure to Endosulfan and Aroclor 1254, gill D2 activity remained the same in treated and control tilapia.

Contrary to a decline in kidney D1 activity as observed in rats exposed to Aroclor 1254 (Hood and Klaassen, 2000), tilapia kidney D1 activity was not affected by this compound neither by Endosulfan.

Brain D2 and D3 activities, muscle D2 and ovaries D2 activity were not affected in a clear way by these contaminants, while the increase in testes D2 activity was related to the time of exposure.

Both T4 and rT3 levels were depressed by the low Endosulfan dose after 21 days, while T3 levels and liver D2 and kidney D1 activities, described as the main enzymes responsible for the regulation of circulating T3 concentrations (Mol et al., 1993, 1997), remained unaltered. The observation that neither of these activities was affected may therefore explain why plasma T3 remained unaltered despite the fact that the changes in other deiodinase activities that were observed are not in agreement with the compensatory changes one would expect to contribute to the maintenance of plasma T3 levels. These facts seem to indicate that Endosulfan interferes either with prior phases of the thyroid system inhibiting T4 production and/or secretion; or by enhancing T4 clearance. The observation that after 35 days of exposure to the same dose no effects on plasma thyroid hormones were found may indicate an adaptation of tilapia to exposure to these compounds. Contrary to what was expected, but in agreement with the observed results to the low dose, the higher Endosulfan dose did not alter thyroid hormones plasma levels or deiodinase activities, with the exception of gill D3 and testes D2; indicating that a higher dose possibly elicits a compensatory mechanism, prior to 21 days of exposure.

On the other hand, Aroclor 1254, as expected due to different chemical characteristics, gives the impression to act in a different way. While the increase observed both in liver and gill D3 activities could favour an increase in T3 elimination and a decrease in plasma T3 levels, this was not observed.

Our experimental results show that when evaluating contaminant effects in tilapia thyroid related mechanisms, plasma thyroid hormone levels measurement is not sufficient. These levels can be found unaltered due to compensatory mechanisms, while the peripheral thyroid hormone metabolism may be disrupted, possibly affecting local T3 availability in specific tissues in response to the pollutants used.

Finally, the differences found between the response to Endosulfan and Aroclor 1254 exposure can, at least partially, be linked to the distinct metabolism of these compounds and sites and ways of action.

Acknowledgments

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References

Adams, B.A., Cyr, D.G., Eales, J.G., 2000. Thyroid hormone deiodination in tissues of American plaice, *Hippoglossoides platessoides*: characterization and short-term responses to polychlorinated biphenyls (PCBs) 77 and 126. Comp. Biochem. Physiol., C 127, 367–378.

Bhattacharya, L., 1995. Histological and histochemical alterations in the thyroid activity of endosulfan treated *Oreochromis mossambicus*. J. Environ. Biol. 16, 347–351.

Brown, S.B., Adams, B.A., Cyr, D.G., Eales, J.G., 2004a. Contaminant effects on the teleost fish thyroid. Environ. Toxicol. Chem. 27, 1680–1701.

Brown, S.B., Evans, R.E., Vandenbyllardt, L., Finnson, K.W., Palace, V.P., Kane, A.S., Yarecheweski, A.Y., Muir, D.C.G., 2004b. Altered thyroid status in lake trout (*Salvelinus namaycush*) exposed to co-planar 3,3′, 4,4′,5-pentachlorobiphenyl. Aquat. Toxicol. 67, 75–85.

Byamungu, N., Mol, K., Kühn, E.R., 1992. Evidence for the kidney as an important source of 5′-monodeiodination activity and stimulation by somatostatin in *Oreochromis niloticus* L. Gen. Comp. Endocrinol. 88, 199–208.

Colborn, T., 2002. Clues from wildlife to create an assay for thyroid system disruptors. Environ. Health Perspect. 110, 363–367.

Ferrando, M.D., Sancho, E., Andreu-Moliner, E., 1991. Comparative acute toxicities of selected pesticides to *Anguilla anguilla*. J. Environ. Sci. Health 26B, 491–498.

Fisher, B.E., 1999. Most unwanted: persistent organic pollutants. Environ. Health Perspect. 107, A18–A23.

Gould, J.C., Cooper, K.R., Scanes, C.G., 1999. Effects of polychlorinated biphenyls on thyroid hormones and liver type I monodeiodinase in the chick embryo. Ecotoxicol. Environ. Saf. 43, 195–203.

Hood, A., Klaassen, C.D., 2000. Effects of microsomal enzyme inducers on outer-ring deiodinase activity toward thyroid hormones in various rat tissues. Toxicol. Appl. Pharmacol. 163, 240–248.

Mol, K., Kaptein, E., Darras, V.M., Greef, W.J., Kühn, E.R., Visser, T.J., 1993. Different thyroid hormone-deiodinating enzymes in tilapia (*Oreochromis niloticus*) liver and kidney. FEBS Lett. 321, 140–144.

Mol, K., Van der Geyten, S., Darras, V.M., Visser, T.J., Kühn, E.R., 1997. Characterization of iodothyronine outer ring and inner ring deiodinase activities in the blue tilapia, *Oreochromis aureus*. Endocrinology 138, 1787–1793.

Mol, K., Van der Geyten, S., Kühn, E.R., Darras, V.M., 1999. Effects of experimental hypo-and hyperthyroidism on iodothyronine deiodinases in Nile tilapia, *Oreochromis niloticus*. Fish Physiol. Biochem. 20, 201–207.

Naqvi, S.M., Vaishnavi, C., 1993. Bioaccumulative potential and toxicity of endosulfan insecticide to non-target animals. Comp. Biochem. Physiol., C 105, 347–361.

Orozco, A., Jeziorski, M.C., Linser, P.J., Greenberg, R.M., Valverde-R, C., 2002. Cloning of the gene and complete cDNA encoding a type 2 deiodinase from *Fundulus heteroclitus*. Gen. Comp. Endocrinol. 128, 162–167.

Orozco, A., Villalobos, P., Jeziorski, M.C., Valverde-R, C., 2003. The liver of *Fundulus heteroclitus* expresses deiodinase type 1 mRNA. Gen. Comp. Endocrinol. 130, 84–91.

Sanders, J.P., Van der Geyten, S., Kaptein, E., Darras, V.M., Kühn, E.R., Leonard, J.L., Visser, T.J., 1997. Characterization of a propylthiouracil-insensitive type I iodothyronine deiodinase. Endocrinology 138, 5153–5159.

Sanders, J.P., Van der Geyten, S., Kaptein, E., Darras, V.M., Kühn, E.R., Leonard, J.L., Visser, T.J., 1999. Cloning and characterization of type III iodothyronine deiodinase from the fish *Oreochromis niloticus*. Endocrinology 140, 3666–3673.

Sinha, N., Lal, B., Singh, T.P., 1991a. Effect of endosulfan on thyroid physiology in the freshwater catfish, *Clarias batrachus*. Toxicology 65, 187–197.

Sinha, N., Lal, B., Singh, T.P., 1991b. Pesticides induced changes in circulating thyroid hormones in the freshwater catfish, *Clarias batrachus*. Comp. Biochem. Physiol., C 100, 107–110.

St. Germain, D.L., Galton, V.A., 1997. The deiodinase family of selenoproteins. Thyroid 7, 655–688.

Sutija, M., Longhurst, T.J., Joss, J.M., 2003. Deiodinase type II and tissue specific mRNA alternative splicing in the Australian lungfish, *Neoceratodus forsteri*. Gen. Comp. Endocrinol. 132, 409–417.

Sutija, M., Longhurst, T.J., Joss, J.M., 2004. Deiodinase type III in the Australian lungfish, *Neoceratodus forsteri*. Gen. Comp. Endocrinol. 136, 152–161.

Tagawa, M., Tanaka, M., Matsumoto, S., Hirano, T., 1990. Thyroid hormones in eggs of various freshwater, marine and diadromous teleosts and their changes during egg development. Fish Physiol. Biochem. 8, 515–520.

Valverde, C., Croteau, W., Lafleu Jr., G.J., Orozco, A., Germain, D.L., 1997. Cloning and expression of a 5′-iodothyronine deiodinase from the liver of *Fundulus heteroclitus*. Endocrinology 138, 642–648.

Van der Geyten, S., Mol, K.A., Pluymers, W., Kühn, E.R., Darras, V.M., 1998. Changes in plasma T3 during fasting/refeeding in tilapia (*Oreochromis niloticus*) are mainly regulated through changes in hepatic type II iodothyronine deiodinase. Fish Physiol. Biochem. 19, 135–143.

Van der Geyten, S., Toguyeni, A., Baroiller, J.F., Fauconneau, B., Fostier, A., Sanders, J.P., Visser, T.J., Kühn, E.R., Darras, V.M., 2001. Hypothyroidism induces type I iodothyronine deiodinase expression in tilapia liver. Gen. Comp. Endocrinol. 124, 333–342.

Visser, T.J., 1996. Pathways of thyroid hormone metabolism. Acta Med. Austriaca 23, 10–16.

Visser, T.J., Docter, R., Hennemann, G., 1977. Radioimmunoassay of reverse tri-iodothyronine. J. Endocrinol. 73, 395–396.

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CAPÍTULO 4

Coimbra AMª, Reis-Henriques MªA, Almeida MªR. Effects of dietary exposure to Endosulfan and Aroclor 1254 on Thyroid hormones binding and transport by plasma proteins on Nile tilapia. (Submitted to *Comparative Biochemistry and Physiology C*).



ABSTRACT

Polychlorinated compounds, as Endosulfan and Aroclor 1254, are known to interfere with the thyroidal status of different organisms. They might induce alterations at different levels namely, synthesis, transport, metabolism and clearance or action of the thyroid hormones. The objectives of this study were to evaluate the effects of these compounds on tilapia thyroid hormones distribution and transport by plasma proteins and to identify tilapia thyroid hormones binding plasma proteins. Polyacrilamide gel electrophoresis (PAGE) analyses were undertaken to evaluate tilapia thyroid hormones binding plasma proteins in presence and absence of the polychlorinated compounds; competition assays were also performed. ^{125}I-T_4 injections on tilapias exposed, through the diet, to each compound (0.5 $\mu g.g^{-1}$ of food) allowed us to evaluate thyroxine (T_4) distribution among tissues and binding to plasma proteins. The results showed the presence of two thyroid hormones binding proteins in tilapia plasma, one identified as "albumin" and the other one as an apolipoprotein-like (apo-like) protein. Transthyretin (TTR), a thyroid hormone binding plasma protein recently identified in fish, was not detected in tilapia plasma. Concerning the effects of Endosulfan and Aroclor 1254, with the used doses, interference with T_4 distribution to tissues or with transport by plasma proteins was not observed.

INTRODUCTION

Since the 1920's, a considerable global environmental pollution developed, due to the extended use of several polychlorinated organic compounds. These compounds have been used as pesticides, insecticides (e.g.: Endosulfan), defoliants and multipurpose industrial chemicals generally designated as polychlorinated biphenyls (PCBs), some of them originated as byproducts of large scale chemical industrial production (Langer, 1998).

PCBs are, perhaps, the most widespread global pollutants. PCBs and polychlorinated insecticides show multiple metabolic and toxic activities, including mutagenic, immunotoxic and carcinogenic effects (McKinney and Waller, 1994). They also mimic several lipophilic natural hormones and thus strongly affect endocrine homeostasis (Birnbaum, 1994; Whitlock, 1994; Li and Hansen, 1997). From such reason these compounds have been called endocrine disruptors (Kavlock et al., 1996).

Animal and human exposure occurs usually from secondary sources, such as food and water intake, inhalation and skin contact. In general, species living in water contain higher levels of PCBs than terrestrial ones (Langer, 1998); PCBs, among other environmental contaminants, are known for being able to affect fish thyroidal status (reviewed in Brucker-Davis, 1998; Rolland, 2000; Brown et al., 2004).

Thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3) are fundamental for fish early development, growth and reproduction. Besides these functions, others were reviewed by Leatherland (Leatherland, 1994). These hormones were also found to be present in eggs and embryos of a high variety of fish species (Tagawa et al., 1990), showing their importance even at early life stages.

In large mammals, the most important thyroid hormones binding proteins in plasma are: thyroxine binding globulin (TBG), transthyretin (TTR) and albumin (Alb) (Larsson et al., 1985), whose main function is to assure the appropriate distribution of thyroid hormones to and within organs and tissues (Mendel et al., 1987). In fish, it was thought that only Alb bound T_4 (Richardson et al., 1994, Scheiber and Richardson, 1997); until the TTR gene was cloned in two teleost fish species (Santos and Power. 1999; Funkenstein et al., 1999; Yamauchi et al., 1999). Contrary to what happens with mammals TTR, fish TTR seems to have a higher binding affinity for T3 than for T_4 (Santos and Power, 1999; Yamauchi et al., 1999). Besides Alb and TTR, in trout plasma, lipoproteins were described as the major transporters of thyroid hormones (Babin, 1992).

One of the most frequent effects of organochlorines on thyroid hormone metabolism is the decrease of plasma T_4 level due to the displacement of T_4 from binding proteins (Van den Berg et al., 1991; Lans et al., 1993 and 1994) and to the increased hepatic

metabolism of T_4 resulting from the induction of UDP-glucuronosyltransferase (Van den Berg et al., 1991; Lans et al., 1993), thus contributing to the decrease of blood thyroid hormone levels.

Several organochlorines inhibit the binding of T_4 to human plasma carrier proteins such as TTR, TBG and Alb (Mckinney and Waller, 1994). Binding of PCBs to TTR is a major contributing factor for reduction in circulating hormones and possibly for certain biological effects related to PCB exposure (Chauhan et al., 2000). PCBs can bind to TTR, although in general, they do it with less affinity than their hydroxylated metabolites (Lans et al., 1993; Chauhan et al., 2000). Decrease of T_4 binding to TTR was observed in Sprague-Dawley rats exposed to Aroclor 1254, a PCBs mixture, (Hallgren and Darnerud, 2002).

Endosulfan is a polycyclic organochlorine insecticide used on a wide variety of crops and as a wood preservative and is known for altering thyroid hormones levels in the freshwater catfish (*Clarias batrachus*) (Sinha et al., 1991a,b) and Nile tilapia (*Oreochromis niloticus*) (Coimbra et al., 2005).

In a previous work with Nile tilapia, we studied the effects of dietary exposure to Aroclor 1254 and Endosulfan (Coimbra et al., 2005). Endosulfan exposure decreased thyroid hormone levels, namely T_4 and rT_3 (reverse T_3) and interfered with iodothyronine deiodinases activities, specially decreasing liver type I deiodinase activity and increasing liver type III deiodinase activity. Aroclor 1254 only interfered with activities of deiodinases, while thyroid hormones plasma concentrations remain unaltered. In the Endosulfan study, the deiodinases activities variations were not sufficient to explain alterations observed on thyroid hormones plasma levels and it has been hypothesized that these compounds interfere with thyroid hormones transport proteins in plasma, increasing their clearance (Coimbra et al., 2005).

Plasma transport of thyroid hormones in fish is still poorly studied; especially regarding TTR as a thyroid hormones carrier protein. Until the recent discover of TTR gene in fish, this protein was thought to be absent in fish (Schreiber and Richardson, 1997) and contradictions regarding its ontogeny and tissue expression are found in the literature available (Funkenstein et al., 1999; Richardson et al., 2005).

The objective of this work was to contribute to the understanding of the effects of polychlorinated compounds on T_4 binding to tilapia plasma proteins, mainly on transport and distribution of thyroid hormones.

MATERIALS AND METHODS

Chemical compounds

Aroclor 1254 was purchased from NSI Solutions, Inc. (Raleigh, USA) as a neat standard. Endosulfan used in these assays was of technical grade (± 95 %) and supplied by AGROQUISA (Lisboa, Portugal).

Animals

Adult Nile tilapias (*Oreochromis niloticus*) were maintained in freshwater tanks of 70 litres with water recirculation and aeration at 21 °C, under a 12:12 hours light:dark photoperiod.

Thyroid hormone binding to plasma proteins *ex vivo* assays

Native polyacrylamide gel electrophoresis (PAGE)

T_3 and T_4 binding to tilapia plasma proteins was assessed by PAGE: 10 µL whole tilapia plasma were incubated with 1 µL ^{125}I-T_4 (1300 µCi µg^{-1}) or ^{125}I-T_3 (1200 µCi µg^{-1}) (Perkin Elmer Life Sciences, Inc, Belgium), for at least one hour at room temperature. The samples were then subjected to electrophoresis on an 8 % polyacrylamide gel in a glycine-acetate buffer system (200 mM glycine, 130 mM sodium acetate, pH 8.6) for 3-4 h at 40 mA at room temperature for the separation of the plasma proteins (Saraiva et al., 1988). Using the same PAGE system, qualitative displacement of T_4 or T_3 from tilapia plasma proteins was also evaluated by addition to the samples prepared as described above either non-radioactive hormones or polychlorinated compounds (Endosulfan, Aroclor 1254); human plasma, recombinant wild-type TTR (Furuya et al., 1991) and recombinant sea bream (SB) TTR (Santos and Power, 1999, Eneqvist et al., 2003 and 2004) were used as control. The migration of human albumin was determined in a serum sample incubated with bromophenol blue run in a different lane. Gels were dried and subjected to phosphor imaging (Typhoon 8600; Molecular Diagnostics, Amersham Biosciences) or to autoradiography.

Protein analysis

A tilapia plasma protein fraction was isolated using a PAGE system described by Altland et al. (Altland et al., 1999). A band with a migration corresponding to the migration

of human TTR was removed and electroeluted overnight in Tris-glycine buffer (Tris 5 mM, Glycine 38 mM, pH 8.3). Analysis of thyroid hormones affinity to the isolated protein fraction and to tilapia larvae homogenates (in PBS-Phosphate Saline Buffer) was performed by PAGE as described for whole plasma samples.

Proteins from the excised gel band were separated by 15 % SDS-PAGE and electrophoretically transferred to PVDF membrane (Hybond-P, Amersham Pharmacia Biotech). Before semi-dry transference, the gel and membrane were equilibrated on a 3-Cyclohexylamino)-1-propanesulfonic acid (CAPS) 10 mM, methanol 15 % solution. The membrane was stained with Coomassie blue for localization of protein bands. The protein band with 14 kDa was excised and N-terminal sequencing was performed at the Protein Chemistry Core Facility of Columbia University, NY, USA.

Thyroid hormones binding assays

Binding of T_4 to plasma proteins and competition of polychlorinated compounds were also assessed.

Tilapia plasma dilution most adequate for the quantitative competition assays was previously determined on a T_4 binding saturation study. Tilapia plasma dilutions (1:5, 1:10 and 1:20) in assay buffer (TNE- 100 mM Tris, 100 mM NaCl and 1 mM EDTA, pH 8.0) were incubated with $2x10^5$ cpm of ^{125}I-T_4 and 1000 nM of unlabelled T_4 and with serial 1:2 dilutions of this T_4 mixture. Samples were incubated overnight at 4 °C. ^{125}I-T_4, bound to tilapia plasma proteins, was separated from unbound by gel filtration chromatography (Almeida et al., 1997) through a 1 ml Bio-Gel P6-DG (Bio-Rad, Hercules, CA, USA) column. All samples were run in duplicate.

T_4 competition assays were performed using basically the same procedure (Lans et al., 1994). In these assays tilapia plasma was diluted 1:10 with TNE buffer. Whole human plasma, diluted 1:40 was used as control. The data were analysed using GraphPad Prism 2.01.

Thyroid hormone binding to plasma proteins *in vivo* assays

Animal treatment

Adult Nile tilapias with average weight and length of 441.52±47.35 g and 28.45±1.24 cm respectively were fed once a day, for 21 days, with a commercial fish diet, to which Endosulfan or Aroclor 1254 (0.5 $\mu g.g^{-1}$) dissolved in ethanol were added, followed by drying at room temperature under air current. Each group consisted of 6 tilapias.

Thyroid hormones plasma levels

Total T_4 and T_3 plasma levels were determined in control and in tilapias treated with Endosulfan or Aroclor 1254. Tilapias were anaesthetised in iced water and a blood sample was collected for Total T_4 and Total T_3 determinations using commercially available radioimmunoassay (RIA) kits (DPC–Diagnostic Products Corporation, Los Angeles, CA, USA).

^{125}I-T_4 injections and distribution

Tilapias were injected in the caudal vessel with $8x10^6$ cpm of purified ^{125}I-T_4. Two hours after ^{125}I-T_4 injection, a blood sample was collected from the caudal vein. Total ^{125}I-T_4 remaining in the plasma was determined by measuring the amount of radioactivity in a known volume and assuming that the total volume of plasma is 4 % of the total body weight (Okimoto et al., 1994). Free ^{125}I-T_4 was separated from the protein bound fraction by gel filtration chromatography through Bio-Gel P6-DG (Bio-Rad Hercules, CA, USA) columns and their percentage was established by measuring the amount of radioactivity before and after elution.

In order to evaluate the distribution of labelled T_4 per tissues and per amount of tissue, tilapias were sacrificed and circulating radioactive hormone (^{125}I-T_4) was removed by perfusion with NaCl 0.9 %, until gills's total discoloration. Liver, kidney, gonads, brain, gills and muscle were collected, weighted and homogenized in PBS. Homogenates were centrifuged at 5000 rpm for 30 min and supernatant radioactivity was measured.

Statistical analysis

After testing ANOVA assumptions, statistical analysis was performed by one-way analysis of variance (ANOVA), followed by the multiple comparison LSD test ($p<0.05$).

RESULTS

Thyroid hormone binding to plasma proteins *ex vivo* assays

The pattern of proteins binding thyroid hormones was established after incubation of tilapia plasma with the labelled hormones, either ^{125}I-T_3 or ^{125}I-T_4, followed by electrophoretic separation of the proteins in native polyacrylamide gel using a glycine-acetate buffer system (Fig.1).

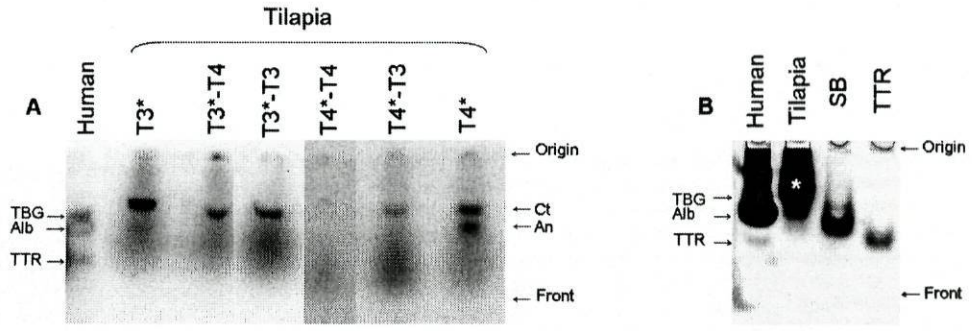


Fig 1: A: Thyroid hormone binding proteins in adult *Oreochromis niloticus* plasma. Plasma proteins were separated by electrophoresis in non-denaturing polyacrylamide gel (PAGE). Tilapia plasma samples were incubated with ^{125}I-T_3 (T_3*) or ^{125}I-T_4 (T_4*) in the presence or absence of unlabelled T_3 or T_4. The protein bands were visualized by autoradiography. B: Electrophoresis of tilapia and human plasma proteins and comparison with human and sea bream recombinant TTRs, as visualized by Coomassie Brilliant Blue staining. Bands corresponding to human TBG, Alb and TTR, to tilapia cathodic (Ct) and anodic (An) proteins, as well as tilapia "albumin" (*) are indicated.

Two protein bands binding thyroid hormones, ^{125}I-T_3 or ^{125}I-T_4, were always visualized in tilapia plasma. One of the proteins, indicated as Ct, presented a more cathodic migration than human TBG and the other one, indicated as An, presented a more anodic migration than human albumin. Although both proteins bind T_3 and T_4, the intensity of the corresponding bands is different reflecting different binding affinities and/or different binding capacities. Protein Ct seemed to be tilapia albumin according to the migration (Fig. 1A) and abundance of the protein (Fig. 1B). Both protein bands presented similar intensity concerning T_4 binding. Protein An, although it can not be visualized in the Coomassie blue stained gel, it presented a migration similar to recombinant sea bream TTR leading us to hypothesize that it could be tilapia TTR. In this sense another native PAGE system (Altland et al, 1999) was used to isolate the protein according to its

migration. The semipurified fraction from the excised gel band was analyzed concerning thyroid hormones binding and molecular weight. The results are presented in Fig. 2A and 2B, respectively.

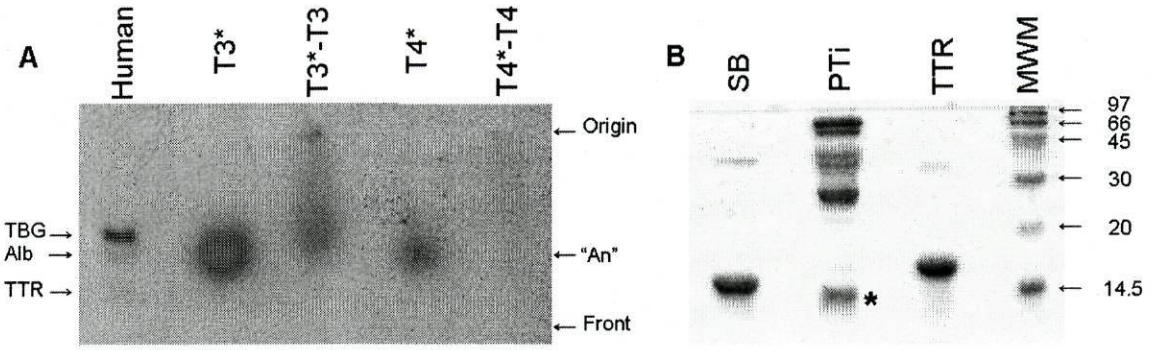


Fig. 2: A- Analysis of T_4 and T_3 binding to an isolated tilapia plasma fraction. The analysis was performed by separation in non-denaturing PAGE. Tilapia plasma fraction incubated with ^{125}I-T_3 and ^{125}I-T_4 in the presence of unlabelled T_3 and T_4, as visualized by autoradiography. B- Tilapia plasma fraction presenting the protein band send to N-terminal sequencing (*). SDS-PAGE analysis as visualized by Coomassie Brilliant Blue staining and comparison with human TTR and recombinant SB. Molecular weight markers (MWM-Amershan Pharmacia Biotech) are indicated.

This protein fraction from tilapia plasma presented affinity for both radiolabelled thyroid hormones and both unlabelled hormones competed for protein binding confirming the specificity of the interaction. In addition, it presented an electrophoretic migration similar to the one shown by sea bream TTR and by tilapia protein An, in native PAGE (Fig. 2A). When analyzed by SDS-PAGE (Fig. 2B) only a single band presented a molecular weight correspondent to the weight of the TTR monomer. N-terminal sequence of the protein indicated with an asterisk (*) revealed, after a BLAST search against NCBI translated DNA data base, a match with *O. mossambicus* 14 kDa apolipoprotein-like (Gen Bank accession no. **AY522608**) and, contrary to what was expected, no identity with TTRs.

As previous literature describes fish TTR as being present in early development phases, tilapia larvae homogenates were also incubated with ^{125}I-T_3 in the presence and absence of unlabelled T_3 (data not shown). A protein with the same characteristics as the isolated tilapia plasma protein was observed.

Although, these results do not confirm the presence of TTR on tilapia plasma, its existence can not be excluded.

Effects of polychlorinated compounds on thyroid hormone binding to plasma proteins *in vitro*

In order to evaluate the effect of the polychlorinated compounds on thyroid hormone binding to plasma proteins, Tilapia plasma was incubated with ^{125}I-T_4 in the presence and absence of either Endosulfan or Aroclor 1254 and the samples were analyzed by PAGE as described above. The results are shown in Fig. 3.

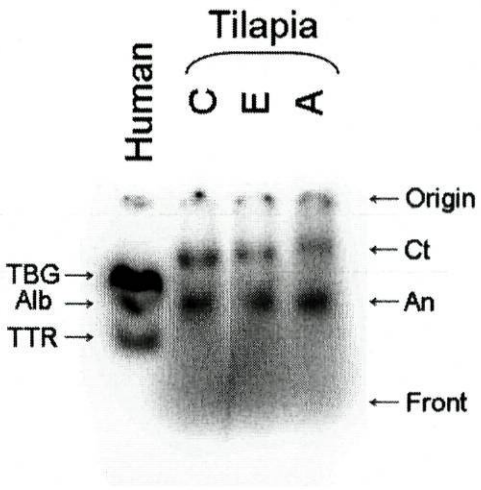


Fig 3: Analysis of competition with ^{125}I-T_4 for binding to plasma proteins. The pattern of plasma proteins was analyzed by separation in nondenaturing PAGE and visualized by phosphor image. Tilapia plasma incubated with ^{125}I-T_4 (C), in the presence of Endosulfan (E) and Aroclor 1254 (A) Signals corresponding to human TBG, Alb and TTR and to tilapia Ct and An proteins are indicated.

Endosulfan and Aroclor 1254 did not show the capacity to displace ^{125}I-T_4 from any of tilapia plasma thyroid hormones binding proteins. Further assays with higher doses of both polychlorinated compounds showed that Aroclor 1254 in higher doses is able to displace ^{125}I-T_4 from tilapia Ct protein (presumably to be tilapia albumin). Concerning its effects on human plasma, Aroclor 1254 displaces the hormone from TBG increasing the binding of ^{125}I-T_4 to TTR (data not shown).

In addition, competition of T_4, T_3, Aroclor 1254 and Endosulfan with ^{125}I-T_4 for the binding to tilapia plasma proteins was assayed in whole tilapia plasma diluted 1:10 (Fig.4).

This plasma dilution (1:10) was chosen after trying lower and higher plasma dilutions (1:5, 1:20, 1:40) without getting results that could fit a competition curve.

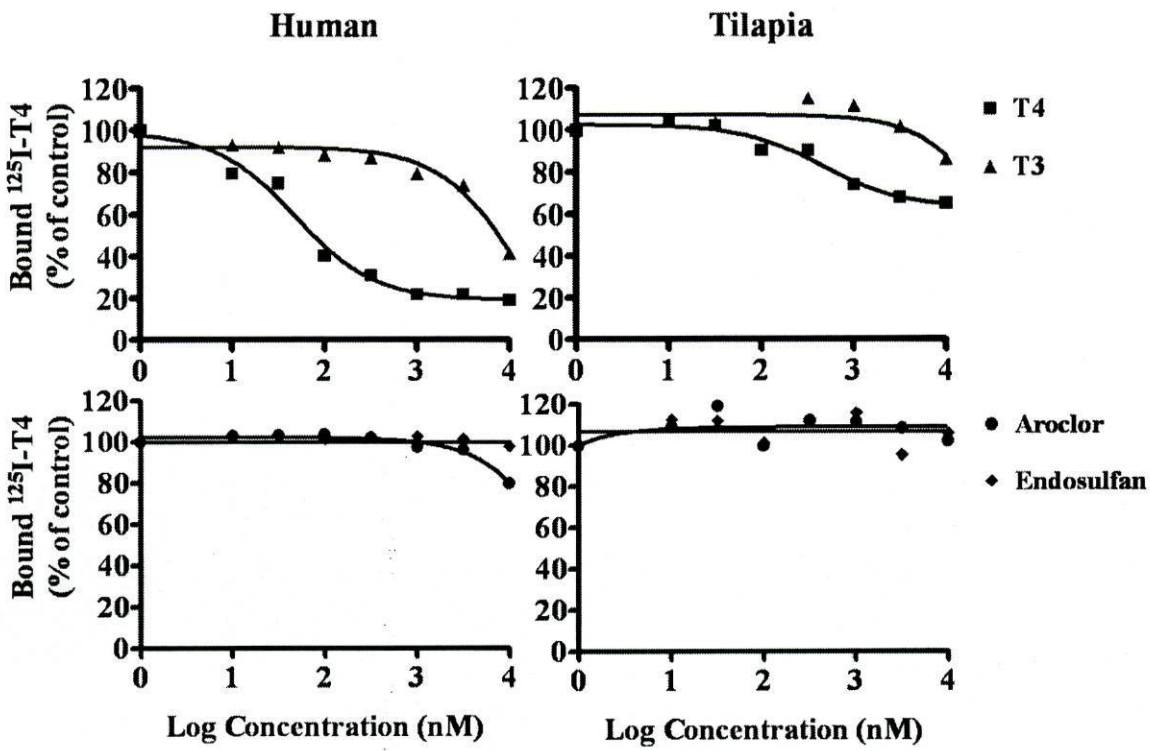


Fig 4: Competition with ^{125}I-T_4 for the binding to human and tilapia TTR. Unlabelled T_4 and T_3, Endosulfan and Aroclor 1254 were incubated with human (40 times diluted) or tilapia plasma (10 times diluted) in the presence of ^{125}I-T_4. Bound ^{125}I-T_4 was separated from unbound ^{125}I-T_4 by gel filtration.

None of the polychlorinated compounds competed with T_4 for the binding to tilapia proteins. Aroclor 1254 showed a small competition for the binding to human TTR not evident by PAGE analysis.

In vivo assays

To study possible alterations caused by the exposure to Aroclor 1254 or Endosulfan on the distribution of T_4 by tilapia tissues, ^{125}I-T_4 was injected in the blood stream of control and exposed tilapias. Previously, and to know if these pollutants interfere with the plasma levels of T_4 and T_3, these hormone concentrations were determined by RIA (Fig. 5).

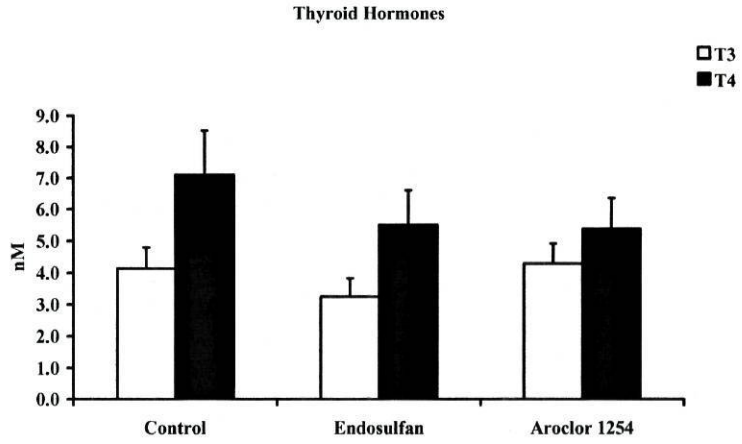


Fig 5: T_3 (□) and T_4 (■) total plasma concentration on control tilapias and tilapias fed with Endosulfan or Aroclor 1254 for 21 days. Results presented as Mean±SE (n=6).

After 21 days of exposure to 0.5 $\mu g.g^{-1}$ of Endosulfan or Aroclor 1254 through the diet, T_4 and T_3 total plasma concentration were the same on control and treated tilapias. Meaning that, the concentration used did not interfere with the total amount of thyroid hormones present in blood.

Possible alterations on T_4 tissue distribution and uptake were evaluated by measuring the amount of radiolabelled T_4 in plasma and tissues two hours after injection. Radioactivity distribution by tilapia tissues is shown on Fig. 6 and Table 1.

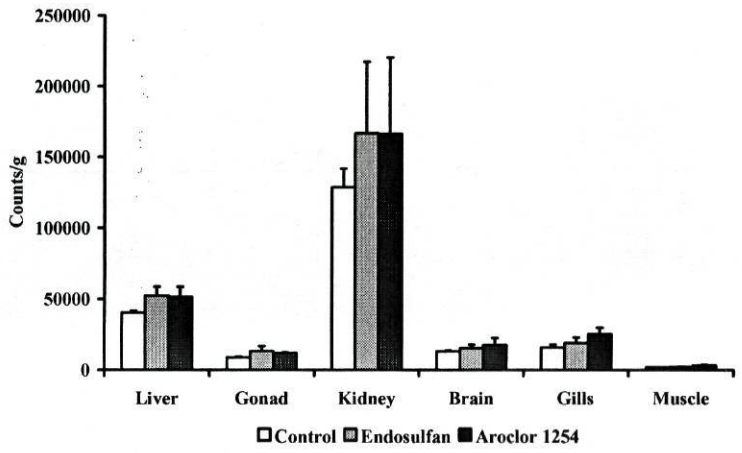


Fig 6: Radioactive counts per gram of organ two hours after the injection of ^{125}I-T_4 on Control Endosulfan and Aroclor 1254 fed tilapias. Results presented as Mean±SE.

Table 1: Percentage of the injected radioactivity present in the organs two hours after injection. Results presented as Mean±SE.

	LIVER	GONAD	KIDNEY	BRAIN	GILLS
CONTROL (N=4)	**6.40±0.608**	**0.63±0.082**	**2.12±0.321**	**0.06±0.007**	**1.94±0.284**
ENDOSULFAN (N=5)	**8.25±1.181**	**0.68±0.172**	**3.51±1.196**	**0.08±0.011**	**2.62±0.538**
AROCLOR 1254 (N=2)	**10.53±1.973**	**0.83±0.018**	**2.64±0.931**	**0.07±0.009**	**3.09±0.050**

The results show that two hours after injection ^{125}I-T_4 was distributed by all analyzed organs. Organs that presented the higher amounts of ^{125}I-T_4 were liver, kidney and gills, since they are highly irrigated. Liver, kidney and brain, as preferential organs on thyroid hormones metabolism presented the highest radioactivity values per gram of tissue, as expected. No differences were found between control and treated tilapias for a $p<0.05$.

Radioactivity percentage present in blood two hours after injection was estimated based on tilapia weight (Okimoto et al., 1994), values found were of 5-7 % of total injected ^{125}I-T_4 in all tilapia and most of the radiolabeled hormone present was found to be bound to plasma proteins (81-85 %). Endosulfan and Aroclor 1254 exposures did not interfere with the binding of T_4 to its transport proteins.

DISCUSSION

Effects of polychlorinated compounds on thyroid hormones transport and distribution on lower vertebrates is still a controversial and poorly studied area. The main objective of the present study was to contribute to understand the effects of these compounds in Nile tilapia thyroid hormones transport by plasma proteins and in thyroid hormones tissue distribution.

In the present study, after dietary exposure to Endosulfan or Aroclor 1254 (0.5 $\mu g.g^{-1}$), tilapias thyroid hormones plasma concentration remained unaltered; this was in accordance with a previous study performed in similar conditions (Coimbra et al., 2005). In that study, it was demonstrated that deiodinases activities changed in the presence of these compounds and only the 0.1 $\mu g.g^{-1}$ Endosulfan dose led to a decrease of T_4 plasma concentration, while T_3 levels were unaffected (Coimbra et al., 2005). This could suggest an interference with T_4 transport by plasma proteins, increasing its clearance. PCBs are known for interfering with T_4 binding to TTR (Lans et al., 1993) and also with TBG and Alb in mammals (Mckinney and Waller, 1994). So, in the current study we proposed to evaluate the actions, *ex vivo* and *in vivo*, of Endosulfan and Aroclor 1254 in the distribution and transport of thyroid hormones, especially T_4. We also tried to identify tilapia thyroid hormones transport plasma proteins and to isolate tilapia TTR.

Since fish TTR studies reported until now were performed in salmonids and sparids, we chose to use a cyclid fish that is employed as biological model in many studies and is native from countries with known contamination by polychlorinated compounds.

Previous studies where fish plasma incubated with labeled thyroid hormones were analyzed by PAGE (Santos and Power, 1999; Richardson et al., 2005) showed that fish thyroid hormones binding plasma proteins present a migration pattern different from proteins of other vertebrate classes and concluded that the anodic protein observed should be TTR due to its migration on native PAGE and affinity to bind thyroid hormones.

Two T_4 binding proteins were observed to be present both, *in vivo* and *ex vivo*, assays. One of the proteins, Ct, we believe to be tilapia Alb, as it is the most abundant and the most common to be present on fish plasma. The other protein, An, according to its migration pattern in native PAGE at pH 8.3 (anodal to Alb and similar migration to recombinant SB) and to its binding to T_4 and T_3 (Richardson et al., 1994 and 2005, Schreiber and Richardson, 1997) could be tilapia TTR. This protein fraction when excised from the native gel and analyzed by SDS-PAGE demonstrated the presence of more than one protein band in the sample though the only protein of aproximately 14 kDa (molecular weight of the TTR monomer) was identified as apolipoprotein-like by N-terminal sequencing. For that reason, our results indicated that the apolipoprotein-like is another

thyroid hormone transport protein in tilapia however we could not confirm the occurrence of tilapia TTR. Therefore, if present, tilapia TTR either occurs at very low concentrations or has a different migration in native gel electrophoresis reflecting perhaps differences in the protein molecular structure.

Apolipoproteins, especially apo A1 (25 kDa) and apo A2 (13kDa) were previously described as the major thyroid hormones transporters in trout (*Onchorynchus mykiss*) plasma (Babin, 1992). In addition, apo A1 binds to TTR in human blood (Sousa et al., 2000). Then, TTR might also occur associated with lipoproteins in tilapia plasma.

Both polychlorinated compounds tested did not compete with T_4 for the binding to tilapia An protein and to human TTR.

Interestingly, different fish species showed different T_4 and T_3 plasma binding proteins (data not shown) suggesting that plasma thyroid hormones transport proteins in fish may vary between species, increasing the difficulty in protein analysis and contaminants effects evaluation.

Endosulfan or Aroclor 1254 exposure did not disturb T_4 distribution by tilapia tissues. In addition, the levels of bound T_4 and free T_4 present in exposed tilapias were equal to the ones presented by control tilapias, indicating that *in vivo* these two compounds do not interfere with the transport of T_4. The *in vivo* tilapia T_4 distribution assay was not sufficient to ascertain Endosulfan and Aroclor 1254 effects in thyroid hormone transport by plasma proteins.

In conclusion, this study showed the presence of two thyroid hormones binding proteins in plasma and identified an apolipoprotein-like as a possible T_4 and T_3 transport protein in tilapia plasma. Also, it has been demonstrated that Endosulfan does not interfere with the transport and distribution of T_4 by tilapia plasma proteins, while Aroclor 1254 displaces this hormone from tilapia Ct, "albumin", though not observed in this study, possibly increasing this hormone clearance.

REFERENCES

Almeida, M.R., Damas, A.M., Lans, M.C., Brouwer, A., Saraiva, M.J., 1997.Thyroxine binding to transthyretin Met 119. Comparative studies of different heterozygotic carriers and structural analysis. Endocrine. 6, 309-315.

Altland, K., Winter, P., Sauerborn, M.K., 1999. Electrically neutral microheterogeneity of human plasma transthyretin (prealbumin) detected by isoelectric focusing in urea gradients. Electrophoresis 20, 1349-1364.

Babin, P.J., 1992. Binding of thyroxine and 3,5,3'-triiodothyronine to trout plasma lipoproteins. Am. J. Physiol. 262, E712-E720.

Birnbaum, L.S., 1994. Endocrine effects of prenatal exposure to PCBs, dioxins, and other xenobiotics: Implications for policy and future research. Environ. Health Perspect. 102, 676-678.

Brown, S.B., Adams, B.A., Cyr, D.G., Eales, J.G., 2004. Contaminant effects on the teleost fish thyroid. Environ. Toxicol. Chem. 23, 1680–1701.

Brucker-Davis, F., 1998. Effects of environmental synthetic chemicals on thyroid function. Thyroid 8, 827-56.

Chauhan, K.R., Kodavanti, P.R., McKinney, J.D., 2000. Assessing the role of ortho-substitution on polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. Toxicol. Appl. Pharmacol. 162, 10-21.

Coimbra, A.M., Reis-Henriques, M.A., Darras, V.M., 2005. Circulating thyroid hormone levels and iodothyronine deiodinase activities in Nile tilapia (*Oreochromis niloticus*) following dietary exposure to Endosulfan and Aroclor 1254. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 141, 8-14.

Eneqvist, T., Lundberg, E., Nilsson, L., Abagyan, R., Sauer-Eriksson, A.E., 2003. The transthyretin-related protein family. Eur. J. Biochem. 270, 518-32.

Eneqvist, T., Lundberg, E., Karlsson, A., Huang, S., Santos, C.R., Power, D.M., Sauer-Eriksson, A.E., 2004. High resolution crystal structures of piscine transthyretin reveal different binding modes for triiodothyronine and thyroxine. J. Biol. Chem. 279, 26411-26416.

Folli, C., Pasquato, N., Ramazzina, I., Battistutta, R., Zanotti, G., Berni, R., 2003. Distinctive binding and structural properties of piscine transthyretin. FEBS Lett. 555, 279-284.Funkenstein, B., Perrot, V., Brown, C.L., 1999. Cloning of putative piscine (*Sparus*

aurata) transthyretin: developmental expression and tissue distribution. Mol. Cell. Endocrinol. 157, 67-73.

Furuya, H., Saraiva, M.J., Gawinowicz, M.A., Alves, I.L., Costa, P.P., Sasaki, H., Goto, I., Sakaki, Y., 1991. Production of recombinant human transthyretin with biological activities toward the understanding of the molecular basis of familial amyloidotic polyneuropathy (FAP). Biochemistry 30, 2415-2421.

Hallgren, S., Darnerud, P.O., 2002. Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats-testing interactions and mechanisms for thyroid hormone effects. Toxicology 177, 227-243.

Kavlock R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D.M., Sinks, T., Tilson, H.A., 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored workshop. Environ. Health Perspect. 104, suppl. 4, 715-740.

Langer, P., 1998. Polychlorinated biphenyls and the thyroid gland –minireview. Endocrine Reg. 32, 193-203.

Lans, M.C., Klasson-Wehler E., Willemsen, M., Meussen, E., Safe, S., Brouwer, A., 1993. Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-p-dioxins and -dibenzofurans with human transthyretin. Chem.-Biol. Interactions 88, 7-21.

Lans, M.C., Spierz, C., Brouwer, A., Koeman, J.H., 1994. Different competition of thyroxine binding to transthyretin and thyroxine-binding globulin by hydroxy-PCBs, PCDDs and PCDFs. Eur. J. Pharmacol. 270, 129-136.

Larsson, M., Pettersson, T., Carlstrom, A., 1985. Thyroid hormone binding in serum of 15 vertebrate species: isolation of thyroxine-binding globulin and prealbumin analogs. Gen. Comp. Endocrinol. 58, 360-75.

Leatherland, J.F., 1994. Reflections on the thyroidology of fishes: from molecules to humanking. Guelph Ichthyology Reviews 2, 1-67.

Li, M.H., Hansen, L.G. 1997. Consideration of enzyme and endocrine interactions in the risk assessment of PCBs. Reviews in Toxicology 1, 71-156.

McKinney, J.D., Waller, C.L., 1994. Polychlorinated biphenyls as hormonally active structural analogues. Environ. Health Perspect. 102, 290-297.

Mendel, C.M., Weisiger, R.A., Jones, A.L., Cavalieri, R.R., 1987. Thyroid hormone-binding proteins in plasma facilitate uniform distribution of thyroxine within tissues: a perfused rat liver study. Endocrinology 120, 1742-1749.

Okimoto, D. K., DiStefano, J. J., Kuwaye, T. T., Ron, B., Weber, G. M., Nguyen, T. T. and Grau, E. G., 1994. On plasma volume measurement and the effect of experimental stress in male tilapia, *Oreochromis mossambicus*, maintained in fresh water. Fish Physiol. Biochem. 12, 431-438.

Richardson, S. J., Bradley, A.J., Duan, W., Wettenhall, R.E.H., Harms, P.J., Babon J.J., Southwell, B.R., Nicol, S., Donnellan, S.C., Schreiber, G., 1994. Evolution of marsupial and other vertebrate thyroxine-binding plasma proteins. Am. J. Physiol. 266, R1359-R1370.

Richardson, S.J., Monk, J.A., Shepherdley, C.A., Ebbesson, L.O., Sin, F., Power, D.M., Frappell, P.B., Kohrle, J., Renfree, M.B., 2005. Developmentally regulated thyroid hormone distributor proteins in marsupials, a reptile, and fish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 288, R1264-R1272.

Rolland, R.M., 2000. A review of chemically-induced alterations in thyroid and vitamin A status from field studies of wildlife and fish. J. Wildl. Dis. 36, 615-635.

Santos, C.R., Power, D.M., 1999. Identification of transthyretin in fish (*Sparus aurata*): cDNA cloning and characterisation. Endocrinology 140, 2430-2433.

Saraiva, M.J.M., Costa, P.P., Goodman, D.S., 1988. Transthyretin (prealbumin) in familial amyloidotic polineuropathy: genetic and functional aspects. In: Adv. Neurol. DiDonato, S., Mamoli, A., DiMauro, S., Rowland, L.P. (eds). 48, 189-200, Raven Press, New York.

Schreiber, G., Richardson, S.J., 1997. The evolution of gene expression, structure and function of transthyretin. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 116, 137-160.

Sinha, N., Lal, B., Singh, T.P., 1991a. Effect of endosulfan on thyroid physiology in the freshwater catfish, Clarias batrachus. Toxicology 67, 187-197.

Sinha, N., Lal, B., Singh, T.P., 1991b. Pesticides induced changes in circulating thyroid hormones in the freshwater catfish Clarias batrachus. Comp. Biochem. Physiol. C. 100, 107-110.Sousa, M.M., Berglund, L., Saraiva, M.J., 2000. Transthyretin in high density lipoproteins: association with apolipoprotein A-I. J. Lipid. Res. 41, 58-65.

Tagawa, M., Tanaka, M., Matsumoto, S., Hirano, T., 1990. Thyroid hormones in eggs of various freshwater, marine and diadromous teleosts and their changes during egg development. Fish Physiol. Biochem 8, 515-520.

Van den Berg, K.J., van Raaij, J.A.G.M., Bragt, P.C., Notten, W.R.F., 1991. Interactions of halogenated industrial chemicals with transthyretin and effects on thyroid hormone levels in vivo. Arch. Toxicol. 65, 15-19.

Whitlock, J.P., 1994. The aromatic hydrocarbon receptor, dioxin action, and endocrine homeostasis. Trends Endocrinol. Metab. 5, 183-188.

Yamauchi, K., Nakajima, J.I., Hayashi, H., Hara, A., 1999. Purification and characterization of thyroid-hormone-binding protein from masu salmon serum: A homolog of higher-vertebrate transthyretin. FEBS J. 265, 944-949

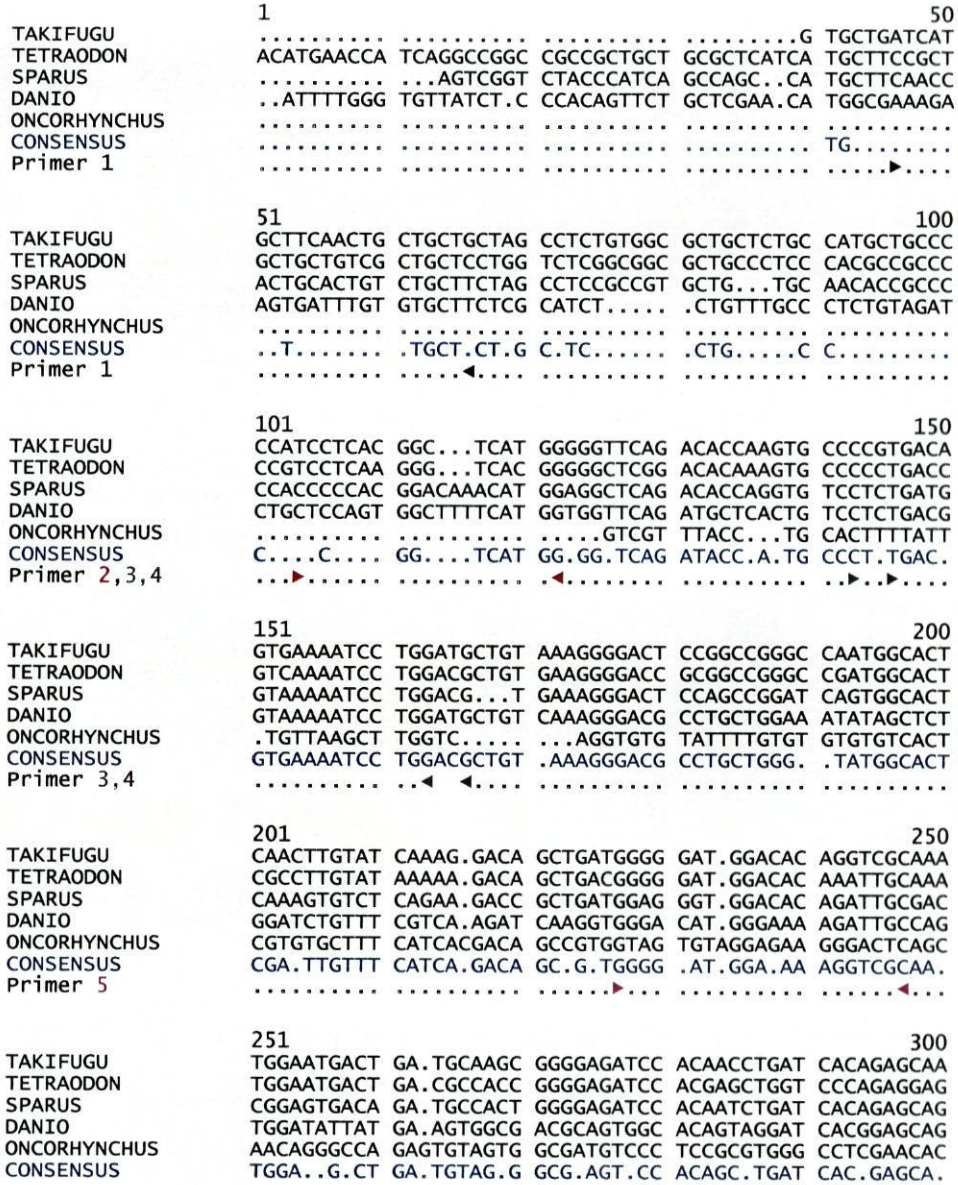
CAPÍTULO 4-TRABALHO COMPLEMENTAR

Search for tilapia transthyretin gene by RT-PCR



Since the efforts to isolate tilapia's TTR protein from the plasma were unsuccessful, a different approach was taken. Thus, a strategy was devised to isolate tilapia's TTR gene.

Recently, some fish TTR gene sequences have become available. Based on those, several sets of primers were designed, both on conserved and non conserved regions (Fig. 1).



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                    301                                                 350
TAKIFUGU            AAGTTCCTCC CAGGGGTGTA CCGGGTGGAC TTTGACACCA AGTCTTACTG
TETRAODON           GCCTTCCCCC CAGGGCTCTA CCGGCTGGAC TTCGACACCA AGGCCTACTG
SPARUS              CAGTTCCCAG CAGGAGTGTA TCGTGTTGAG TTTGACACCA AAGCTTACTG
DANIO               GAGTTCACTC CTGGTGTGTA TCGGGTGGAG TTTGACACTT TAACCTACTG
ONCORHYNCHUS        CACTTCAGCA GTCTCGTGGA AAGG.TGTGG TT...CCCTG AGACTTCCAG
CONSENSUS           .AGTTCA.CC CTGG.GTGTA .CGGGTGGAG TTTGACACT. AGACTTACTG

                    351                                                 400
TAKIFUGU            GAAGAATGAG GGAAGTGTAC CGTTTCATGA AGTGACTAAT ......GTTG
TETRAODON           GATGAACGAG GGAAATACCC CCTTTCATGA AGTGACCAA. .....CGTTG
SPARUS              GACGAATCAG GGCAGCACGC CGTTCCATGA AGTAGCTGA. .....GGTGG
DANIO               GAAGACAGAG GGTCGCACAC CTTTCCACCA GCTGGCTGAT GCAATGGTGG
ONCORHYNCHUS        TAGGCTTTAG TGTCAAACTC AACTCGGTAC ACCCCCGACT GGA..AGTCC
CONSENSUS           GA.GA.TGAG GGTCG.AC.C C.TTCCAT.A ACTG.CTAAT G.A...GT.G

                    401                                                 450
TAKIFUGU            TCTTTGAAGC TCACTCTGAA GGCCATCGTC ACTACACGTT GGCCATGCTG
TETRAODON           TGTTTGAAGC TCACGCTGGA GGCCACCGCC ACTACACGCT GGCCATGCTG
SPARUS              TGTTTGACGC CCATCCTGAA GGCCATCGTC ACTACACCTT GGCTCTGCTG
DANIO               TGTTTGAAGC TCATGCAGAG GGACATCGTC ATTACACACT GGCTCTCCTT
ONCORHYNCHUS        TGATCTGAGA TCA.GATTAT GGACTTCCCC TGTCAAGTCT GTCACTCCAC
CONSENSUS           TGTTTGAAGC TCA.GCTGA. GGACATCGTC A.TACAC.CT GGC.CTCCT.
Primer 6,7,         .......... .......... .........► ..►....... ........◄.

                    451                                                 500
TAKIFUGU            CTGAGTCCAT ACTCGTTCAC CACCACAGCT CTGGTCACAG ACGTTCAACA
TETRAODON           CTGAGCCCGT ACTCCTTCAC CACCACGGCT CTGGTCTCCG ACGTTCCCCA
SPARUS              CTCAGTCCGT TCTCCTACAC CACCACCGCC GTGGTCTCCA GCGTACACGA
DANIO               CTGAGCCCTT TCTCGTACAC CACAACTGCT GTGGTCGTCA AGGCACATGA
ONCORHYNCHUS        TAGCAACCT. ....GTGCCC AGGTCATTCC ATTGACTC.G TCGGGAGACA
CONSENSUS           CTGAG.CCTT .CTCGT.CAC CAC.ACTGCT .TGGTCTC.G ACG..CAACA
Primer 7,8,9,10     .◄.►..... .......... ..◄....... ..►..►.... ..........

                    501                                                 550
TAKIFUGU            CTGA..... .......... .......... .......... ..........
TETRAODON           CTGAC..CC. .........G CCCGTGCACG CCCCCGTGCA CGCCCACCCA
SPARUS              GTGAC..CC. .........G C........G TCACACTGAG ATCTTGTTT.
DANIO               CTGACATCCT TTAAAAGATG GCCCATATCA ACAACTTGAA ACCAAATACT
ONCORHYNCHUS        CTCAGTGCC. .......... ....ACGGCG CCCGCTGGTA CACCCTTCAC
CONSENSUS           CTGA..... .........G ..CCA...CG .C..CT.G.A ..C...T...
Primer 9,10         .◄.◄..... .......... .......... .......... ..........

                    551                                                 600
TAKIFUGU            .......... .......... .......... .......... ..........
TETRAODON           ..CGCACTCA CCTCCAGAAG GCCC..CAGG CAG..ACGCA GCCGTTCCCT
SPARUS              ....CTCTCA TGTTCTGAT. ...C..CAAA CCA..ACATA GTATTTTTT
DANIO               GTACATCCCG AGTTCAAGAT TACCTTCTTG CAGTAACCAA GTCATTCGAT
ONCORHYNCHUS        ..CGCATCCA AAATCTTAAC CATCAACGGG CAG....TGA GTGTCTGACT
CONSENSUS           ....C..CCA A..TC..AA. .A.C..C..G CAGT.....A GT...T...T

                    601                                                 650
TAKIFUGU            .......... .......... .......... .......... ..........
TETRAODON           CGCTCTGACC GACTGCATCA GGGCGGCAAA .....GAAAC AA....GAGG
SPARUS              TTCCATG... ..CATCTTCT TTTC..CTCT .....GTGCC AT....AAAG
DANIO               TGCCATTTCA ATAAGTATTT ATGTAGGTAT TTGTGGTAAC TTTTTGATAG
ONCORHYNCHUS        CACCATGCCT GTC..CACTG GGGCGGCGCT ACAGAGTAGG ACAGCAGTGG
CONSENSUS           ..CCATG.C. .TC..CA.T. ..GC.GC..T .....GTA.. A......T.G

                    651                                                 700
TAKIFUGU            .......... .......... .......... .......... ..........
TETRAODON           CACACGTCCG CTCTGACCTC CAGAGTACGG CAACGACACT TTTATTTGAA
SPARUS              CTC....... ......CATC CAAACAAAGA AAA..ATGAT TAAAAT...A
DANIO               CTTTAAACAT TTCATCACAC CTGGTTTTCA TAAATCTATT TACAAGTGAG
ONCORHYNCHUS        CTAG...... ..CAGCACAC AGAGCAGTGA ACTGTCCATT TGGAGCGATA
CONSENSUS           CT........ ..CA.CACAC ...G...TGA ....TC.ATT T..A.....A

                    701                                                 750
TAKIFUGU            .......... .......... .......... .......... ..........
TETRAODON           ....ATTTGA TGATTTAAAA ATCAAA.ATA AAAACGT... TTAAAAACGA
SPARUS              ....CATTGA TGAGCCAAAA AAAAAA.AAA AA........ ..........
DANIO               GGACACTAGT TACGCTTCCA GCCAAAGATG CGAATGTGAC TTATGTGCGA
ONCORHYNCHUS        ....ACTGGT TGGCGGCTCT AACACAGTCA AACCTATAAA CTTAGACTGA
CONSENSUS           ....ACT.GT TG......C. A.CA.AG..A AA..T.T.A. .T..G...GA
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                751                                                       800
TAKIFUGU        .......... .......... .......... .......... ..........
TETRAODON       CA........ .......... .......... .......... ..........
SPARUS          .......... .......... .......... .......... ..........
DANIO           AACTGGAATA TCATTTAAAA CATTTGCGAA TAAAGCACCA TTTCTATCCA
ONCORHYNCHUS    CCCTCGTGC. .......... .......... .......... ..........
CONSENSUS       ..CT.G.... .......... .......... .......... ..........

                801             820
TAKIFUGU        .......... ..........
TETRAODON       .......... ..........
SPARUS          .......... ..........
DANIO           ATGAGCAAAA GAGAATGAAA
ONCORHYNCHUS    .......... ..........
CONSENSUS       .......... ..........
```

Fig. 1: Multiple fish transthyretin gene sequence alignment showing the consensus sequence and the location of the designed primers. Ensemble gene ID: *Takifugu rubripes* (**NEWSINFRUG00000149824**), *Tetraodon nigroviridis* (GSTENG00019814001). GenBank accession numbers: *Sparus aurata* (AF059193), *Danio rerio* (BC081488), *Oncorhynchus mykiss* (CB497711).

When there were different nucleotides for the same position, the option taken was to use the one from the *Sparus aurata* sequence, since phylogenetically it is the species with higher proximity to tilapia (Fig. 2).

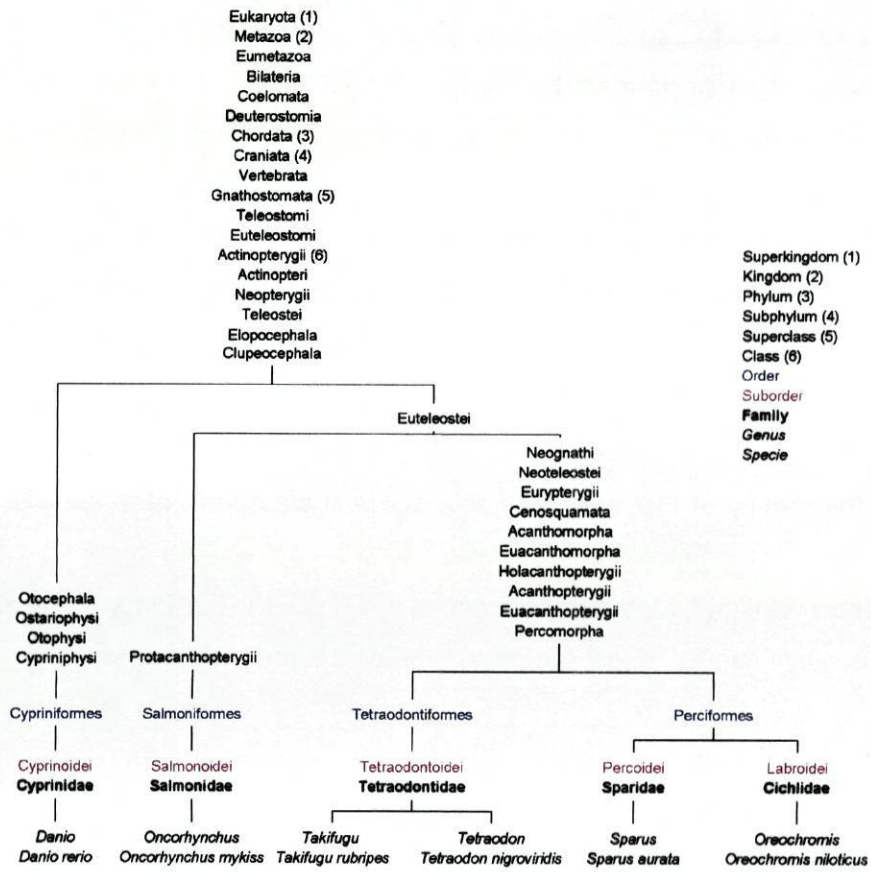


Fig. 2: Taxonomic tree representing the fish species where the TTR gene sequence is known and tilapia (adapted from NCBI Taxonomy Browser).

MATERIAL AND METHODS

Animal tissue for RNA extraction

Adult tilapia liver or 10 days tilapia larvae were collected to RNA later (Sigma) and frozen at -80 °C until RNA was extracted.

RNA extraction was performed using the "RNeasy Midi Kit (QIAGEN).

cDNA

Tilapia liver and larvae cDNA was prepared using the "SuperScript. First-Strand Synthesis System for RT-PCR" (Invitrogen).

Polymerase Chain Reaction (PCR)

PCR was performed on a 20 µl solution with: 1 µl of cDNA, 0.4 µM of each oligonucleotide primer (Table), 0.2 mM of each dNTP, 1 unit of Taq polymerase (Eppendorf) and 1 X Taq polymerase self adjusting magnesium buffer. The PCR program started with a 2 min denaturation at 94 °C, followed by cycles (30-45): 45 s at 93 °C; 30 s at annealing temperature (50-60 °C); 30 s at 72°C; and ended with 10 min at 72 °C The number of cycles and the annealing temperature depended on the set of primers used.

The analysis was performed by loading 5-10µl of the PCR product on a 1.5-2 % agarose gel with 0.4 $\mu g.ml^{-1}$ of ethidium bromide in TAE buffer (Tris base 40 mM; EDTA-Na_2 1 mM; Acetic acid 20 mM).

Gel bands, with the expected number of base pairs, were excised, purified using the "QIAquick Gel Extraction Kit" (QUIAGEN) and sent to be sequenced (StabVida).

Controls

To test the quality of the isolated tilapia cDNA it was performed a PCR using β-actin primers.

A pBluescipt plasmid containing sea bream TTR cDNA (Santos and Power, 1999) was used as a positive control to test the several sets of primers.

Table 1: Primers sequences.

	Primer	Sequence (5'→3')
Forward	**1**	CAA CCA CTG CAC TGT CTG CT
	2	CCC CCA CGG ACA AAC ATG G
	3	CTG ATG GTA AAA ATC CTG GA
	4	CTC TGA TGG TAA AAA TCC TGG
	5	GGA GGG TGG ACA CAG ATT GC
Reverse	**6**	CAC TAC ACC CTG GCT CTG CT
	7	TAC ACC TTG GCT CTG CTG CT
	8	AGT CCG TTC TCC TAG ACC AC
	9	CTC CAG CGT ACA CGA GTG A
	10	GGT CTC CAG CGT ACA CGA GT

RESULTS

None of the analyzed tilapia sequences presented homology with the known TTRs sequences and therefore were not identified as tilapia TTR.

Tilapia β-actin amplification was successful, indicating a good cDNA quality and the expected sequences were always amplified with the positive control.

DISCUSSION

The attempt to identify the tilapia TTR gene was unsuccessful and since the tilapia cDNA isolation was successful as shown by β-actin amplification and the primers used functioned with a positive control. Three hypotheses to justify this result can be put forward:

a) The primers used for RT-PCR with tilapia larvae or adult liver cDNA were designed both on conserved and non conserved regions of the fish TTR gene and assuming that sea bream TTR sequence would be the one with higher homology to tilapia TTR; as none of the primer combinations amplified a tilapia sequence homologue to fish TTRs the first hypothesis is that the tilapia TTR gene sequence is highly divergent from the known fish TTR's, thus the primers were not appropriate for tilapia. This could be tested by designing degenerated primers in the most conserved TTR gene regions;

b) In sea bream TTR is expressed in larvae and in liver of both immature and adult fish as shown by Northern analysis (Funkenstein et al., 1999, Santos and Power, 1999, Power et al., 2000). Assuming that the homology between tilapia TTR and other fish TTRs is high and that the primers were appropriate, the third hypothesis is that tilapia TTR gene, in opposition to sea bream, is not expressed on larvae and adult tilapia liver.

c) The fourth hypothesis, probably the most unlikely, is that tilapia does not have the TTR gene. The use of Southern blot analysis would allow the detection of the tilapia TTR gene, even if it is not expressed. However, this would imply some similarity between tilapia and other fish TTRs, for the occurrence of cross-hybridization.

REFERENCES

FUNKENSTEIN B, PERROT V, BROWN CL (1999). Cloning of putative piscine (Sparus aurata) transthyretin: developmental expression and tissue distribution. *Molecular and Cellular Endocrinology.* **157**:67-73.

NCBI Taxonomy Browser- http://www.ncbi.nlm.nih.gov/entrez/query.fcgi

POWER DM, ELIAS NP, RICHARDSON SJ, MENDES J, SOARES CM, SANTOS CRA (2000). Evolution of the thyroid hormone-binding protein, transthyretin. *General and Comparative Endocrinology.* **119**:241-255.

SANTOS CRA, POWER DM (1999). Identification of transthyretin in fish (Sparus aurata): cDNA cloning and characterisation. *Endocrinology.* **140**:2430-2433.

ICBAS

CAPÍTULO 5

COIMBRA AM[a], Reis-HENRIQUES M[a]A (2005). Nile tilapia, *Oreochromis niloticus* L., reproduction inhibition by dietary exposure to Aroclor 1254. *Bulletin of Environmental Contamination and Toxicolology* **75**:407-412.



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Bulletin of Environmental Contamination and Toxicology

Nile Tilapia, *Oreochromis niloticus* L., Reproduction Inhibition by Dietary Exposure to Aroclor 1254

A. M. Coimbra, M. A. Reis-Henriques

ICBAS, Department of Applied Physiology, Abel Salazar Institute for Biomedical Sciences, University of Porto, Largo do Prof. Abel Salazar number 2, 4099-003, Porto, Portugal, and CIIMAR, Laboratory of Environmental Toxicology, Center of Marine and Environmental Research of University of Porto, Rua dos Bragas 289, 4050-123, Porto, Portugal

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Polychlorinated biphenyls (PCBs) belong to a family of environmental contaminants that persist for a long period in ecosystems. Their lipophilic properties are responsible for bioaccumulation in food chains. PCBs are associated with a large variety of biologic and toxicological effects (Dragnev et al. 1994), being capable of reproductive and endocrine disruption in all classes of vertebrates (Alston et al. 2003). Usually, PCBs are present as complex mixtures of several PCB congeners, like Aroclor 1254 which is a commercial mixture of PCBs congeners with 54% chlorine in weight. Reports on PCBs exposure, both in wild and laboratory fish populations, describe a wide variety of alterations connected with reproduction, such as decreased hatching success, low survival rates, impaired development of eggs and embryos, reduced and delayed spawning, inhibition of spermatogenesis, testicular abnormalities and disruption of the reproductive endocrine function (review in Örn et al. 1998).

Tilapia belong to one of the most commercially important groups of freshwater fish in world aquaculture (Coward and Bromage 2000). These factors affecting reproduction may cause severe ecological effects, as well as economical ones.

In order to determine if dietary exposure to PCBs interferes with tilapia reproduction, tilapia were exposed to Aroclor 1254 through the diet. We tested the effects of two doses of this compound at two exposure periods. In this study, progesterone and testosterone plasma levels were measured to evaluate PCBs impact on gonadal steroidogenesis. Gonadal histology was performed.

MATERIALS AND METHODS

Aroclor 1254 was purchased from NSI Solutions, Inc. (Raleigh, USA) as a pure standard.

One week before the beginning of the assay, 8 adult tilapia, <1 year old, (average size and weight: 19.9±0.4cm and 170.3±10.8g) were randomly allocated, in five 70L tanks (Control and Aroclor 1254 two doses-two exposure times), with an individual mechanical and biological filtration system to each dose group.

Correspondence to: A. M. Coimbra, CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas 289, 4050-123, Porto, Portugal

Supplemental aeration was provided to maintain dissolved oxygen near saturation and water temperature was kept at 17°C, since at this temperature tilapia gonads remain immature. The water source was the public water supply. Before use water was de-chlorinated and an approximate 3L renovation occurs per hour in each system.

Throughout the assay, specimens were exposed through the diet to two different Aroclor 1254 doses (0.5 and 4.5μg/g - Aroclor 1254/g food). Animals were fed with approximately 2% of their body weight once a day with the contaminated diet. During the assay they were kept under ideal reproduction conditions: 27°C water temperature and a 12-hour light, 12-hour dark photoperiod. Control tilapia experienced the same conditions and were fed with a commercial fish diet.

Five tilapia from each group were sacrificed at the end of 10 and 20 days of exposure (control was sacrificed at the end of 20 days). Simultaneously, the specimens were anaesthetised, blood was collected from the caudal vein, centrifuged and plasma was stored, until further analysis, at -80°C. All the organs collected were frozen and stored at -80°C. Whenever possible, a part of the gonad was fixed in Bouin Liquor (Panreac) and after dehydration included in paraffin, sectioned at 5μm, mounted on glass slides and stained with haematoxylin & eosin.

Testosterone and progesterone plasma levels were measured by solid-phase radio immuno assay (RIA) using ^{125}I Coat-a-Count kits (DPC, Los Angeles,USA), with antibody precoated tubes as was described for tilapia hormones (Rocha and Reis-Henriques 1996, Fontaínhas-Fernandes et al. 2000). The sensitivities of the standard curve were 0.02ng/ml for progesterone and 0.004ng/ml for testosterone. The antibodies are highly specific for both hormones, showing a 100% binding to progesterone, 50% binding for testosterone and a low cross-reactivity with other naturally occurring plasma steroids, such 3.4% to 17α-hydroxyprogesterone and 0.1% to pregnenolone and testosterone with the progesterone kit and 16% to 11-ketotestosterone and 0.02% to estradiol with the testosterone kit. The intra-assay coefficients were 6.7% for progesterone and 19.1% for testosterone. Inter-assay variation was avoided by measuring all samples in the same assay. All samples were analysed in duplicate.

All results are expressed as mean±standard error of the mean (SEM). Statistical analysis between control and exposed tilapia data was performed by analysis of variance (ANOVA) using the LSD test ($p<0.05$). Correlation coefficients were calculated using the Pearson correlation method ($p<0.05$).

RESULTS AND DISCUSSION

Although ideal reproduction conditions were maintained during the assay, reproduction did not occur, in either the control or in Aroclor 1254 exposed tilapia.

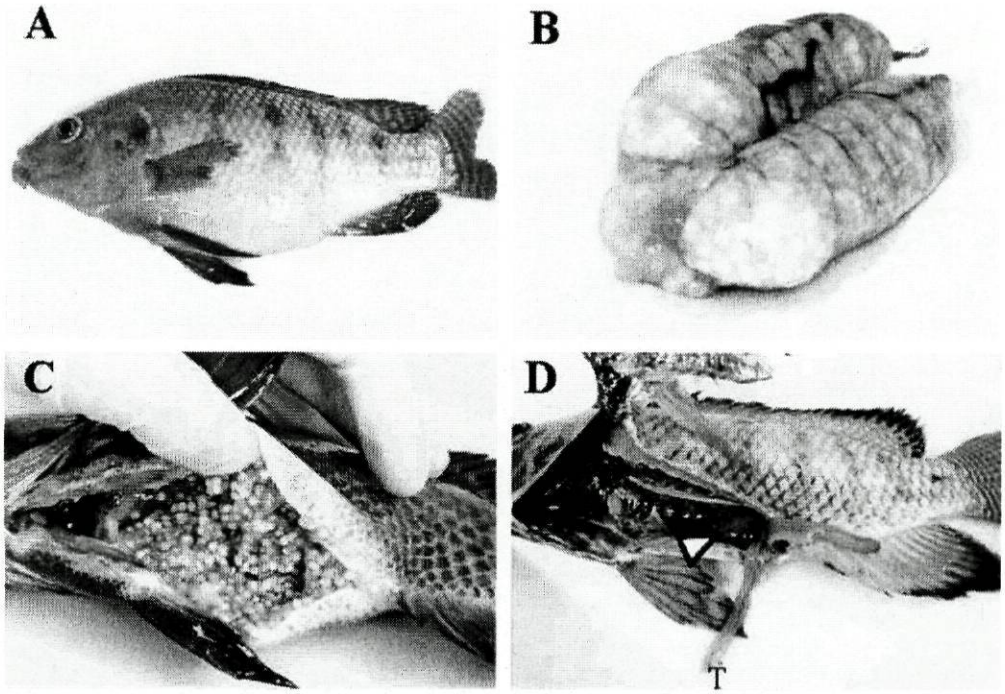


Figure 1. A-Female tilapia after being fed for 20 days with a diet containing 4.5μg/g of Aroclor 1254 presenting an increase in abdomen volume. B-Ovary of a tilapia fed for 20 days with a diet containing 4.5μg/g of Aroclor 1254, in which can be observed a high percentage of atretic oocytes. C-Ovary of a tilapia fed for 20 days with a diet containing 4.5μg/g of Aroclor 1254; in this case the ovary membrane had been damaged and a mass of atretic oocytes was found scattered in the abdominal cavity. D- Male tilapia fed for 20 days with a diet containing 4.5μg/g of Aroclor 1254. The arrowhead indicates the bladder full of urine, T-testis.

At the end of 20 days of exposure to Aroclor 1254, some tilapia from the group fed with 4.5μg/g of Aroclor 1254 presented an increased abdomen volume (Fig. 1A); during sampling it was observed that these tilapia were all females. The increase was due to an abnormally high number of oocytes in ovaries; also, the percentage of atretic oocytes was found to be greater than 40% (Fig. 1B), a value described as the maximum reached by Nile tilapia (review in Coward and Bromage 2000). Some females presented due to destruction of the ovary membrane, oocytes dispersed inside the abdominal cavity (Fig. 1C). These results indicate that while Aroclor 1254 does not interfere with tilapia oogenesis, spawning seems to be inhibited, resulting in accumulation of oocytes in the interior and rupture of ovary walls.

In male tilapia, the urinary bladder was visible and full of urine (Fig. 1D). This observation contrasts with that observed by Rocha and Reis-Henriques (1996) which described this organ as being compressed and empty between the gonads and the abdominal cavity wall upon manipulation.

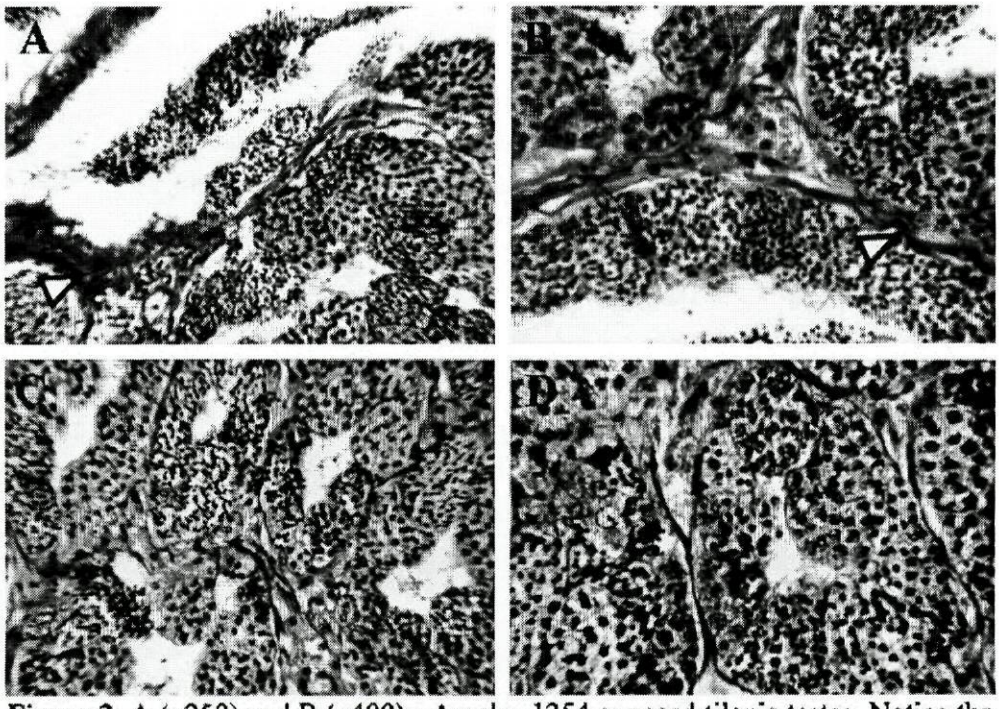


Figure 2. A (x250) and B (x400) - Aroclor 1254 exposed tilapia testes. Notice the spermatozoa in the lumen of the seminiferous tubules, the thickness of connective tissue of the walls (arrowheads) and the decline of the germinal epithelium. C (x250) and D (x400) - Control tilapia testes.

In female tilapia, the genital pore is separated from the urinary pore, while in males they form a common orifice. In this study, we observed no release of female gametes, while in males there was urine accumulation in the urinary bladder. These results suggest that exposure to Aroclor 1254 might cause obstruction of the genital pores. We speculate that the connective tissue surrounding the pore may have increased, thereby diminishing pore elasticity. Further studies on the morphology of tilapia genital pores, after Aroclor 1254 exposure are necessary to confirm this hypothesis.

Due to the severe damage of the ovaries, it was impossible to perform a histological evaluation of this organ. However, testes histology (Fig. 2) showed that Aroclor fed males presented a thicker seminiferous tubule wall compared to equivalently mature control males. Also we detected a decrease in germinal epithelial layers. Similar results were observed in mammals and birds, where the same testicular damages were described after Aroclor 1242 (Ahmad et al. 2003) and Aroclor 1254 exposure (Alston et al. 2003; Zhang and Qiao 2004).

Aroclor 1254 exposure significantly depressed progesterone levels in male tilapia (Table 1). Significant testosterone level depression was observed for males exposed to the lower dose for 20 days and to the higher dose for 10 days.

Progesterone and testosterone plasma concentrations were found to be positively

Table 1. Progesterone and testosterone (ng/ml) plasma levels of tilapia exposed to Aroclor 1254 and control. Values presented as Mean±SEM. (n) number of animals. Statistical differences between treatment and control:*p<0.05; **p<0.01; ***p<0.001.

Aroclor 1254 (µg/g)	Day	Male		Female	
		Progesterone	Testosterone	Progesterone	Testosterone
Control		2.34±0.71 (2)	51.17±5.23 (2)	0.75±0.06 (2)	37.47±1.91 (2)
0.5	10	0.56±0.36** (2)	23.26±15.68 (2)	0.17±0.07 (2)	9.63±2.88 (2)
0.5	20	0.49±0.24** (3)	17.11±6.57* (3)	0.44 (1)	19.46 (1)
4.5	10	0.25±0.14*** (3)	9.27±3.40** (3)	0.56 (1)	44.4 (1)
4.5	20	0.64±0.26** (2)	22.78±12.52 (2)	0.14±0.02 (2)	5.1±4.87 (2)

correlated (r=0.93; p<0.0001). In this study, control male tilapia presented plasma concentrations of 2.34±0.71 and 51.17±5.23ng/ml of progesterone and testosterone, respectively. In exposed tilapia (4.5µg/g for 10 days) a significant decrease of progesterone (0.25±0.14ng/ml) and testosterone (9.27±3.40ng/ml) was observed.

In male tilapia, no differences were found in testosterone and progesterone levels between 10 and 20 days of exposure to the lower Aroclor 1254 dose. Tilapia exposed 10 days to the higher dose presented significant (p<0.05) lower levels when compared to tilapia exposed 20 days. The observation that in the male both hormone levels at the end of 20 days were similar to those measured after exposure to 0.5µg/g, suggests a slight recovery of testes.

The decrease in progesterone plasma levels observed in male exposed tilapia, may explain the decline in testosterone levels, as progesterone is the precursor for testosterone production and a strong positive correlation was observed. Aroclor 1254 exposure inhibited tilapia testicular steroidogenesis. This was also described in rats where both *in vivo* and *in vitro* Aroclor 1248 exposure inhibited testicular steroidogenesis (Andric et al. 2000).

Due to the number of female specimens analyzed and the high variability among the hormone concentrations, it was not possible to observe significant differences. However, progesterone levels for females exposed to Aroclor 1254 were always lower than those of the control. The data on plasma hormone levels and ovarian morphology observations, suggest that ovarian steroidogenesis was also inhibited. The destruction of the ovary tissue, as demonstrated by the high number of atretic oocytes, is the likely cause.

In conclusion, a short exposure period to a low dose of Aroclor 1254 through the diet interrupts tilapia reproduction. The inhibiting mechanisms seem to result in alterations of sex steroid synthesis due to morphological alterations of the testicular and ovarian germinative tissues. Globally, our results do not discard the hypothesis of Aroclor 1254 being capable of producing effects at other levels of the reproductive axis.

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REFERENCES

Ahmad SU, Tariq S, Jalali S, Ahmad MM (2003) Environmental pollutant Aroclor 1242 (PCB) disrupts reproduction in adult male rhesus monkey (*Macaca mullata*). Environ Res 93:272-278

Alston DA, Tandler B, Gentles B, Smith EE (2003) Testicular histopathology in deer mice (*Peromyscus maniculatus*) following exposure to polychlorinated biphenyl. Chemosphere 52:283-285

Andric SA, Kostic TS, Stojilkovic SS, Kovacevic RZ (2000) Inhibition of rat testicular androgenesis by a polychlorinated biphenyl mixture Aroclor 1248. Biol Reprod 62:1882-1888

Coward K, Bromage NR (2000) Reproductive physiology of female tilapia broodstock. Rev Fish Physiol 10:1–25

Dragnev KH, Beebe LE, Jones CR, Fox SD, Thomas PE, Nims RW, Lubet RA (1994) Subchronic dietary exposure to Aroclor 1254 in rats: accumulation of PCBs in liver, blood, and adipose tissue and its relationship to induction of various hepatic drug-metabolizing enzymes. Toxicol Appl Pharmacol 125:111-122

Fontaínhas-Fernandes A, Monteiro M, Figueiredo A, Gomes E, Coimbra J, Reis-Henriques MA (2000) Partial or total replacement of fish meal by plant protein affects gonadal development and plasma 17β-estradiol levels in female Nile tilapia. Aquacult Int 8:299-313

Örn S, Andersson PL, Forlin L, Tysklind M, Norrgren L (1998) The impact on reproduction of an orally administered mixture of selected PCBs in zebrafish (*Danio rerio*). Arch Environ Contam Toxicol 35:52-57

Rocha MJ, Reis-Henriques MA (1996) Plasma and urine levels of C18, C19 and C21 steroids in an asynchronous fish, the tilapia *Oreochromis mossambicus* (Teleostei, Cichlidae). Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 115:257-264

Zhang CQ, Qiao HL (2004) Effect of polychlorinated biphenyls on spermatogenesis and testosterone secretion in adult cocks. J Zhejiang Univ Sci 5(2):193-197

CAPÍTULO 5-TRABALHO COMPLEMENTAR

Aroclor 1254 effects in Nile tilapia reproduction, at two temperatures



OBJECTIVES

To investigate which was the minimal Aroclor 1254 dose that inhibits reproduction other exposures to Aroclor 1254, using adult tilapia, were performed (assay 1 and 2). Additionally, the effects of Aroclor 1254, associated to temperature, were tested (assay 2). To achieve these objectives it was performed: 1) progesterone and testosterone plasma concentrations determination; 2) testis histology (assay 1); 3) spermatozoa counting (assay 2). Also, CYP1A EROD activity, which is related with the reproductive stage of fish (Stegeman and Hahn, 1994), was measured.

MATERIALS AND METHODS

Animals

Assay 1

Tilapia used in this assay were maintained in the same temperature (27 °C) and water conditions described in our published work (Coimbra and Reis-Henriques, 2005). Tilapias were fed with a diet containing 1.5 $\mu g.g^{-1}$ food of Aroclor 1254 (A) or with a Control diet (C). The exposure period was of 21 days.

Assay 2

In this assay besides testing the exposure to Aroclor 1254, it was simultaneously tested the effects of temperature, as a factor responsible for gonad development. Four tilapia groups were formed, two groups were fed with a diet containing 2.5 $\mu g.g^{-1}$ food of Aroclor 1254, one of which was maintained at 18 °C (A-18) and the other at 27 °C (A-27). The other two groups were fed with a control diet and maintained at the same two temperatures (C-18 and C-27). In this assay tilapia were exposed to Aroclor 1254 for a period of 30 days.

Radio Imuno Assay (RIA)

Testosterona and progesterone plasma levels were determined by RIA, as described for tilapias (Rocha and Reis-Henriques 1996, Fontaínhas-Fernandes et al., 2000), using ^{125}I Coat-a-Count kits (DPC, Los Angeles,USA). All samples were analyzed in duplicates.

Testis histology

In order to evaluate effects of Aroclor 1254 exposure on testis morphology, a portion of this tissue was fixed on Bouin's liquor (Panreac). After dehydratation, it was included on paraffin and cut on 5 µm sections. Testis slides were prepared to optic microscopy, stained with Hemathoxilin-Eosin and qualitative slide analysis was undertaken.

Sperm counts

Total spermatozoa number (per ml) and flagellum presence/absence were determined in male tilapia from assay 2. Sperm was diluted on a saline solution and dyed with Rose

Bengal (McAllister and Kime, 2003). The total numbers of sperm with or without flagella were calculated based on two sub-samples and the concentration of spermatozoa was determined in a Neubauer hemocytometer chamber.

Statistical Analyses

All results are presented as Mean±standard error of the mean (SE). Statistical analysis was performed by analysis of variance (ANOVA), using the multiple comparison LSD test ($p<0.05$). Correlation coefficients were calculated using Pearson correlation method ($p<0.05$) and Spearman correlation method ($p<0.05$) (STATISTICA 6.0).

RESULTS

Assay 1

Reproductive success

In this assay no reproduction occurred on 1.5 $\mu g.g^{-1}$ Aroclor 1254 exposed (A) tilapias. At the end of 21 days females from this group presented the same ovarian alterations described in the published work (Coimbra and Reis-Henriques, 2005). Aroclor 1254 exposed female presented a gonad somatic index (GSI-percentage of gonad weight.total $weight^{-1}$) significantly higher than control female ($p < 0.01$); while, male GSI was not affected by Aroclor 1254 exposure.

Testis histological analysis showed the occurrence on the exposed animals of similar lesions to the ones described in the published work (Fig.1). Namely, an increase in the seminiferous tubules walls thickness.

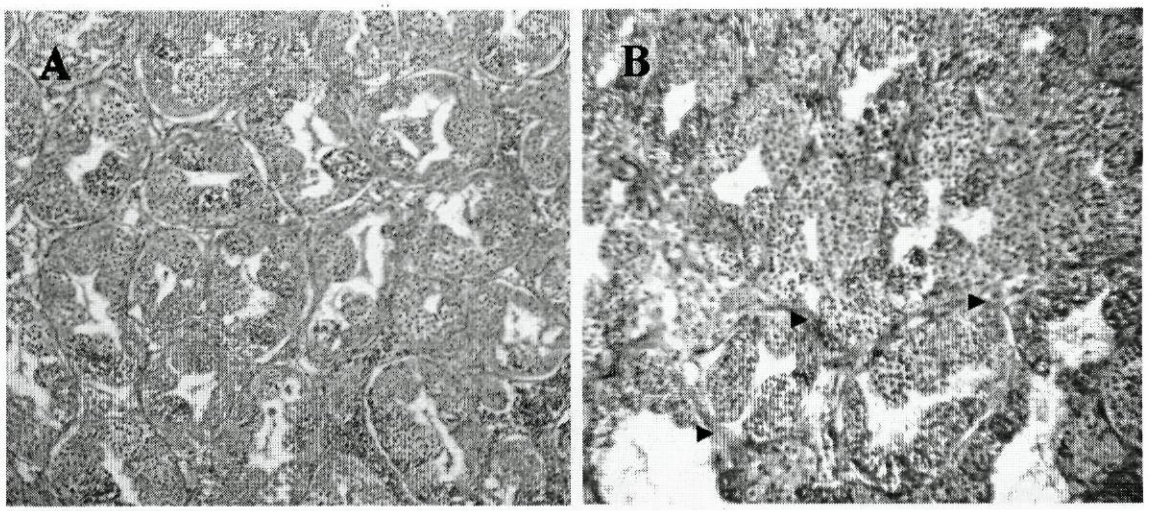


Fig. 1: Histological sections of testes of Control (A) and Aroclor 1254 exposed (B) tilapia (X 100). The arrows indicate the seminiferous tubules thicken walls.

Plasma progesterone and testosterone levels

Both male and female progesterone and testosterone concentrations were not significantly affected by the exposure to Aroclor 1254 (Table 1).

Table 1: Progesterone and testosterone plasma concentration (nM) tilapia control (C) and exposed to Aroclor 1254 (A) 21 days. Values presented as Mean±SE.

	Progesterone		Testosterone	
Group	**Males**	**Females**	**Males**	**Females**
C	7.4 ± 2.27	2.4 ± 0.20	179.1 ± 18.30	131.1 ± 6.67
A	3.4 ± 2.27	1.5 ± 0.36	125.4 ± 74.34	92.4 ± 29.48

Also, in this assay, a positive correlation between both hormone levels was observed ($r = 0.81$, $p < 0.001$).

EROD activity

CYP1A EROD activity increased both in tilapia male and female at the end of 21 days of exposure to 1.5 $\mu g.g^{-1}$ of Aroclor 1254 (Fig. 2).

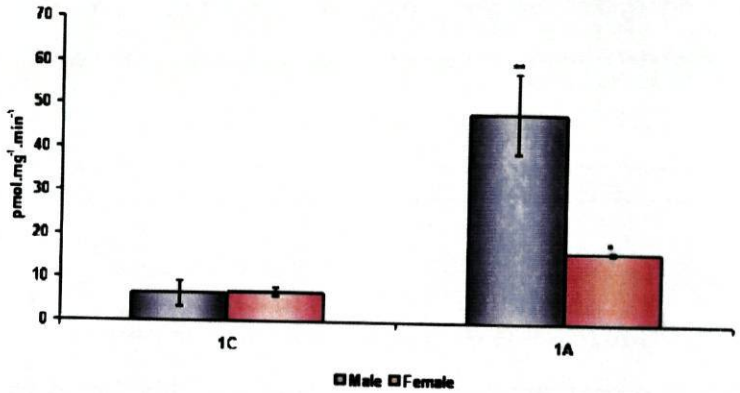


Fig. 2: Liver CYP1A EROD activity ($pmol.mg^{-1}$ protein.min^{-1}) in tilapia control (C) and exposed to Aroclor 1254 (A) for 21 days. Values presented as Mean±SE. Statistical differences: ** $p<0.01$.

Assay 2

The objectives of this assay were to evaluate the effects of temperature associated with the exposure to Aroclor 1254 (2.5 $\mu g.g^{-1}$) during 30 days.

Reproductive success

Reproduction did not occur on the Aroclor 1254 exposed tilapia groups.

Ovary lesions identical to the ones described before were only observed on tilapia from group exposed to Aroclor 1254 at 27 °C (A-27).

Total spermatozoa count was affected by temperature, due to maturation induction, and also by Aroclor 1254 exposure. While, male from the groups maintained at 27 °C showed higher spermatozoa counts than the groups maintained at 18 °C, male from the groups exposed to Aroclor 1254 presented lower spermatozoa counts that control male. The percentages of spermatozoa with/without flagella were unaffected, either by Aroclor 1254 exposure or by temperature (Table 2).

Table 2: Spermatozoa concentration (number.ml^{-1}) and percentage of spermatozoa with and without flagella in tilapias control and exposed to Aroclor 1254 for 30 days at 18 °C and 27 °C. Values presented as Mean±SE.

		Flagella	
Group	**Concentration**	**With**	**Without**
2C-18	96 667 ± 92 919.5	73 ± 1.5	30 ± 1.4
2A-18	45 417 ± 17 337.5	72 ± 2.0	27 ± 1.6
2C-27	275 000 ± 146 498.8	85 ± 1.6	15 ± 1.6
2A-27	155 000 ± 51 981.6	90 ± 1.3	10 ± 1.2

Plasma progesterone and testosterone levels

Concentrations of progesterone in male increased with the increase of the water temperature and no differences were observed between control and Aroclor 1254 exposed male at both temperatures. By the contrary, 2A-18 female presented higher progesterone values when compared with 2C-18 female; no differences resulting from the water temperature were observed on female progesterone (Fig. 3).

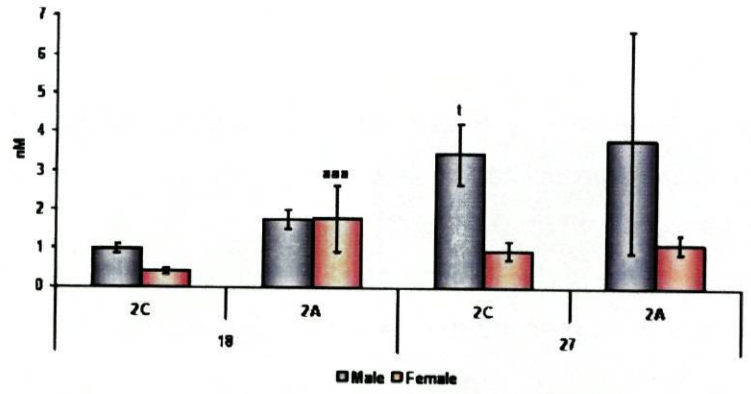


Fig. 3:. Progesterone plasma concentration (nM) in tilapia control (2C) and exposed to Aroclor 1254 (2A) for 30 days at 18 °C and 27 °C. Values presented as Mean±SE. Statistical differences due to Aroclor 1254 exposure ^{aaa} $p < 0.001$ and to temperature ^{t} $p < 0.05$.

Testosterone plasma concentration increased in control female with the higher water temperature, female from this group presented values five times higher than the other control females. Female from group A-18 also presented higher values when compared with female from group C-18. Male tilapia testosterone plasma levels were not affected neither by temperature nor Aroclor 1254 exposure (Fig. 4).

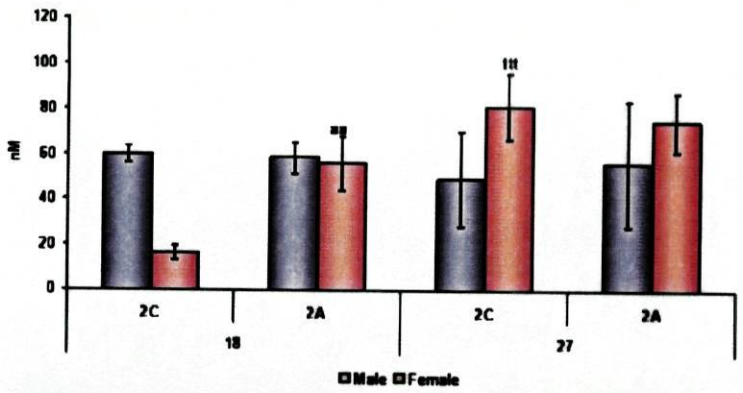


Fig. 4:. Testosterone plasma concentration (nM) in tilapias control (C) and exposed to Aroclor 1254 (A) for 30 days at 18 °C and 27 °C. Values presented as Mean±SE. Statistical differences due to Aroclor 1254 exposure ^{aa} $p < 0.01$ and to temperature ^{ttt} $p < 0.001$.

A positive correlation between both hormone levels was observed ($r = 0.52$, $p < 0.01$). In this assay, testosterone levels were also found to be correlated with temperature ($r = 0.39$, $p < 0.05$).

EROD activity

When the two control groups were compared, we observed that the hepatic CYP1A EROD activity was depressed by higher water temperature in female; in male from the group C-27 also showed a decline in this enzyme activity when compared to control at 18 °C, although not significant ($p>0.05$).

No differences were found due to Aroclor 1254 exposure at 27 °C, but tilapia exposed to this contaminant at 18 °C presented a decline of this enzyme activity (Fig. 5).

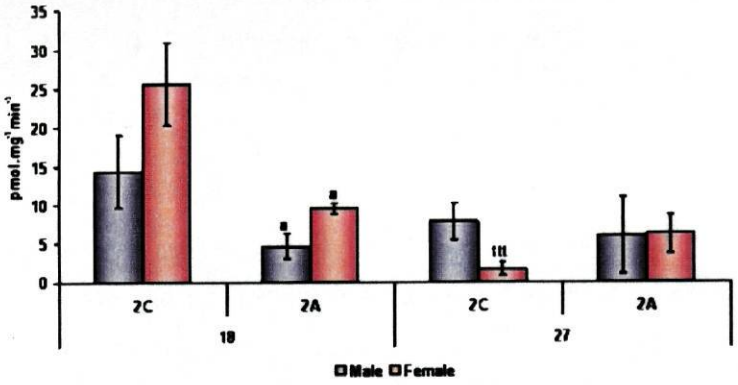


Fig. 5:. Liver CYP1A EROD activity (pmol.mg^{-1} protein.min^{-1}) in tilapia control (C) and exposed to Aroclor 1254 (A) for 30 days at 18 °C and 27 °C. Values presented as Mean±SE. Statistical differences due to Aroclor 1254 exposure ^{a} $p < 0.05$ and to temperature ^{ttt} $p < 0.001$.

CONCLUSION

The results of this complementary assays, taken together with the published results (Coimbra and Reis-Henriques, 2005), showed that Aroclor 1254 disturbed tilapia reproduction and that it was an effect dose related, since higher Aroclor 1254 concentrations were more effective.

Furthermore, female tilapia seem to be more susceptible to Aroclor 1254 than male tilapia, as gonad and hormone alterations appear earlier. Identically, to our results (assay 2), a study in male barbel, *Barbus barbus*, showed that spermatozoa number was not altered, in that case exposed to Aroclor 1260, and also that female barbel failed to spawn (Hugla and Thomé, 1999). Although, female barbel one year after exposure to the contaminant were able to spawn; this probably would not be possible in our assays, due to the ovary wall rupture.

Because, in the previous study (Coimbra and Reis-Henriques, 2005), evaluation of progesterone and testosterone in plasma of female tilapia was not be performed, due to the low number of females in that assay, these complementary assays permitted to determine that Aroclor 1254 action is dependent on water temperature, that is responsible for the gonadal development.

At the same time, liver CYP1A EROD activity also presented a temperature dependent response. Also, the results showed that after a higher dose and an extended exposure period other detoxification mechanism should already be active, only in tilapia at 27° C, since EROD activity was not different, while it was depressed at the lower temperature.

These observed results could be explained by the Aroclor 1254 interference at central level at the hypothalamus-hypophise-gonads axis, since this compound is known for interfere with the synthesis and release of the luteinizing hormone (LH) in the Atlantic croaker, *Micropogonias undulates*, inhibiting gonadal growth (Khan and Thomas, 2000, 2001; Khan et al., 2001).

As final conclusion, it was confirmed that Aroclor 1254 inhibits tilapia reproduction.

REFERENCES

COIMBRA AM, REIS-HENRIQUES MA (2005). Nile tilapia, Oreochromis niloticus L., reproduction inhibition by dietary exposure to Aroclor 1254. *Bulletin of Environmental Contamination and Toxicology.* **75:**407-412.

FONTAINHAS-FERNANDES A, MONTEIRO M, FIGUEIREDO A, GOMES E, COIMBRA J, REIS-HENRIQUES MA (2000). Partial or total replacement of fish meal by plant protein affects gonadal development and plasma 17 beta-estradiol levels in female Nile tilapia. *Aquaculture International.* **8:**299-313.

HUGLA JL, THOME JP (1999). Effects of polychlorinated biphenyls on liver ultrastructure, hepatic monooxygenases, and reproductive success in the barbel. *Ecotoxicology and Environmental Safety.* **42:**265-273.

KHAN IA, MATHEWS S, OKUZAWA K, KAGAWA H, THOMAS P (2001). Alterations in the GnRH-LH system in relation to gonadal stage and Aroclor 1254 exposure in Atlantic croaker. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology.* **129:**251-259.

KHAN IA, THOMAS P (2000). Lead and Aroclor 1254 disrupt reproductive neuroendocrine function in Atlantic croaker. *Marine Environmental Research.* **50:**119-123.

KHAN IA, THOMAS P (2001). Disruption of neuroendocrine control of luteinizing hormone secretion by Aroclor 1254 involves inhibition of hypothalamic tryptophan hydroxylase activity. *Biology of Reproduction.* **64:**955-964.

MCALLISTER BG, KIME DE (2003). Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (Danio rerio). *Aquatic Toxicology.* **65:**309-316.

ROCHA MJ, REIS-HENRIQUES MA (1996). Plasma and urine levels of C-18, C-19 and C-21 steroids in an asynchronous fish, the tilapia Oreochromis mossambicus (Teleostei, cichlidae). *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology.* **115:**257-264.

STEGEMAN JJ, HAHN ME (1994). Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species In: DC MALINS and GK OSTRANDER (Eds.). *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives.* Lewis Publishers.

CAPÍTULO 6

COIMBRA AM[a], Reis-HENRIQUES M[a]A. Tilapia larvae Aroclor 1254 exposure: effects on gonads and circulating thyroid hormones during adulthood. (Submitted to *Bulletin of Environmental Contamination and Toxicology*).



Tilapia larvae Aroclor 1254 exposure: effects on gonads and circulating thyroid hormones during adulthood

A. Mª. Coimbra[1,2], Mª. A. Reis-Henriques[1,2]

[1]ICBAS- Instituto de Ciências Biomédicas de Abel Salazar, Laboratório de Fisiologia Aplicada, Largo do Prof. Abel Salazar nº2, 4099-003, Porto, Portugal
[2]CIIMAR- Centro Interdisciplinar de Investigação Marinha e Ambiental, Laboratório de Toxicologia Ambiental, Rua dos Bragas 289, 4050-123, Porto, Portugal

*Correspondence to Ana Maria Coimbra
Present address: CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas 289, 4050-123, Porto, Portugal
Tel: +351 22 340 18 00
Fax: +351 22 339 06 08
E-mail: anacoimbra@cimar.org

* Correspondence to Ana Maria Coimbra

Present address: CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental,

Rua dos Bragas 289, 4050-123, Porto, Portugal

Anthropogenic chemicals in the environment are a major problem to human and wildlife. Many of these chemicals used in industry and/or agriculture have as final destiny the aquatic environment (Hwang et al., In Press); also some of these chemicals are known to interfere with endocrine functions, leading to reproductive and developmental anomalies in wildlife populations (Cooper and Kavlock, 1997), which may cause malformations on sexual characters and disturb the reproductive function in fish (Harrison et al., 1997, Tyler et al., 1998) and other vertebrates (Colborn et al., 1993, Guillette and Gunderson, 2001).

Polychlorinated biphenyls (PCBs) are usually present in the environment as complex mixtures of several congeners; one of them is Aroclor 1254, which is a commercial PCB mixture with a 54 weight percentage of chlorine. Exposure to these compounds is associated with both biological and toxicological effects and with reproductive and endocrine disruption in vertebrates (Brouwer et al., 1998, Vos et al., 2000). Recently, Coimbra and Reis-Henriques (2005) demonstrated that adult tilapia exposed during 20 days through the diet to 4.5μg.g^{-1} Aroclor 1254 presented an inhibition of reproduction.

Fish early life stages are thought to be more sensitive to environmental chemicals than adult fish (Nguyen and Janssen, 2002) and thyroid hormones importance in fish early development, growth and reproduction is well known (Leatherland, 1994, Power et al., 2001). Furthermore, PCBs are known to accumulate in fish ovary and therefore might be present in descendents of contaminated fish (Hellou et al., 1993, Monosson et al., 2003).

Despite works dealing with the action of PCBs in vertebrates, studies of the effects in adulthood of Aroclor 1254 exposure during early life stages are still lacking. Therefore, the objectives of our study were to evaluate the impact of Aroclor 1254 in gonad development and thyroid function of tilapias exposed to Aroclor 1254 during larval stages, after the vitelline sac absorption.

MATERIAL AND METHODS

Tilapia (*Oreochromis niloticus*) larvae were removed from the mouth of their progenitor, randomly divided in two groups, Control and Aroclor 1254, and

placed in 70 L tanks with individual mechanical and biological filtration. Supplemental aeration was provided to maintain dissolved oxygen near saturation. The water source was the public water supply which was de-chlorinated before entering the tanks; an approximate 3L renewal occurs per hour in each system. Water temperature (20 °C) and photoperiod (12 hour-light: 12 hour-dark) were maintained constant during the assay period.

Larvae, 10 days post-hatch and after absorption of the vitelline sac, were exposed to 0.05 $\mu g.g^{-1}$ of Aroclor 1254 (NSI Solutions, Inc.; Raleigh, USA) through the diet. Aroclor 1254 contaminated diets were prepared by immersion of a commercial fish diet, previously powdered, on an Aroclor 1254-ethanol solution and then allowed to dry. Control tilapias were fed with the same commercial fish diet, which was only immersed in ethanol. Larvae were fed with the contaminated diet for 40 days and from this period up to the end of the experiment tilapia from the exposed group were also fed with the control diet. Eighteen months after the beginning of the exposure, specimens were sacrificed.

Testis slides were prepared for optic microscopy, stained with Hemathoxilin-Eosin and qualitative slide analysis was undertaken.

Plasma concentrations of total T3 and total T4 were determined using commercial available RIA kits (Kit Coat-a-Count, DPC) according to the manufactures instructions. Both RIA kits use antibodies with high specificity (100 % of reactivity) to the correspondent hormone. Cross-reactivity of each kit is very low, the T3 kit presents a reactivity of 0.05% for T4, while the T4 kit presents a 2% reactivity for T3. Inter assay variation was avoided by analysis of all samples in the same assay. All samples were analyzed in duplicate.

All results are presented as mean±standard error (SE). Statistical analysis was performed by analysis of variance (ANOVA), using the multiple comparison LSD test ($p<0.05$) (STATISTICA 6.0).

RESULTS AND DISCUSSION

Eighteen months after 40 days of dietary exposure to Aroclor 1254, specimens presented ovarian and testicular abnormalities. During sampling it was observed that 78 % of exposed females presented ovary lesions, characterized by an abnormal weight and the presence of atretic oocytes within the ovaries. Some of the exposed females presented a 60% content of atretic oocytes, a value superior to the 40% described as the maximum reached by Nile tilapia (Coward and Bromage, 2000); the average gonad somatic index (as % of total body weight) was of 6.4±1.11 in exposed females and of 4.11±0.55 in control females. Also 33 % of males showed a full urinary bladder (Fig. 1), while alterations were not observed in control tilapia. In addition, testis histology showed the occurrence of Leydig cells hyperplasia and a decrease of the germinal epithelium layer of Aroclor 1254 exposed tilapia (Fig. 2).

Gonad macroscopic changes observed in the present study were similar to the

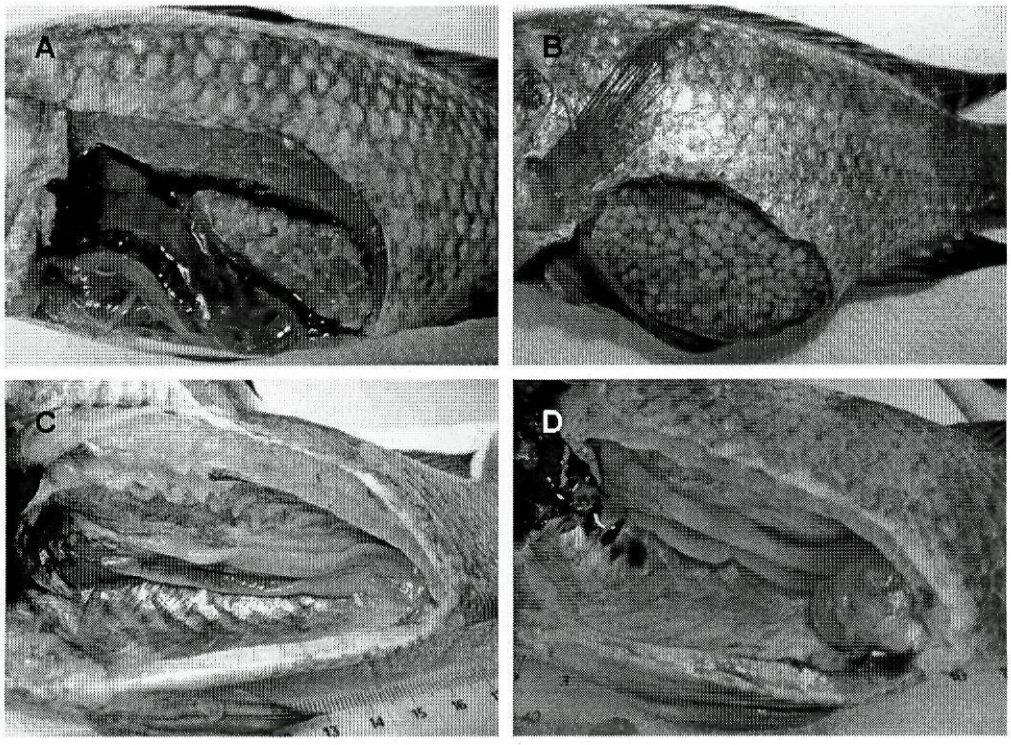


Fig. 1: A-Control female tilapia. B-Aroclor 1254 exposed female tilapia presenting an abnormal ovary size and atretic oocytes. C-Control male tilapia. D-Aroclor 1254 exposed male tilapia presenting a full urinary bladder (arrow).

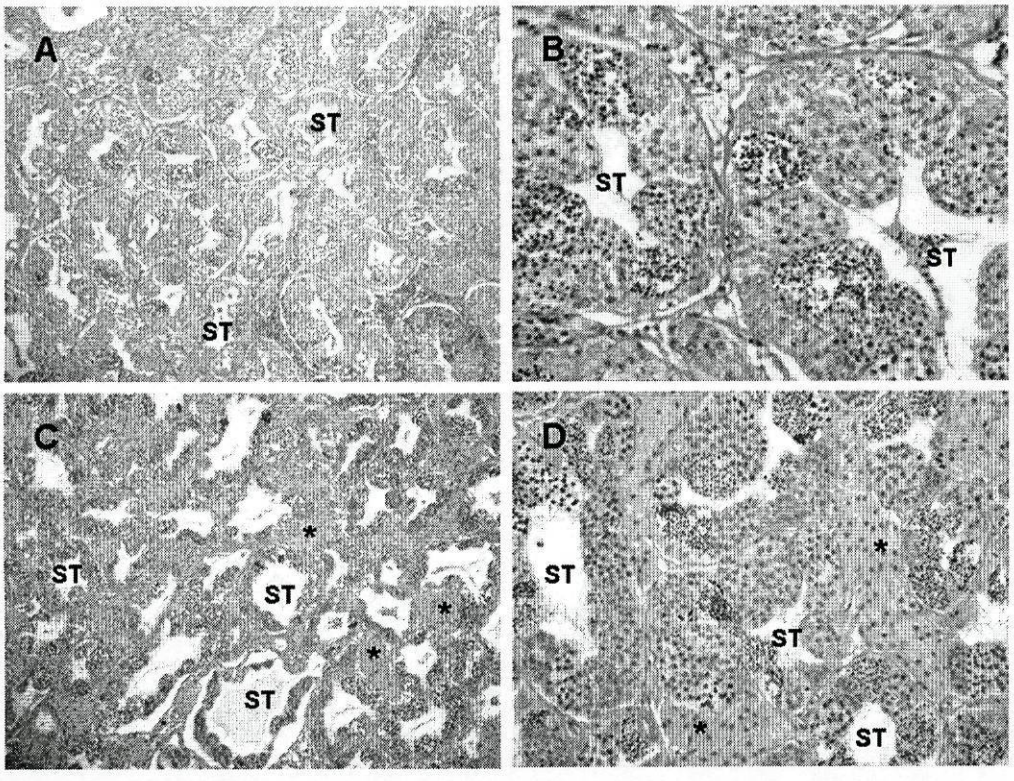


Fig. 2: Histological images of tilapia testis. A-100X control tilapia; B-400X control tilapia; C-100X Aroclor 1254 exposed tilapia; D-400 X Aroclor 1254 exposed tilapia. * Leydig cells hyperplasia; ST-seminiferous tubules lumen.

ones described for adult tilapia exposed to Aroclor 1254 (Coimbra and Reis-Henriques, 2005), although, in the present study, rupture of ovaries walls was not seen. Females presented the ovaries full of oocytes, including a high number of atretic oocytes; a dose dependent increase in atretic oocytes was also observed in zebrafish exposed to doses from 40-270 $ng.g^{-1}$ of food of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) contaminated diets (Heiden et al., 2005).
Zebrafish TCDD exposure did not arrest spawning, but females presented ovarian necrosis and egg production reduction, being these effects caused by the accumulated TCDD, in concentrations as little as 0.6 $ng.g^{-1}$ of fish (Heiden et al., 2005, Heiden et al., 2006). Due to the severe damage observed in tilapia ovaries, it was impossible to perform a histological evaluation of this organ.

Male tilapia exposed to Aroclor 1254 during the larvae stage exhibited full urinary bladders, as was also observed when exposed as adults (Coimbra and Reis-Henriques, 2005). Therefore, as we previously suggested, Aroclor 1254 seems to interfere with the urogenital pore elasticity, blocking female spawning and interfering with male release of urine and possibly of spermatozoa. Uro-genital morphological alterations were also described for LE Hooded and Holtzman rats (Gray and Ostby, 1995) and Syrian hamsters (Wolf et al., 1999) exposed to TCDD *in utero*. Gray and Ostby (1995) observed in rats dosed by gavage with 1 $\mu g.kg^{-1}$ of TCDD, at gestational day 15, a delay of the vaginal opening and in some females a partial vaginal occlusion by a persistent membrane or thread.

The testicular tissue lesions observed in tilapia exposed as larvae are in some degree different from lesions described for adults. Both in adult tilapia (Coimbra and Reis-Henriques, 2005) and in this study it was observed a decrease of the germinal epithelium layer (Fig. 2). Identical results were observed in cod (Sangalang et al., 1981) and in other vertebrates exposed to PCBs mixtures (Ahmad et al., 2003, Zhang and Qiao, 2004); however, while tilapias exposed when adults, presented an increase in the seminiferous tubules walls thickness, due to connective tissue proliferation (Coimbra and Reis-Henriques, 2005), also observed in cod (Sangalang et al., 1981); tilapia exposed during the larvae stage presented a hyperplasia of Leydig cells. Increased Leydig cell number was observed in Sprague Dawley rats exposed during lactation to Aroclor 1242 (Kim et al., 2001). This Leydig cell hyperplasia may be a response to maintain androgens levels, which were shown to be depressed when adults were exposed to this pollutant (Coimbra and Reis-Henriques, 2005); Aroclor 1248 also inhibit *in vivo* and *in vitro* rat testicular stereoidogenesis (Andric et al., 2000).

The results obtained in our study demonstrate that the exposure during the larvae stage to a low and environmental relevant concentration of a PCBs mixture originates effects in the adult stage, that may led to reproductive impairment. The dose used, 0.05 $\mu g.g^{-1}$, is in the range of total PCBs observed in a food web of the Kalamazoo River (Kay et al., 2005) and lower than concentrations found in adult *O. mossambicus* from the Salton Sea where a total PCBs concentrations ranging from 10.9-32.8 $ng.g^{-1}$ wet weight (ww) in muscle, 16.4-114.5 $ng.g^{-1}$ ww in liver and 14.4-98.4 $ng.g^{-1}$ ww in gonads were observed (Sapozhnikova et al., 2004). Thus, we believe that further studies should be performed exposing larvae to even lower Aroclor 1254 concentrations to evaluate their impact in adulthood.

The effects observed in our study reflect either a direct action of Aroclor 1254 in gonad development or a indirect impairment by acting in the hypothalamic-pituitary-gonadal axis, since this compound has the capacity to cause alterations at central level; Aroclor 1254 was found to interfere with reproduction of *Micropogonias undulates,* after a 1 $mg.kg^{-1}$ exposure for 30 days, by decreasing the secretion and release of the luteinizing hormone (LH), through inhibition of serotonin synthesis and disruption of the serotonin-gonadotropin releasing hormone pathway that controls LH secretion and resulting in gonad growth inhibition (Khan and Thomas, 2001). Thus, alterations observed in ovaries could reflect an action of Aroclor 1254 at central level enabling tilapia maturation and/or ovulation.

Adult tilapia, which were exposed to 0.05 $\mu g.g^{-1}$ Aroclor 1254, showed a significant decrease in T4 plasma concentrations from 13.6±1.15 nM in control to 8.0±0.77 nM ($p<0.01$), corresponding to a 59% reduction in this hormone levels. T3 plasma concentration were also depressed with Aroclor 1254 (7.5±1.58 nM in control and 5.8±1.07 nM in Aroclor 1254 dosed animals), although differences between groups were not significant ($p>0.05$).

Aroclor 1254 exposure of tilapia larvae seem to have originated a deficient thyroid hormone status, revealed by plasma T4 depletion in adults. As observed in adult tilapia, plasma T3 concentration in exposed tilapia revealed similar values presented by control, since Aroclor 1254 exposure does not interfere with the deiodinase activities responsible for the maintenance of this hormone plasmatic concentration (Coimbra et al., 2005). T4 levels decline might result from the competition of PCBs and/or of their metabolites for the binding to thyroid hormones transport proteins present in the blood (Lans et al., 1994, Cheek et al., 1999, Chauhan et al., 2000).

In rats, hypothyroidism has been previously associated with an increased of Leydig cell number as a direct increase of Leydig cells precursors (Hardy et al., 1993, Mendishandagama and Sharma, 1994). Moreover, Coimbra et al. (2005) showed that adult tilapia fed with a diet contaminated with 0.5 $\mu g.g^{-1}$ of Aroclor 1254 for 35 days presented an increase in testicular activity of the deiodinase responsible for the conversion of T4 in T3, suggesting the occurrence of an increase in testicular metabolism, probably due to an effort of the cells to compensate on the steroidogenesis disruption.

Studies with other fish species showed that PCBs exposure may lead to population decline due to decreased ovary growth (Khan and Thomas, 2001), induction of testicular lesions (Sangalang et al., 1981), reduction in the number of spawned eggs (Orn et al., 1998, Heiden et al., 2005), or even increase in embryo and larvae abnormalities and mortality (Palstra et al., 2006).

In conclusion, Aroclor 1254 disrupted both the reproductive axis enabling tilapia reproduction during adulthood, since the injuries caused by Aroclor 1254 in tilapia gonads seem to be irreversible; and likewise affected the thyroid axis, as suggested by the decrease in T4 plasmatic concentrations. The current study shows that gonads are sensitive target organs for disruption by PCBs and that effects of the exposure to low levels of PCBs, even for short periods of time, during early life stages, may have serious repercussions in the gonads development and thus affecting the reproductive capability of adults. Thus, since these compounds are still widely distributed in the environment, our results indicate that

even low levels may have a considerable impact in fish populations as PCBs exposure may led to a decrease of larvae recruitment and to reproduction impairment.

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REFERENCES

Ahmad SU, Tariq S, Jalali S, Ahmad MM (2003). Environmental pollutant Aroclor 1242 (PCB) disrupts reproduction in adult male rhesus monkeys (Macaca mulatta). Environmental Research. 93:272-278.

Andric SA, Kostic TS, Stojilkovic SS, Kovacevic RZ (2000). Inhibition of rat testicular androgenesis by a polychlorinated biphenyl mixture Aroclor 1248. Biology of Reproduction. 62:1882-1888.

Brouwer A, Morse DC, Lans MC, Schuur AG, Murk AJ, Klasson-Wehler E, Bergman A, Visser TJ (1998). Interactions of persistent environmental organohalogens with the thyroid hormone system: Mechanisms and possible consequences for animal and human health. Toxicology and Industrial Health. 14:59-84.

Chauhan KR, Kodavanti PRS, McKinney JD (2000). Assessing the role of ortho-substitution on polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. Toxicology and Applied Pharmacology. 162:10-21.

Cheek AO, Kow K, Chen J, McLachlan JA (1999). Potential mechanisms of thyroid disruption in humans: Interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. Environmental Health Perspectives. 107:273-278.

Coimbra AM, Reis-Henriques MA (2005). Nile tilapia, Oreochromis niloticus L., reproduction inhibition by dietary exposure to Aroclor 1254. Bulletin of Environmental Contamination and Toxicology. 75:407-412.

Coimbra AM, Reis-Henriques MA, Darras VA (2005). Circulating thyroid hormone levels and iodothyronine deiodinase activities in Nile tilapia (Oreochromis niloticus) following dietary exposure to Endosulfan and Aroclor 1254. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology. 141:8-14.

Colborn T, Saal FSV, Soto AM (1993). Developmental Effects of Endocrine-Disrupting Chemicals in Wildlife and Humans. Environmental Health Perspectives. 101:378-384.

Cooper RL, Kavlock RJ (1997). Endocrine disruptors and reproductive development: A weight-of-evidence overview. Journal of Endocrinology. 152:159-166.

Coward K, Bromage NR (2000). Reproductive physiology of female tilapia broodstock. Reviews in Fish Biology and Fisheries. 10:1-25.

Gray LE, Ostby JS (1995). In-Utero 2,3,7,8-Tetrachlorodibenzo-P-Dioxin (TCDD) Alters Reproductive Morphology and Function in Female Rat Offspring. Toxicology and Applied Pharmacology. 133:285-294.

Guillette LJ, Gunderson MP (2001). Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. Reproduction. 122:857-864.

Hardy MP, Kirby JD, Hess RA, Cooke PS (1993). Leydig-Cells Increase Their Numbers but Decline in Steroidogenic Function in the Adult-Rat after Neonatal-Hypothyroidism. Endocrinology. 132:2417-2420.

Harrison PTC, Holmes P, Humfrey CDN (1997). Reproductive health in humans and wildlife: are adverse trends associated with environmental chemical exposure? Science of the Total Environment. 205:97-106.

Heiden TK, Carvan MJ, Hutz RJ (2006). Inhibition of follicular development, vitellogenesis, and serum 17 beta-estradiol concentrations in zebrafish following chronic, sublethal dietary exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicological Sciences. 90:490-499.

Heiden TK, Hutz RJ, Carvan MJ (2005). Accumulation, tissue distribution, and maternal transfer of dietary 2,3,7,8,-tetrachlorodibenzo-p-dioxin: Impacts on reproductive success of zebrafish. Toxicological Sciences. 87:497-507.

Hellou J, Warren WG, Payne JF (1993). Organochlorines Including Polychlorinated-Biphenyls in Muscle, Liver, and Ovaries of Cod, Gadus-Morhua. Archives of Environmental Contamination and Toxicology. 25:497-505.

Hwang H-M, Green PG, Young TM (In Press). Tidal salt marsh sediment in California, USA. Part 1: Occurrence and sources of organic contaminants. Chemosphere. doi:10.1016/j.chemosphere.2005.12.024

Kay DP, Blankenship AL, Coady KK, Neigh AM, Zwiernik MJ, Millsap SD, Strause K, Park C, Bradley P, Newsted JL, Jones PD, Giesy JP (2005). Differential accumulation of polychlorinated biphenyl congeners in the aquatic food web at the Kalamazoo River superfund site, Michigan. Environmental Science & Technology. 39:5964-5974.

Khan IA, Thomas P (2001). Disruption of neuroendocrine control of luteinizing hormone secretion by Aroclor 1254 involves inhibition of hypothalamic tryptophan hydroxylase activity. Biology of Reproduction. 64:955-964.

Kim IS, Ariyaratne HBS, Mendis-Handagama S (2001). Effects of continuous and intermittent exposure of lactating mothers to Aroclor 1242 on testicular steroidogenic function in the adult male offspring. Tissue & Cell. 33:169-177.

Lans MC, Spiertz C, Brouwer A, Koeman JH (1994). Different Competition of Thyroxine-Binding to Transthyretin and Thyroxine-Binding Globulin by Hydroxy-Pcbs, Pcdds and Pcdfs. European Journal of Pharmacology-Environmental Toxicology and Pharmacology Section. 270:129-136.

Leatherland JF (1994). Reflections on the thyroidology of fishes : from molecules to humankind Guelph Ichthyology Reviews. 1-67.

Mendishandagama S, Sharma OP (1994). Effects of Neonatal Administration of the Reversible Goitrogen Propylthiouracil on the Testis Interstitium in Adult-Rats. Journal of Reproduction and Fertility. 100:85-92.

Monosson E, Ashley JTF, McElroy AE, Woltering D, Elskus AA (2003). PCB congener distributions in muscle, liver and gonad of Fundulus heteroclitus from the lower Hudson River Estuary and Newark Bay. Chemosphere. 52:777-787.

Nguyen LTH, Janssen CR (2002). Embryo-larval toxicity tests with the African catfish (Clarias gariepinus): Comparative sensitivity of endpoints. Archives of Environmental Contamination and Toxicology. 42:256-262.

Orn S, Andersson PL, Forlin L, Tysklind M, Norrgren L (1998). The impact on reproduction of an orally administered mixture of selected PCBs in zebrafish (Danio rerio). Archives of Environmental Contamination and Toxicology. 35:52-57.

Palstra A, van Ginneken V, Murk A, van den Thillart G (2006). Are dioxin-like contaminants responsible for the eel (Anguilla anguilla) drama? Naturwissenschaften. 93:145-148.

Power DM, Llewellyn L, Faustino M, Nowell MA, Bjornsson BT, Einarsdottir IE, Canario AVM, Sweeney GE (2001). Thyroid hormones in growth and development of fish.

Comparative Biochemistry and Physiology C-Toxicology & Pharmacology. 130:447-459.

Sangalang GB, Freeman HC, Crowell R (1981). Testicular Abnormalities in Cod (Gadus-Morhua) Fed Aroclor-1254. Archives of Environmental Contamination and Toxicology. 10:617-626.

Sapozhnikova Y, Bawardi O, Schlenk D (2004). Pesticides and PCBs in sediments and fish from the Salton Sea, California, USA. Chemosphere. 55:797-809.

Tyler CR, Jobling S, Sumpter JP (1998). Endocrine disruption in wildlife: A critical review of the evidence. Critical Reviews in Toxicology. 28:319-361.

Vos JG, Dybing E, Greim HA, Ladefoged O, Lambre C, Tarazona JV, Brandt I, Vethaak AD (2000). Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Critical Reviews in Toxicology. 30:71-133.

Wolf CJ, Ostby JS, Gray LE (1999). Gestational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) severely alters reproductive function of female hamster offspring. Toxicological Sciences. 51:259-264.

Zhang CQ, Qiao HL (2004). Effect of polychlorinated biphenyls on spermatogenesis and testosterone secretion in adult cocks. Journal Zhejiang University Science. 5:193-197.

DISCUSSÃO GERAL



A contaminação dos ecossistemas aquáticos por xenobióticos utilizados nas actividades agrícolas e industriais e os seus efeitos sobre os biotas têm vindo, há várias décadas, a ser alvo de estudo da comunidade científica. A dispersão destes compostos pelos vários compartimentos do meio aquático faz com que os organismos contactem com eles pela água e/ou por via alimentar.

Devido às propriedades físico-químicas que lhes conferem capacidade de persistência e dispersão nos ecossistemas, de bioacumulação nos tecidos animais e de actuação como disruptores endócrinos, os compostos denominados de "Persistent Organic Pollutants-POPs" são os que têm originado maior preocupação. Para além destes, estão presentes nos ecossistemas aquáticos outros xenobióticos que não possuem todas estas propriedades, mas que também induzem efeitos deletérios nos organismos que neles habitam. Para a execução deste trabalho foram escolhidos dois xenobióticos: o Aroclor 1254, uma mistura de congéneres de PCBs pertencente à lista dos POPs e o Endosulfan, um insecticida hexaclorado usado à escala mundial e que não pertence a esta lista.

Os peixes encontram-se nos níveis superiores da cadeia trófica aquática e devido à bioacumulação e bioconcentração de alguns poluentes podem estar expostos a concentrações mais elevadas do que as que ocorrem na água.

As propriedades físico-químicas destes contaminantes são responsáveis pela baixa solubilidade em água, o que faz com que adiram às paredes dos tanques de ensaio, diminuindo a sua biodisponibilidade. Por isso e porque se sabe que estão presentes nas cadeias tróficas dos ecossistemas aquáticos, a exposição da tilápia do Nilo foi realizada por incorporação de Endosulfan e de Aroclor 1254 na dieta.

Esta espécie foi seleccionada para a realização deste trabalho pela sua actual dispersão geográfica e importância como fonte proteica a nível mundial, assim como pelas suas características biológicas - como reprodução contínua e capacidade de resistência - que a caracterizam como um bom organismo para o tipo de ensaios efectuados.

Em suma, neste trabalho foram estudados os efeitos da exposição, por via alimentar, ao Endosulfan na regulação osmótica e na função da tiróide e ao Aroclor 1254 na função da tiróide, na reprodução e desenvolvimento larvar.

EIXO HIPOTÁLAMO-HIPÓFISE-RIM

Exposição ao Endosulfan

O eixo hipotálamo-hipófise-rim da tilápia do Nilo foi um dos sistemas afectados pelo Endosulfan. A exposição a uma dieta, contendo 1 μg de Endosulfan por grama de peso, originou um decréscimo dos níveis plasmáticos de cortisol, uma hormona envolvida na regulação osmótica. Resultado idêntico foi obtido, também com este composto, em ensaios *in vitro* usando células do rim cefálico de truta (Leblond et al., 2001, Bisson e Hontela, 2002).

Esta alteração nos níveis de cortisol deu origem a um decréscimo da actividade da ATPase Na^+,K^+ dependente no rim e na brânquia, diminuindo o transporte de iões (Na^+, Cl^-) e, consequentemente, provocando uma diminuição da capacidade de regulação osmótica na tilápia.

Sabe-se que este composto quando administrado na água destrói o epitélio das brânquias causando edema, separação do epitélio das lamelas branquiais e fusão das lamelas, como foi observado em trutas expostas a 2,6 e 23,7 $\mu g.l^{-1}$ (Capkin et al., 2006); neste estudo o Endosulfan chega às brânquias por via sanguínea, onde interfere com as células com função de osmoregulação.

Acção na Função da Tiróide

A acção das HT nos peixes está sob o controlo do eixo hipotálamo-hipófise-tiróide (controlo central), que regula a produção de T4, e sob o controlo de todos os outros tecidos onde a síntese de T3 e sua disponibilidade (controlo periférico) é regulada pela actividade local das desiodases (Eales et al., 1999).

Estas hormonas estão envolvidas numa grande variedade de processos fisiológicos, tais como: a ontogenia, o desenvolvimento, o crescimento, a reprodução, o metabolismo, a regulação osmótica, o desenvolvimento e funcionamento do sistema nervoso (Leatherland, 1994, Parhar et al., 2000, Power et al., 2001, Biswas et al., 2006).

Devido à vasta acção destas hormonas, um desequilíbrio no funcionamento do eixo hipotálamo-hipófise-tiróide a nível central e/ou a nível periférico, causado pela exposição a xenobióticos, pode alterar a disponibilidade das hormonas e modificar os processos fisiológicos anteriormente referidos, originando efeitos nefastos nos organismos expostos.

De acordo com Eales et al. (1999), a avaliação dos efeitos da exposição a xenobióticos na função da tiróide deve ser realizada em três níveis: no controlo central da secreção de T4 para o plasma, no controlo periférico da conversão de T4 em T3 e no controlo dos efeitos pós-receptores de T3. Neste trabalho foram avaliados efeitos da exposição a compostos policlorados nos dois primeiros níveis.

Controlo Central e Periférico das HT

Exposição ao Endosulfan

De entre as várias concentrações de Endosulfan utilizadas, apenas a dose de 0,1 $\mu g.g^{-1}$ de alimento alterou significativamente a concentração plasmática de T4. A dose mais baixa a que as tilápias foram expostas (0,001 $\mu g.g^{-1}$) parece não ter sido suficiente para induzir alterações na concentração desta hormona. Assim, esperava-se que doses superiores a 0,1 $\mu g.g^{-1}$ de Endosulfan (0,5 e 1 $\mu g.g^{-1}$) tivessem também provocado decréscimos significativos da concentração plasmática de T4, mas tais resultados não foram observados.

A mesma dose de Endosulfan (0,1 $\mu g.g^{-1}$), que originou uma diminuição da concentração plasmática de T4, causou igualmente uma diminuição nos níveis de rT3 circulante. No entanto, a concentração plasmática de T3 não foi significativamente alterada por nenhuma das doses utilizadas neste trabalho. A manutenção da concentração de T3 circulante, apesar das concentrações de T4 e rT3 estarem diminuidas, mostrou que a tilápia é capaz de manter os níveis de T3 circulantes relativamente constantes, apesar das alterações na concentração de T4, tal como tinha sido verificado anteriormente num trabalho realizado em tilápia (Mol et al., 1999).

As actividades das desiodases em alguns tecidos foram afectadas pela exposição ao Endosulfan. Verificou-se, nomeadamente no fígado, uma redução da actividade da D1 e um aumento da actividade da D3 para a dose de 0,1 $\mu g.g^{-1}$, ao fim de 21 e 35 dias de exposição. Seria de esperar que estas alterações fossem responsáveis por uma menor disponibilidade de T3, no entanto, os níveis circulantes desta hormona não foram diferentes entre tilápias expostas e tilápias controlo, o que é indicativo do papel predominante das actividades da D2 hepática e da D1 renal na manutenção da concentração de T3 circulante.

Ao fim de 21 dias de exposição a 0,1 $\mu g.g^{-1}$ de Endosulfan as tilápias apresentaram concentrações de T4 e rT3 circulantes inferiores aos das tilápias controlo. Simultaneamente, mantiveram-se constantes a concentração de T3 e as actividades da D2 hepática e da D1 renal, enzimas descritas como principais responsáveis pela regulação da concentração de T3 circulante (Mol et al., 1993, Mol et al., 1997). O facto de nenhuma destas actividades ter sido afectada, explica porque é que a concentração de T3 não foi modificada, apesar das alterações na actividade das outras desiodases (D1 e D3 hepáticas) não estarem de acordo com as alterações compensatórias esperadas para a manutenção dos níveis de T3 circulantes. Os resultados obtidos na actividade das desiodases não justificam a diminuição observada na concentração de T4, indicando que o Endosulfan exerceu a sua acção ao nível do eixo hipotálamo-hipófise-tiróide. Desta forma, a diminuição da síntese de T4 pode resultar de efeitos deste ao nível: do hipotálamo, visto que é um inibidor dos canais dependentes do GABA (Bloomquist, 1996, Bloomquist, 2003); da hipófise diminuindo a síntese e/ou a libertação da TSH; ou da tiróide diminuindo a síntese de T4. Embora não se conheçam efeitos deste composto sobre a síntese e libertação de TSH, sabe-se que na tilápia mossâmbica a exposição através da água originou a degenerescência dos folículos da tiróide (Bhattacharya, 1995), o que indica que esta pode ser a razão pela qual a exposição ao Endosulfan origina uma diminuição da concentração plasmática de T4.

Ao fim de 35 dias de exposição à mesma dose de Endosulfan (0,1 $\mu g.g^{-1}$) não foram observadas alterações nas concentrações plasmáticas das HT, o que poderá indicar uma adaptação dos animais à exposição a este composto. Contrariamente ao que seria esperado, mas de acordo com os resultados observados para a dose de 0,1 $\mu g.g^{-1}$, a dose de 0,5 $\mu g.g^{-1}$ não afectou a concentração das HT nem a actividade das desiodases, com excepção da D3 na brânquia e da D2 no testículo, o que indica que uma dose mais elevada poderá induzir mecanismos compensatórios antes dos 21 dias de exposição.

Doses mais elevadas ou tempos de exposição mais prolongados podem ter como consequência a ocorrência de lesões do epitélio intestinal, descritas para outras espécies de peixes expostas ao Endosulfan por via alimentar (Braunbeck e Appelbaum, 1999;

Cengiz et al., 2001). Estas lesões originaram uma menor absorção deste composto, não se verificando assim o efeito descrito para a dose de 0,1 $\mu g.g^{-1}$.

Exposição ao Aroclor 1254

A exposição ao Aroclor 1254 não interferiu com as concentrações plasmáticas das HT, o que está de acordo com resultados de estudos anteriores, realizados em trutas expostas a uma dose 1000 vezes superior, onde não foram observadas alterações das HT circulantes (Brown et al., 2004a). No entanto, quando a exposição ao Aroclor 1254 foi efectuada numa fase do ciclo de vida mais precoce, em que os órgãos ainda não estão completamente formados, verificou-se que esses animais apresentavam concentrações plasmáticas de T4 inferiores às observadas em animais controlo. Este resultado pode ser consequência de uma acção sobre os órgãos responsáveis pela manutenção da concentração de T4, impedindo a sua síntese e/ou libertação ou de uma competição dos congéneres de PCBs presentes no Aroclor 1254 com as HT para a ligação às proteínas plasmáticas de transporte (Van den Berg et al., 1990, Lans et al., 1993, Lans et al., 1994; Cheek et al., 1999, Chauhan et al., 2000), aumentando a concentração de T4 livre no plasma e promovendo a excreção desta hormona.

A exposição ao Aroclor 1254 não causou alterações na actividade da D2 hepática, o que está de acordo com os resultados de estudos realizados em *Salvelinus namaycush* expostos ao PCB 126 na alimentação (Brown et al., 2004b) e em *Hipoglossoides platessoides* injectados com os congéneres 77 e 126 (Adams et al., 2000).

O aumento da actividade das D3 hepática e branquial induzido por este poluente poderia ter favorecido um aumento da eliminação da T3 e uma diminuição dos níveis circulantes desta hormona, contudo este efeito não foi observado. Por conseguinte, as alterações observadas na actividade das desiodases não estão relacionadas com a manutenção da concentração plasmática de T3, mas podem reflectir efeitos da exposição aos xenobióticos ao nível dos tecidos, de forma a manterem-se os níveis de T3 intracelulares necessários à função de cada tecido.

Os resultados obtidos mostraram que, a actividade das desiodases foi afectada, principalmente no fígado, o que pode ser atribuído à função metabólica deste órgão e às propriedades físico-químicas destes compostos. Como o fígado possui um conteúdo elevado de lípidos e estes compostos são lipofílicos, existe uma tendência para se acumularem no tecido hepático. Estas alterações podem também dever-se ao facto do Endosulfan e do Aroclor 1254 terem sido administrados através da dieta e, desta forma, o fígado, em comparação com os outros órgãos estudados, ter sido um dos primeiros órgãos a estar em contacto com estes compostos e de ser sujeito a concentrações mais

elevadas, não só destes poluentes como também dos seus metabolitos que, em alguns casos, são mais tóxicos que os próprios produtos (Lans et al., 1993, Naqvi e Vaishnavi, 1993, Lans et al., 1994).

Proteínas plasmáticas de transporte das hormonas da tiróide

Como já foi referido, alguns xenobióticos competem com as HT para a ligação às proteínas plasmáticas de transporte, aumentando a excreção destas hormonas. Estudos anteriores, em que o plasma de peixe foi incubado com HT marcadas radioactivamente e as proteínas plasmáticas separadas por PAGE (Santos e Power, 1999, Richardson et al., 2005), mostraram que as proteínas de transporte das HT apresentam um padrão de migração diferente das presentes noutras classes de vertebrados e concluiram que a proteína anódica observada deveria ser a TTR, devido à sua migração em PAGE nativo e à afinidade para as HT.

A TTR é uma proteína de transporte das HT presente noutros vertebrados e recentemente isolada do plasma de salmonídeos (Yamauchi et al., 1999). O gene foi identificado em *Sparus aurata* (Funkestein et al., 1999, Santos et al., 1999), conhecendo-se também a sua sequência em outras espécies íctias em que o genoma está sequenciado. Apesar de se terem realizado vários estudos no sentido de pesquisar a presença de TTR no plasma de tilápia, não foi possível identificar esta proteína nesta espécie. A TTR ou não é expressa ou é expressa em quantidades muito baixas, de forma que os métodos utilizados não tiveram a sensibilidade necessária para determinar a sua presença. Também é possível que a sequência do gene não apresente grande homologia com as sequências conhecidas de outros peixes.

Os resultados deste trabalho permitiram identificar duas proteínas de transporte de HT no plasma de tilápia, a albumina, uma vez que esta proteína está presente em todas as espécies estudadas de vertebrados (Richardson et al., 2005) e outra identificada como uma apolipoproteína de 14 kDa. As lipoproteínas foram anteriormente descritas como fundamentais para o transporte das HT no plasma dos peixes, nomeadamente a apo A1 (25 kDa) e a apo A2 (13 kDa) que foram descritas como responsáveis pelo transporte em truta (Babin, 1992). Para além disso, a apo A1 nos humanos liga-se à TTR (Sousa et al., 2000), podendo ocorrer interacções semelhantes no plasma de tilápia.

Os trabalhos realizados, *ex vivo* e *in vivo*, com o objectivo de determinar se o Endosulfan ou o Aroclor 1254 competem com as HT para a ligação às proteínas plasmáticas de transporte, mostraram que a exposição ao Endosulfan não interfere com a ligação das HT a esta classe de proteínas. O Endosulfan apresenta uma estrutura química muito diferente da estrutura das HT, o que pode justificar a ausência de efeito na ligação às proteínas plasmáticas de transporte. No entanto, o Aroclor 1254 é uma mistura

de PCBs, alguns dos quais apresentam uma estrutura química semelhante à estrutura do anel bifenilo das HT. Trabalhos anteriores mostraram que alguns dos metabolitos hidroxilados dos PCBs competem fortemente com as HT para a ligação a estas proteínas (Van den Berg et al., 1990, Lans et al., 1993, Lans et al., 1994, Cheek et al., 1999; Chauhan et al., 2000). Neste estudo, os resultados dos trabalhos *ex vivo* mostraram que o Aroclor 1254 compete com as HT para a ligação à albumina de tilápia, apenas quando em quantidades muito elevadas, ao mesmo tempo que aumenta a ligação das HT à apolipoproteína. Isto sugere a presença de um sistema de compensação e justifica a ausência de alterações na distribuição da T4 pelos tecidos analisados (cérebro, brânquias, fígado, rim, gónadas e músculo) no ensaio *in vivo*.

Incubações de plasma de várias espécies de peixe com HT radioactivas foram analisadas por PAGE nativo e mostraram que existem diferenças no padrão de migração e distribuição das HT pelas proteínas plasmáticas de transporte. Esta variabilidade entre espécies íctias aumenta a dificuldade na análise destas proteínas e a avaliação dos efeitos dos contaminantes.

Embora não tenham sido avaliados efeitos da exposição ao Endosulfan ou ao Aroclor 1254 no controlo dos efeitos pós-receptores de T3, a observação de que os níveis plasmáticos de T3 se mantiveram constantes pode sugerir que estes efeitos não ocorreriam. Contudo, a observação de alterações das actividades das desiodases em alguns tecidos indica, como já foi referido, necessidades locais de T3 e a possibilidade de ocorrerem efeitos pós-receptores.

ACÇÃO SOBRE A REPRODUÇÃO

Um grande número de trabalhos realizados sobre os efeitos da exposição a xenobióticos em organismos aquáticos analisou a sua acção no sistema reprodutivo, que é o responsável pela perpetuação das espécies. Do ponto de vista ecológico, alterações na reprodução a nível individual podem reflectir-se ao nível populacional, embora na maioria dos casos estes não tenham sido atingidos (Taylor e Harrison, 1999). Estas alterações observadas em animais selvagens e atribuídas a compostos com capacidade de actuar como disruptores endócrinos ocorrem normalmente em locais com um elevado grau de poluição (Ferreira et al., 2004, Rodrigues et al., 2006). Os efeitos incluem, entre outros: alterações no desenvolvimento das gónadas, intersexo, alterações das concentrações plasmáticas dos esteróides sexuais, indução da produção de vitelogenina, alteração do comportamento reprodutivo e, em alguns casos, redução do número de descendentes viáveis (Tyler et al., 1998, Van Der Kraak, 1998; Taylor e Harrison, 1999; Vos et al., 2000). Algumas das alterações causadas pela exposição a xenobióticos são específicas para determinados tipos de composto, sendo os exemplos mais representativos a produção de vitelogenina pelos machos e juvenis de peixes expostos a xenoestrogénios, e a masculinização (imposex) de fêmeas de neogastrópodes quando expostas ao trifenilestanho (TBT) (Matthiessen e Gibbs, 1998, Santos et al., 2005).

No presente trabalho a introdução do Aroclor 1254 na dieta induziu alterações no sistema reprodutivo, dos machos e das fêmeas de tilápia, que podem levar ao impedimento da reprodução.

De acordo com trabalhos publicados anteriormente, os PCBs, congéneres isolados ou misturas de congéneres podem actuar em diferentes locais do eixo do sistema reprodutivo, como no cérebro de peixes (Khan e Thomas, 2000, Khan e Thomas, 2001, Khan et al., 2001) e de ratos (Hany et al., 1999, Mariussen e Fonnum, 2001, Muthuvel et al., 2006) e nas gónadas de peixe (Sangalang et al., 1981) e de outros vertebrados (Andric et al., 2000, Gandolfi et al., 2002, Ahmad et al., 2003, Alston et al., 2003). Em ensaios *in vivo*, este facto dificulta a identificação dos locais de actuação e a avaliação dos efeitos destes compostos, uma vez que ao interferirem com um ou mais locais do eixo hipotálamo-hipófise-gónada podem desencadear efeitos ao longo de todo o eixo reprodutivo.

Exposição ao Aroclor 1254

Um dos objectivos iniciais deste trabalho foi o estudo dos efeitos nos descendentes de animais adultos expostos ao Aroclor 1254. No entanto, quando tilápias adultas foram alimentadas com dietas contendo este composto, observaram-se alterações morfológicas

nos ovários e nos testículos, que impediram a obtenção de descendentes destes animais, por impedimento da emissão de gâmetas. Assim, para avaliar os efeitos do Aroclor 1254 no desenvolvimento da tilápia, optou-se por expor as larvas de tilápia, durante um curto espaço de tempo (40 dias), a este composto logo que se iniciasse a alimentação externa, seguindo esta população até à fase adulta.

Nas tilápias expostas durante a fase adulta, o aparecimento de alterações nas gónadas foi comparado com a dose de Aroclor 1254 e com o tempo de exposição, tendo-se observado que os animais expostos às doses mais elevadas apresentavam lesões após um período de exposição mais curto. Verificou-se ainda que as larvas expostas a este composto, apresentavam na fase adulta alterações das gónadas semelhantes às observadas anteriormente.

Assim, as fêmeas expostas durante a fase adulta ou durante a fase larvar apresentavam um aumento do conteúdo do ovário e do número de oócitos atrésicos, que em alguns animais levou à ruptura da parede deste órgão e à libertação do conteúdo na cavidade abdominal. A percentagem de oócitos atrésicos observado foi superior ao valor descrito como o máximo (40%) atingido pela tilápia do Nilo (Coward e Bromage, 2000). Isto indica que o Aroclor 1254 não interfere com a oogénese, mas que inibe a desova, resultando na acumulação de oócitos e na ruptura das paredes dos ovários, que podem levar à morte destes indivíduos. Noutro trabalho efectuado em *Danio rerio,* onde os animais foram expostos a uma mistura de 20 congéneres de PCBs administrados na dieta ocorreu, e dependendo da dose usada, uma inibição da maturação dos oócitos, afectando negativamente a capacidade reprodutiva desta espécie (Örn et al., 1998).

Nos machos, a primeira observação foi o não esvaziamento da bexiga urinária, contrariamente ao que acontece quando as tilápias são manipuladas (Rocha e Reis-Henriques, 1996); verificando-se também que os testículos apresentavam alterações morfológicas e, neste caso, observaram-se diferenças entre os animais expostos durante a fase adulta e a fase larvar. Nos animais expostos durante a fase adulta ocorreu um aumento da espessura da parede dos tubos seminíferos devido a um aumento do tecido conjuntivo, enquanto que, nos animais expostos durante a fase larvar se observou a ocorrência de uma hiperplasia das células de Leydig. Em ambos os casos, os animais expostos, quando comparados com os animais controlo, apresentavam uma diminuição do epitélio germinativo. Estas alterações foram também observadas noutros vertebrados, incluíndo os peixes, expostos a misturas de PCBs (Sangalang et at., 1981, Ahmad et al., 2003, Alston et al., 2003, Zhang e Quiao, 2004).

Um dos possíveis locais de actuação deste composto é o poro urogenital. Nas fêmeas, o poro genital encontra-se separado do poro urinário, enquanto que, nos machos formam um orifício comum. O facto de os machos apresentarem a bexiga urinária cheia e de as

fêmeas serem incapazes de desovar, sugere que a exposição ao Aroclor 1254 pode originar a obstrução do poro genital.

As alterações observadas podem indicar uma acção directa do Aroclor 1254 nas gónadas ou uma acção indirecta através do eixo hipotálamo-hipófise-gónada. Assim, este composto pode actuar directamente nas gónadas, interferindo com alguns tipos celulares, nomeadamente no macho, com as células de Leydig produtoras de androgénios, e nas fêmeas com as células da teca e/ou da granulosa, produtoras de estrogénios e progestagénios. A interferência deste composto com estas células pode ser responsável pela diminuição nas concentrações plasmáticas de progesterona e testosterona observadas nos animais expostos, uma vez que ao actuar sobre estas células interfere com a actividade esteroidogénica. Por outro lado, pode também destruir as células de Leydig, como é sugerido pela proliferação de tecido conjuntivo nos machos expostos durante a fase adulta, que vai ocupar o espaço anteriormente ocupado por estas células. A diminuição da síntese dos esteróides sexuais pode levar, no caso do testículo, à diminuição do epitélio germinativo, uma vez que estas hormonas são necessárias à proliferação das células germinativas e à formação de gâmetas viáveis. A inibição da esteroidogénese devido à exposição a PCBs foi descrita em várias espécies de vertebrados (Kovacevic et al., 1995, Andric et al., 2000, Kim et al., 2001, Benninghoff e Thomas, 2005, Murugesan et al., 2005a,b).

De acordo com estudos anteriores, a hiperplasia das células de Leydig observada nos machos expostos ao Aroclor 1254, durante a fase larvar, pode dever-se ao facto deste composto ter induzido uma diminuição da concentração plasmática de T4, uma vez que a ocorrência de situações de hipotiroidismo, durante as fases iniciais do desenvolvimento do testículo, induz a proliferação de células precursoras das células de Leydig (Hardy et al., 1993, Mendishandagama e Sharma, 1994, Cooke at al, 2004) e consequente aumento do número destas células. Embora a concentração plasmática de T3 se tenha mantido dentro dos valores normais, indicando que esta hormona aparentemente está disponível da mesma forma que nos animais controlo, verificou-se, no entanto, que este composto pode alterar o metabolismo das HT no testículo, nomeadamente aumentando a actividade da D2, podendo assim ocorrer um aumento local de T3.

O Aroclor 1254 pode também exercer a sua acção ao nível central do eixo hipotálamo--hipófise-gónada. Sabe-se que em *Micropogonias undulatas* este composto interfere com a síntese da LH, diminuindo a produção desta hormona através da inibição da síntese de serotonina (Khan e Thomas, 2001). Os níveis mais elevados de LH foram observados em tilápia, quando os níveis de esteróides sexuais estavam elevados (Melamed et al., 2000). Noutras espécies de peixes sabe-se que as concentrações de estradiol e de testosterona apresentam um máximo no início da fase final da reprodução, seguido pelo aparecimento

de um máximo de LH (Kobayashy et al., 1989). Uma diminuição da concentração da LH poderia explicar porque é que as fêmeas de tilápia foram incapazes de desovar, uma vez que esta hormona é necessária para a maturação dos oócitos e para a ovulação.

Neste trabalho, mostrou-se que o Aroclor 1254 actua directamente nas gónadas, causando lesões no testículo e no ovário de tilápia, que parecem ter um carácter irreversível e que tornam os animais expostos incapazes de se reproduzirem.

É importante referir que as concentrações de Aroclor 1254 usadas estão dentro da gama de concentrações que podem ser encontradas em organismos aquáticos em ambientes poluídos (Sapozhnikova et al., 2004, Kay et al., 2005). Larvas expostas a estas concentrações durante uma pequena parte do seu ciclo de vida apresentam lesões das gónadas que podem inibir ou reduzir a sua capacidade reprodutiva; sugerindo que populações de tilápia, que habitem em locais contaminados ou que tenham contactado durante um período do ciclo de vida com estes ou com compostos semelhantes, poderão vir a ser reduzidas ou mesmo, em casos extremos, ocorrer uma extinção da espécie no local.

Em face aos resultados obtidos seria de interesse no futuro avaliar o efeito de doses mais baixas de Endosulfan no eixo hipotálamo-hipófise-rim, por determinação da concentração plasmática de cortisol e da actividade da ATPase Na^+,K^+ no rim e na brânquia, bem como realizar transferências de tilápias para ambientes de salinidade superior, de forma a avaliar o impacto da exposição a este composto na capacidade de osmoregulação desta espécie e determinar o grau de destruição dos tecidos, quer do rim, quer da brânquia.

No que respeita o eixo hipotálamo-hipófise-tiróide, a avaliação dos níveis de TSH e a análise morfológica do tecido da tiróide poderão ser a forma de completar a avaliação da acção destes compostos no controlo central da função da tiróide. A análise da interferência destes poluentes nos mecanismos de conjugação poderão esclarecer se existe um aumento da excreção das HT. A determinação de efeitos pós-receptores por ensaios de competição com a T3 para a ligação aos receptores nucleares poderá ajudar a clarificar a acção sobre alguns genes alvo.

A acção do Aroclor 1254 sobre o eixo reprodutivo poderá ser aprofundada estudando, ao nível central, a síntese ou expressão de FSH e de LH, assim como dos vários neurotransmissores e ao nível das gónadas, a síntese de outros esteróides sexuais, quer por determinação da concentração destas hormonas, quer por determinação da actividade das enzimas responsáveis pela esteróidogénese e estudando a acção deste composto nas morfologia das células germinativas e do poro uro-genital.

O estudo destes sistemas endócrinos, complementado com a realização de estudos *in vitro* e a avaliação da distribuição destes compostos, assim como dos seus metabolitos, poderá ajudar a determinar os locais de acção directa e esclarecer os efeitos destes poluentes nos diversos órgãos.

CONCLUSÕES



Os resultados obtidos neste trabalho permitiram chegar às seguintes conclusões:

+ A exposição, por via alimentar, ao Endosulfan origina uma disrupção do eixo hipotálamo-hipófise-rim que resulta numa diminuição da concentração plasmática de cortisol e numa consequente diminuição da actividade da ATPase Na^+, K^+ da brânquia e do rim, alterando a capacidade de osmoregulação das tilápias expostas.

+ O Endosulfan interfere com o funcionamento do eixo da tiróide causando uma diminuição da concentração plasmática de T4, sem, no entanto, interferir significativamente com a concentração plasmática de T3.

+ O metabolismo periférico das HT é afectado por este insecticida, pois altera a actividade das deiodinases em alguns tecidos, embora, a actividade das desiodases responsáveis pela manutenção da concentração plasmática de T3, D1 renal e D2 hepática, não sofra alterações por exposição ao Endosulfan.

+ O transporte e distribuição das HT pelas proteínas plasmáticas de transporte na tilápia, a albumina e a apolipoproteína de 14 kDa não são afectados pela exposição a este insecticida.

+ A exposição de adultos de tilápia ao Aroclor 1254 não promove alterações na concentração plasmática das hormonas da tiróide. No entanto, quando expostas durante a fase larvar, as tilápias apresentam uma menor concentração plasmática de T4, o que sugere que sofreram uma alteração no eixo da tiróide que se manteve até ao estado adulto.

+ Esta mistura de PCBs interfere com o metabolismo periférico das HT em alguns tecidos, sendo de realçar a sua acção no testículo.

+ O Aroclor 1254 compete com as hormonas da tiróide para a ligação à albumina, sendo este efeito apenas observado nas doses mais elevadas e nos ensaios *ex vivo*.

+ A exposição ao Aroclor 1254, durante as fases larvar ou adulta do ciclo de vida, induz alterações morfológicas do tecido das gónadas com consequente diminuição da produção dos esteróides sexuais (progesterona e testosterona), originando uma deficiente capacidade reprodutiva dos animais expostos. Estas alterações podem resultar

de uma acção directa sobre as gónadas ou de uma acção indirecta através do hipotálamo e da hipófise.

✦ E finalmente, este trabalho mostrou que estes dois compostos organoclorados, embora quimicamente diferentes, promovem alterações em vários sistemas endócrinos e alterações morfológicas em vários órgãos, que causam alterações no desenvolvimento e na capacidade reprodutiva da tilápia do Nilo.

BIBLIOGRAFIA GERAL



A

ABBASSY MS, IBRAHIM HZ, ABU EL-AMAYEM MM (1999). Occurrence of pesticides and polychlorinated biphenyls in water of the Nile river at the estuaries of Rosetta and Damiatta branches, North of Delta, Egypt. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes.* **34:**255-267.

ABELSOHN A, GIBSON BL, SANBORN MD, WEIR E (2002). Identifying and managing adverse environmental health effects: 5. Persistent organic pollutants. *Canadian Medical Association Journal.* **166:**1549-1554.

ADAMS BA, CYR DG, EALES JG (2000). Thyroid hormone deiodination in tissues of American plaice, *Hippoglossoides platessoides*: characterization and short-term responses to polychlorinated biphenyls (PCBs) 77 and 126. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology.* **127:**367-378.

AHMAD SU, TARIQ S, JALALI S, AHMAD MM (2003). Environmental pollutant Aroclor 1242 (PCB) disrupts reproduction in adult male rhesus monkeys (*Macaca mulatta*). *Environmental Research.* **93:**272-278.

ALMEIDA MR, DAMAS AM, LANS MC, BROUWER A, SARAIVA MJ (1997). Thyroxine binding to transthyretin Met 119 - Comparative studies of different heterozygotic carriers and structural analysis. *Endocrine.* **6:**309-315.

ALSTON DA, TANDLER B, GENTLES B, SMITH EE (2003). Testicular histopathology in deer mice (*Peromyscus maniculatus*) following exposure to polychlorinated biphenyl. *Chemosphere.* **52:**283-285.

ALTLAND K, WINTER P, SAUERBORN MK (1999). Electrically neutral microheterogeneity of human plasma transthyretin (prealbumin) detected by isoelectric focusing in urea gradients. *Electrophoresis.* **20:**1349-1364.

ANDRIC SA, KOSTIC TS, STOJILKOVIC SS, KOVACEVIC RZ (2000). Inhibition of rat testicular androgenesis by a polychlorinated biphenyl mixture Aroclor 1248. *Biology of Reproduction.* **62:**1882-1888.

ANKLEY G, MIHAICH E, STAHL R, TILLITT D, COLBORN T, MCMASTER S, MILLER R, BANTLE J, CAMPBELL P, DENSLOW N, DICKERSON R, FOLMAR L, FRY M, GIESY J, GRAY LE, GUINEY P, HUTCHINSON T, KENNEDY S, KRAMER V, LEBLANC G, MAYES M, NIMROD A, PATINO R, PETERSON R, PURDY R, RINGER R, THOMAS P, TOUART L, VAN DER KRAAK G, ZACHAREWSKI T (1998). Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. *Environmental Toxicology and Chemistry.* **17:**68-87.

ANTUNES P, GIL O, FERREIRA M, VALE C, REIS-HENRIQUES MA (2004). Depuration of PCBs and DDTs in mullet under captivity conditions. *Dioxin 2004, 24th International Symposium on Halogenated Environmental Organic Pollutants and POPs.* (*Berlim*). 1892-1898.

ANTUNES P, GIL O, REIS-HENRIQUES MA (2005). Kinetics of PCN accumulation and elimination in sea bass (*Dicentrarchus labrax*) of two size classes. *ECOTOX 2005 Advances and Trends in Ecotoxicology.* (*Czech Republic*). 16-18.

ANTUNES P, GIL O, FERREIRA M, VALE C, REIS-HENRIQUES MA (in press). Depuration of PCBs and DDTs in mullet under captivity clean conditions. *Chemosphere.*

ATSDR (1995). Toxicological profile for polychlorinated biphenyls. Draft for public comment (Update). Research Triangle Institute. *US Department of Heath and Human Services-Public Health Service.* Contract No. 205-93-0606.

B

BABIN PJ (1992). Binding of thyroxine and 3,5,3'-triiodothyronine to trout plasma-lipoproteins. *American Journal of Physiology.* **262:**E712-E720.

BARSOTTI DA, MARLAR RJ, ALLEN JR (1976). Reproductive dysfunction in rhesus-monkeys exposed to low-levels of polychlorinated biphenyls (Aroclor 1248). *Food and Cosmetics Toxicology.* **14:**99-103.

BECKER KB, STEPHENS KC, DAVEY JC, SCHNEIDER MJ, GALTON VA (1997). The type 2 and type 3 iodothyronine deiodinases play important roles in coordinating development in *Rana catesbeiana* tadpoles. *Endocrinology.* **138**:2989-2997.

BEMIS JC, SEEGAL RF (2004). PCB-induced inhibition of the vesicular monoamine transporter predicts reductions in synaptosomal dopamine content. *Toxicological Sciences.* **80**:288-295.

BENNINGHOFF AD, THOMAS P (2005). Involvement of calcium and calmodulin in the regulation of ovarian steroidogenesis in Atlantic croaker (*Micropogonias undulatus*) and modulation by Aroclor 1254. *General and Comparative Endocrinology.* **144**:211-223.

BESSELINK HT, VANBEUSEKOM S, ROEX E, VETHAAK AD, KOEMAN JH, BROUWER A (1996). Low hepatic 7-ethoxyresorufin-O-deethylase (EROD) activity and minor alterations in retinoid and thyroid hormone levels in flounder (*Platichthys flesus*) exposed to the polychlorinated biphenyl (PCB) mixture, Clophen A50. *Environmental Pollution.* **92**:267-274.

BHATTACHARYA L (1995). Histological and histochemical alterations in the thyroid-activity of endosulphan treated *Oreochromis mossambicus*. *Journal of Environmental Biology.* **16**:347-351.

BIANCO AC, SALVATORE D, GEREBEN B, BERRY MJ, LARSEN PR (2002). Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocrine Reviews.* **23**:38-89.

BINELLI A, PROVINI A (2004). Risk for human health of some POPs due to fish from Lake Iseo. *Ecotoxicology and Environmental Safety.* **58**:139-145.

BIRNBAUM LS (1994). Endocrine effects of prenatal exposure to PCBs, dioxins, and other xenobiotics - implications for policy and future-research. *Environmental Health Perspectives.* **102**:676-679.

BISSON M, HONTELA A (2002). Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed in vitro. *Toxicology and Applied Pharmacology.* **180**:110-117.

BISWAS A, KUNDU S, ROY S, DE J, PRAMANIK M, RAY AK (2006). Thyroid hormone profile during annual reproductive cycle of diploid and triploid catfish, *Heteropneustes fossilis* (Bloch). *General and Comparative Endocrinology.* **147**:126-132.

BLOOMQUIST JR (1996). Ion channels as targets for insecticides. *Annual Review of Entomology.* **41**:163-190.

BLOOMQUIST JR (2003). Chloride channels as tools for developing selective insecticides. *Archives of Insect Biochemistry and Physiology.* **54**:145-156.

BOSMA PT, KOLK SM, REBERS FEM, LESCROART O, ROELANTS I, WILLEMS P, SCHULZ RW (1997). Gonadotrophs but not somatotrophs carry gonadotrophin-releasing hormone receptors: Receptor localisation, intracellular calcium, and gonadotrophin and GH release. *Journal of Endocrinology.* **152**:437-446.

BRANN DW, MAHESH VB (1994). Excitatory amino-acids - function and significance in reproduction and neuroendocrine regulation. *Frontiers in Neuroendocrinology.* **15**:3-49.

BRAUNBECK T, APPELBAUM S (1999). Ultrastructural alterations in the liver and intestine of carp *Cyprinus carpio* induced orally by ultra-low doses of endosulfan. *Diseases of Aquatic Organisms.* **36**:183-200.

BRETON B, MIKOLAJCZYK T, DANGER JM, GONNET F, SAINTPIERRE S, VAUDRY H (1989). Neuropeptide-Y (NPY) modulates *in vitro* gonadotropin in release from rainbow-trout pituitary glands. *Fish Physiology and Biochemistry.* **7**:77-83.

BRETON B, MIKOLAJCZYK T, POPEK W, BIENIARZ K, EPLER P (1991). Neuropeptide-Y stimulates *in vivo* gonadotropin-secretion in teleost fish. *General and Comparative Endocrinology.* **84**:277-283.

BROUWER A (1991). Role of biotransformation in PCB-induced alterations in vitamin-a and thyroid--hormone metabolism in laboratory and wildlife species. *Biochemical Society Transactions.* **19**:731-737.

BROUWER A, MORSE DC, LANS MC, SCHUUR AG, MURK AJ, KLASSON-WEHLER E, BERGMAN A, VISSER TJ (1998). Interactions of persistent environmental organohalogens with the thyroid hormone

system: Mechanisms and possible consequences for animal and human health. *Toxicology and Industrial Health.* **14:**59-84.

Brown SB, Adams BA, Cyr DG, Eales JG (2004a). Contaminant effects on the teleost fish thyroid. *Environmental Toxicology and Chemistry.* **23:**1680-1701.

Brown SB, Evans RE, Vandenbyllardt L, Finnson KW, Palace VP, Kane AS, Yarechewski AY, Muir DCG (2004b). Altered thyroid status in lake trout (*Salvelinus namaycush*) exposed to co-planar 3,3',4,4 ',5-pentachlorobiphenyl. *Aquatic Toxicology.* **67:**75-85.

Brucker-Davis F (1998). Effects of environmental synthetic chemicals on thyroid function. *Thyroid.* **8:**827-856.

Byamungu N, Corneillie S, Mol K, Darras V, Kuhn ER (1990). Stimulation of thyroid-function by several pituitary-hormones results in an increase in plasma thyroxine and reverse triiodothyronine in tilapia (*Tilapia nilotica*). *General and Comparative Endocrinology.* **80:**33-40.

Byamungu N, Mol K, Kuhn ER (1992). Evidence for the kidney as an important source of 5'-monodeiodination activity and stimulation by somatostatin in *Oreochromis niloticus* L. *General and Comparative Endocrinology.* **88:**199-208.

Byrne JJ, Carbone JP, Hanson EA (1987). Hypothyroidism and abnormalities in the kinetics of thyroid-hormone metabolism in rats treated chronically with polychlorinated biphenyl and polybrominated biphenyl. *Endocrinology.* **121:**520-527.

C

Callard GV (1983). Androgen and estrogen actions in the vertebrate brain. *American Zoologist.* **23:**607-620.

Capkin E, Altinok I, Karahan S (2006). Water quality and fish size affect toxicity of endosulfan, an organochlorine pesticide, to rainbow trout. *Chemosphere.* **64:**1793-1800.

Cengiz EI, Unlu E, Balci K (2001). The histopathological effects of Thiodan (R) on the liver and gut of mosquitofish, *Gambusia affinis*. *Journal of Environmental Science and Health B.* **36:** 75-85.

Cerda-Reverter JM, Larhammar D (2000). Neuropeptide Y family of peptides: Structure, anatomical expression, function, and molecular evolution. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire.* **78:**371-392.

Cerejeira MJ, Viana P, Batista S, Pereira T, Silva E, Valerio MJ, Silva A, Ferreira M, Silva-Fernandes AM (2003). Pesticides in Portuguese surface and ground waters. *Water Research.* **37:**1055-1063.

Chanoine JP, Braverman LE, Farwell AP, Safran M, Alex S, Dubord S, Leonard JL (1993). The thyroid-gland is a major source of circulating-T3 in the rat. *Journal of Clinical Investigation.* **91:**2709-2713.

Chauhan KR, Kodavanti PRS, McKinney JD (2000). Assessing the role of ortho-substitution on polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. *Toxicology and Applied Pharmacology.* **162:**10-21.

Cheek AO, Kow K, Chen J, McLachlan JA (1999). Potential mechanisms of thyroid disruption in humans: Interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid--binding globulin. *Environmental Health Perspectives.* **107:**273-278.

Chiba A, Oka S, Honma Y (1996). Ontogenetic changes in neuropeptide Y-like-immunoreactivity in the terminal nerve of the chum salmon and the cloudy dogfish, with special reference to colocalization with gonadotropin-releasing hormone-immunoreactivity. *Neuroscience Letters.* **213:**49-52.

Chung YW, Clemens LG (1999). Effects of perinatal exposure to polychlorinated biphenyls on development of female sexual behavior. *Bulletin of Environmental Contamination and Toxicology.* **62:**664-670.

Coimbra AM, Almeida MR, Silva L, Reis-Henriques MA (2002). Some effects of endosulfan on Nile tilapia (*Oreochromis niloticus*) In: R Keller, H Dircksen, D Sedlmeier and H Vaudry (Eds.).

Proceedings of the 21st Conference of European Comparative Endocrinologists (Bonn-Germany). 75-79.

COIMBRA AM, REIS-HENRIQUES MA (2005). Nile tilapia, *Oreochromis niloticus* L., reproduction inhibition by dietary exposure to Aroclor 1254. *Bulletin of Environmental Contamination and Toxicology.* **75:**407-412.

COIMBRA AM, REIS-HENRIQUES MA, DARRAS VA (2005). Circulating thyroid hormone levels and iodothyronine deiodinase activities in Nile tilapia (*Oreochromis niloticus*) following dietary exposure to Endosulfan and Aroclor 1254. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology.* **141:**8-14.

COLBORN T (2002). Clues from wildlife to create an assay for thyroid system disruption. *Environmental Health Perspectives.* **110:**363-367.

COLBORN T, SAAL FSV, SOTO AM (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives.* **101:**378-384.

COOKE PS, HOLSBERGER DR, WITORSCH RJ, SYLVESTER PW, MEREDITH JM, TREINEN KA, CHAPIN RE (2004). Thyroid hormone, glucocorticoids, and prolactin at the nexus of physiology, reproduction, and toxicology. *Toxicology and Applied Pharmacology.* **194:**309-335.

COOPER RL, KAVLOCK RJ (1997). Endocrine disruptors and reproductive development: A weight-of-evidence overview. *Journal of Endocrinology.* **152:**159-166.

COWARD K, BROMAGE NR (2000). Reproductive physiology of female tilapia broodstock. *Reviews in Fish Biology and Fisheries.* **10:**1-25.

D

DALVIE M, CAIRNCROSS E, SOLOMON A, LONDON L (2003). Contamination of rural surface and ground water by endosulfan in farming areas of the Western Cape, South Africa. *Environmental Health: A Global Access Science Source.* **2:**1.

DANG Z, BALM PH, FLIK G, WENDELAAR BONGA SE, LOCK RA (2000). Cortisol increases Na^+/K^+-ATPase density in plasma membranes of gill chloride cells in the freshwater tilapia *Oreochromis mossambicus*. *Journal of Experimental Biology.* **203:**2349-2355.

DANGER JM, BRETON B, VALLARINO M, FOURNIER A, PELLETIER G, VAUDRY H (1991). Neuropeptide-Y in the trout brain and pituitary - localization, characterization, and action on gonadotropin-release. *Endocrinology.* **128:**2360-2368.

DE LEEUW R, VAN 'T VEER C, GOOS HJT, VAN OORDT PGWJ (1988). The dopaminergic regulation of gonadotropin-releasing hormone receptor binding in the pituitary of the African catfish, *Clarias gariepinus*. *General and Comparative Endocrinology.* **72:**408-415.

DORVAL J, HONTELA A (2003). Role of glutathione redox cycle and catalase in defense against oxidative stress induced by endosulfan in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*). *Toxicology and Applied Pharmacology.* **192:**191-200.

DORVAL J, LEBLOND VS, HONTELA A (2003). Oxidative stress and loss of cortisol secretion in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) exposed *in vitro* to endosulfan, an organochlorine pesticide. *Aquatic Toxicology.* **63:**229-241.

DRAGNEV KH, BEEBE LE, JONES CR, FOX SD, THOMAS PE, NIMS RW, LUBET RA (1994). Subchronic dietary exposure to Aroclor 1254 in rats: accumulation of PCBs in liver, blood, and adipose tissue and its relationship to induction of various hepatic drug-metabolizing enzymes. *Toxicology and Applied Pharmacology.* **125:**111-122.

E

EALES JG, SHOSTAK S (1985). Free T4 and T3 in relation to total hormone, free hormone indexes, and protein in plasma of Rainbow-trout and Arctic charr. *General and Comparative Endocrinology.* **58:**291-302.

EALES JG (1987). Kinetics of T-4-binding and T-3-binding to plasma sites in salmonid teleost fish. *General and Comparative Endocrinology.* **65:**288-299.

EALES JG, FINNSON KR (1991). Response of hepatic thyroxine 5'-deiodinase of rainbow-trout, *Oncorhynchus-mykiss*, to chronic ingestion of 3,5,3'-triiodo-l-thyronine. *Journal of Experimental Zoology.* **257:**230-235.

EALES JG, BROWN SB (1993). Measurement and regulation of thyroidal status in teleost fish. *Reviews in Fish Biology and Fisheries.* **3:**299-347.

EALES JG (1997). Iodine metabolism and thyroid-related functions in organisms lacking thyroid follicles: Are thyroid hormones also vitamins? *Proceedings of the Society for Experimental Biology and Medicine.* **214:**302-317.

EALES JG, BROWN SB, CYR DG, ADAMS BA, FINNSON KR (1999). Deiodination as an index of chemical disruption of thyroid hormone homeostasis and thyroidal status in fish In: DS HENSHEL, MC BLACK and MC HARRASS (Eds.). *Environmental Toxicology and Risk Assessment: Standardization of Biomarkers for Endocrine Disruption and Environmental Assessment: Eighth Volume.* **1364:**136-164.

EDWARDS P (2003). Role of tiapia in rural aquaculture. *Global Aquaculture Advocate.* 59-61.

EL-KABBANY S, RASHED MM, ZAYED MA (2000). Monitoring of the pesticide levels in some water supplies and agricultural land, in El-Haram, Giza (ARE). *Journal of Hazardous Materials.* **72:**11-21.

ENEQVIST T, LUNDBERG E, NILSSON L, ABAGYAN R, SAUER-ERIKSSON AE (2003). The transthyretin--related protein family. *European Journal of Biochemistry.* **270:**518-532.

ENEQVIST T, LUNDBERG E, KARLSSON A, HUANG SH, SANTOS CRA, POWER DM, SAUER-ERIKSSON AE (2004). High resolution crystal structures of piscine transthyretin reveal different binding modes for triiodothyronine and thyroxine. *Journal of Biological Chemistry.* **279:**26411-26416.

EPA http://www.epa.gov/toxteam/pcbid/aroclor_comp.htm.

F

FALKNER NW, EALES JG (1973). Investigation of iodothyronine binding to plasma-proteins in brook trout, *Salvelinus-fontinalis*, using precipitation, dialysis, and electrophoretic methods. *Journal of the Fisheries Research Board of Canada.* **30:**1131-1140.

FAO-FIGIS (2003). http://www.fao.org/figis/servlet/static?dom=root&xml=tseries/index.xml.

FENT K, BUCHELI TD (1994). Inhibition of hepatic-microsomal monooxygenase system by organotins *in-vitro* in fresh-water fish. *Aquatic Toxicology.* **28:**107-126.

FENTON B, OROZCO A, VALVERDE C (1997). Kinetic characterisation of skin inner-ring deiodinative pathway and its correlation with circulating levels of reverse tri-iodothyronine in developing rainbow trout. *Journal of Endocrinology.* **154:**547-554.

FENTON B, VALVERDE C (2000). Hepatic outer-ring deiodinase in a Mexican endemic lizard (*Sceloporus grammicus*). *General and Comparative Endocrinology.* **117:**77-88.

FERRANDO MD, SANCHO E, ANDREUMOLINER E (1991). Comparative acute toxicities of selected pesticides to *Anguilla-anguilla. Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes.* **26:**491-498.

FERREIRA M, ANTUNES P, GIL O, VALE C, REIS-HENRIQUES MA (2004). Organochlorine contaminants in flounder (*Platichthys flesus*) and mullet (*Mugil cephalus*) from Douro estuary, and their use as sentinel species for environmental monitoring. *Aquatic Toxicology.* **69:**347-357.

FIGUEIREDO-FERNANDES A, FONTAINHAS-FERNANDES A, MONTEIRO R, REIS-HENRIQUES MA, ROCHA E (2006). Effects of the fungicide mancozeb on liver structure of Nile tilapia, *Oreochromis niloticus*:

Assessment and quantification of induced cytological changes using qualitative histopathology and the stereological point-sampled intercept method. *Bulletin of Environmental Contamination and Toxicology.* **76:**249-255.

FIGUEIREDO-FERNANDES A, FONTAINHAS-FERNANDES A, MONTEIRO RAF, REIS-HENRIQUES MA, ROCHA E (In press). Spacial relationships of the intrahepatic vascular-biliary tracs and associated pancreatic acini of Nile tilapia, Oreochromis niloticus (Teleostei, Cichlidae): a serial sectioning analysis study by light miscrocopy. *Annals of Anatomy.*

FINGLER S, DREVENKAR V, TKALCEVIC B, SMIT Z (1992). Levels of polychlorinated-biphenyls, organochlorine pesticides, and chlorophenols in the Kupa river water and in drinking waters from different areas in Croatia. *Bulletin of Environmental Contamination and Toxicology.* **49:**805-812.

FINNSON KW, EALES JG (1999). Effect of T3 treatment and food ration on hepatic deiodination and conjugation of thyroid hormones in rainbow trout, *Oncorhynchus mykiss. General and Comparative Endocrinology.* **115:**379-386.

FINNSON KW, MCLEESE JM, EALES JG (1999). Deiodination and deconjugation of thyroid hormone conjugates and type I deiodination in liver of rainbow trout, *Oncorhynchus mykiss. General and Comparative Endocrinology.* **115:**387-397.

FISHER BE (1999). Most unwanted persistent organic pollutants. *Environmental Health Perspectives.* **107:**A18-A23.

FLIK G, KLAREN PHM, VAN DEN BURG EH, METZ JR, HUISING MO (2006). CRF and stress in fish. *General and Comparative Endocrinology.* **146:**36-44.

FOLLI C, PASQUATO N, RAMAZZINA I, BATTISTUTTA R, ZANOTTI G, BERNI R (2003). Distinctive binding and structural properties of piscine transthyretin. *Febs Letters.* **555:**279-284.

FOLMAR LC, HODGINS HO (1982). Effects of Aroclor 1254 and No-2 fuel-oil, singly and in combination, on predator-prey interactions in coho salmon (*Oncorhynchus-kisutch*). *Bulletin of Environmental Contamination and Toxicology.* **29:**24-28.

FONTAÍNHAS FERNANDES AA (1998). Tilápia In: MA REIS-HENRIQUES (Eds.). *Manual de Aquacultura* Editora Marca-Artes Gráficas. 207.

FONTAINHAS-FERNANDES A, MONTEIRO M, FIGUEIREDO A, GOMES E, COIMBRA J, REIS-HENRIQUES MA (2000). Partial or total replacement of fish meal by plant protein affects gonadal development and plasma 17 beta-estradiol levels in female Nile tilapia. *Aquaculture International.* **8:**299-313.

FONTAINHAS-FERNANDES A, GOMES EF, REIS-HENRIQUES MA, COIMBRA J (2003). Effect of cortisol on some osmoregulatory parameters of the teleost, *Oreochromis niloticus* L., after transference from freshwater to seawater. *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia.* **55:**562-567.

FRITH SD, EALES JG (1996). Thyroid hormone deiodination pathways in brain and liver of rainbow trout, *Oncorhynchus mykiss. General and Comparative Endocrinology.* **101:**323-332.

FUNKENSTEIN B, PERROT V, BROWN CL (1999). Cloning of putative piscine (*Sparus aurata*) transthyretin: developmental expression and tissue distribution. *Molecular and Cellular Endocrinology.* **157:**67-73.

FURUYA H, SARAIVA MJM, GAWINOWICZ MA, ALVES IL, COSTA PP, SASAKI H, GOTO I, SAKAKI Y (1991). Production of recombinant human transthyretin with biological-activities toward the understanding of the molecular-basis of familial amyloidotic polyneuropathy (FAP). *Biochemistry.* **30:**2415-2421.

G

GANDOLFI F, POCAR P, BREVINI TAL, FISCHER B (2002). Impact of endocrine disrupters on ovarian function and embryonic development. *Domestic Animal Endocrinology.* **23:**189-201.

GARCIA-G C, JEZIORSKI MC, VALVERDE-R C, OROZCO A (2004). Effects of iodothyronines on the hepatic outer-ring deiodinating pathway in killifish. *General and Comparative Endocrinology.* **135:**201-209.

GOERKE H, WEBER K (2001). Species-specific elimination of polychlorinated biphenyls in estuarine animals and its impact on residue patterns. *Marine Environmental Research.* **51:**131-149.

GOOS HJT, JOY KP, DELEEUW R, VANOORDT P, VANDELFT AML, GIELEN JT (1987). The effect of luteinizing-hormone-releasing hormone analog (LHRHa) in combination with different drugs with anti-dopamine and anti-serotonin properties on gonadotropin-release and ovulation in the african catfish, *Clarias-gariepinus*. *Aquaculture.* **63:**143-156.

GOTHILF Y, MUNOZCUETO JA, SAGRILLO CA, SELMANOFF M, CHEN TT, KAH O, ELIZUR A, ZOHAR Y (1996). Three forms of gonadotropin-releasing hormone in a perciform fish (*Sparus aurata*): Complementary deoxyribonucleic acid characterization and brain localization. *Biology of Reproduction.* **55:**636-645.

GOULD JC, COOPER KR, SCANES CG (1999). Effects of polychlorinated biphenyls on thyroid hormones and liver type I monodeiodinase in the chick embryo. *Ecotoxicology and Environmental Safety.* **43:**195-203.

GRAU EG, HELMS LMH, SHIMODA SK, FORD CA, LEGRAND J, YAMAUCHI K (1986). The thyroid-gland of the hawaiian parrotfish and its use as an *in vitro* model system. *General and Comparative Endocrinology.* **61:**100-108.

GRAY LE, OSTBY JS (1995). *In-utero* 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters reproductive morphology and function in female rat offspring. *Toxicology and Applied Pharmacology.* **133:**285-294.

GUILLETTE LJ, PICKFORD DB, CRAIN DA, ROONEY AA, PERCIVAL HF (1996). Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. *General and Comparative Endocrinology.* **101:**32-42.

GUILLETTE LJ, GUNDERSON MP (2001). Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. *Reproduction.* **122:**857-864.

GUR G, MELAMED P, GISSIS A, YARON Z (2000). Changes along the pituitary-gonadal axis during maturation of the black carp, *Mylopharyngodon piceus*. *Journal of Experimental Zoology.* **286:**405-413.

GUR G, BONFIL D, SAFARIAN H, NAOR Z, YARON Z (2002). GnRH signaling pathways regulate differentially the tilapia gonadotropin subunit genes. *Molecular and Cellular Endocrinology.* **189:**125-134.

GUR G, BONFIL D, SAFARIAN H, NAOR Z, YARON Z (2001a). GnRH receptor signaling in tilapia pituitary cells: role of mitogen-activated protein kinase (MAPK). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology.* **129:**517-524.

GUR G, ROSENFELD H, MELAMED P, MEIRI I, ELIZUR A, YARON Z (2001b). Tilapia glycoprotein hormone alpha subunit: cDNA cloning and hypothalamic regulation. *Molecular and Cellular Endocrinology.* **182:**49-60.

H

HABIBI HR, MARCHANT TA, NAHORNIAK CS, VANDERLOO H, PETER RE, RIVIER JE, VALE WW (1989). Functional-relationship between receptor-binding and biological-activity for analogs of mammalian and salmon gonadotropin-releasing hormones in the pituitary of goldfish (*Carassius-auratus*). *Biology of Reproduction.* **40:**1152-1161.

HALLGREN S, DARNERUD PO (2002). Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats - testing interactions and mechanisms for thyroid hormone effects. *Toxicology.* **177:**227-243.

HANY J, LILIENTHAL H, SARASIN A, ROTH-HARER A, FASTABEND A, DUNEMANN L, LICHTENSTEIGER W, WINNEKE G (1999). Developmental exposure of rats to a reconstituted PCB mixture or Aroclor 1254: Effects on organ weights, aromatase activity, sex hormone levels, and sweet preference behavior. *Toxicology and Applied Pharmacology.* **158:**231-243.

HARDELL L, VAN BAVEL B, LINDSTROM G, ERIKSSON M, CARLBERG M (2006). *In utero* exposure to persistent organic pollutants in relation to testicular cancer risk. *International Journal of Andrology.* **29:**228-234.

HARDY MP, KIRBY JD, HESS RA, COOKE PS (1993). Leydig-cells increase their numbers but decline in steroidogenic function in the adult-rat after neonatal-hypothyroidism. *Endocrinology.* **132:**2417-2420.

HARRISON PTC, HOLMES P, HUMFREY CDN (1997). Reproductive health in humans and wildlife: are adverse trends associated with environmental chemical exposure? *Science of the Total Environment.* **205:**97-106.

HEIDEN TK, HUTZ RJ, CARVAN MJ (2005). Accumulation, tissue distribution, and maternal transfer of dietary 2,3,7,8,-tetrachlorodibenzo-p-dioxin: Impacts on reproductive success of zebrafish. *Toxicological Sciences.* **87:**497-507.

HEIDEN TK, CARVAN MJ, HUTZ RJ (2006). Inhibition of follicular development, vitellogenesis, and serum 17 beta-estradiol concentrations in zebrafish following chronic, sublethal dietary exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicological Sciences.* **90:**490-499.

HENRY L, KISHIMBA MA (2006). Pesticide residues in Nile Tilapia (*Oreochromis niloticus*) and nile perch (*Lates niloticus*) from Southern Lake Victoria, Tanzania. *Environmental Pollution.* **140:**348-354.

HONTELA A, DUMONT P, DUCLOS D, FORTIN R (1995). Endocrine and metabolic dysfunction in yellow perch, *Perca-flavescens*, exposed to organic contaminants and heavy-metals in the St-Lawrence River. *Environmental Toxicology and Chemistry.* **14:**725-731.

HOOD A, KLAASSEN CD (2000). Effects of microsomal enzyme inducers on outer-ring deiodinase activity toward thyroid hormones in various rat tissues. *Toxicology and Applied Pharmacology.* **163:**240-248.

HUANG YS, WANG LH, TSAI CL (2004). Photoperiod affects the development of central neurotransmitter systems of tilapia, *Oreochromis mossambicus*. *Neuroscience Letters.* **355:**201-204.

HUGLA JL, THOME JP (1999). Effects of polychlorinated biphenyls on liver ultrastructure, hepatic monooxygenases, and reproductive success in the barbel. *Ecotoxicology and Environmental Safety.* **42:**265-273.

HWANG H-M, GREEN PG, YOUNG TM (2006). Tidal salt marsh sediment in California, USA. Part 1: Occurrence and sources of organic contaminants. *Chemosphere.* **64:**1383-1392.

HYLLNER SJ, OPPENBERNTSEN DO, HELVIK JV, WALTHER BT, HAUX C (1991). Estradiol-17-beta induces the major vitelline envelope proteins in both sexes in teleosts. *Journal of Endocrinology.* **131:**229-236.

J

JANSEN HT, COOKE PS, PORCELLI J, LIU TC, HANSEN LG (1993). Estrogenic and antiestrogenic actions of PCBs in the female rat – *in vitro* and *in vivo* studies. *Reproductive Toxicology.* **7:**237-248.

JANSSEN PAH, LAMBERT JGD, VETHAAK A, GOOS HJT (1997). Environmental pollution caused elevated concentrations of oestradiol and vitellogenin in the female flounder, *Platichthys flesus* (L). *Aquatic Toxicology.* **39:**195-214.

JOBLING S, NOLAN M, TYLER CR, BRIGHTY G, SUMPTER JP (1998). Widespread sexual disruption in wild fish. *Environmental Science & Technology.* **32:**2498-2506.

JOHNSON W, FINLEY M (1980). Handbook of acute toxicity of chemicals to fish and aquatic invertebrates. UNITED STATES DEPARTMENT OF THE INTERIOR FISH AND WILDLIFE SERVICE (Eds.). *Resource Publication* 137:1-98.

JOHNSTON CE, EALES JG (1995). Effects of acclimation and assay temperature on outer-ring and inner-ring thyroxine and 3,5,3'-triiodo-l-thyronine deiodination by liver-microsomes of rainbow-trout, *Oncorhynchus-mykiss*. *Journal of Experimental Zoology.* **272:**426-434.

K

KAH O, TRUDEAU VL, SLOLEY BD, CHANG JP, DUBOURG P, YU KL, PETER RE (1992). Influence of GABA on gonadotropin-release in the goldfish. *Neuroendocrinology.* **55:**396-404.

KAH O, ANGLADE I, LEPRETRE E, DUBOURG P, DEMONBRISON D (1993). The reproductive brain in fish. *Fish Physiology and Biochemistry.* **11:**85-98.

KAMIJIMA M, CASIDA JE (2000). Regional modification of [H-3]ethynylbicycloorthobenzoate binding in mouse brain GABA(A) receptor by endosulfan, fipronil, and avermectin B-1a. *Toxicology and Applied Pharmacology.* **163:**188-194.

KASZA L, COLLINS WT, CAPEN CC, GARTHOFF LH, FRIEDMAN L (1978). Comparative toxicity of polychlorinated biphenyl and polybrominated biphenyl in the rat thyroid-gland - light and electron--microscopic alterations after sub-acute dietary exposure. *Journal of Environmental Pathology and Toxicology.* **1:**587-599.

KAVLOCK RJ, DASTON GP, DEROSA C, FENNERCRISP P, GRAY LE, KAATTARI S, LUCIER G, LUSTER M, MAC MJ, MACZKA C, MILLER R, MOORE J, ROLLAND R, SCOTT G, SHEEHAN DM, SINKS T, TILSON HA (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the US EPA-sponsored workshop. *Environmental Health Perspectives.* **104:**715-740.

KAY DP, BLANKENSHIP AL, COADY KK, NEIGH AM, ZWIERNIK MJ, MILLSAP SD, STRAUSE K, PARK C, BRADLEY P, NEWSTED JL, JONES PD, GIESY JP (2005). Differential accumulation of polychlorinated biphenyl congeners in the aquatic food web at the Kalamazoo River superfund site, Michigan. *Environmental Science & Technology.* **39:**5964-5974.

KHAN IA, THOMAS P (1997). Aroclor 1254-induced alterations in hypothalamic monoamine metabolism in the Atlantic croaker (*Micropogonias undulatas*): Correlation with pituitary gonadotropin release. *Neurotoxicology.* **18:**553-560.

KHAN IA, THOMAS P (2000). Lead and Aroclor 1254 disrupt reproductive neuroendocrine function in Atlantic croaker. *Marine Environmental Research.* **50:**119-123.

KHAN IA, THOMAS P (2001). Disruption of neuroendocrine control of luteinizing hormone secretion by Aroclor 1254 involves inhibition of hypothalamic tryptophan hydroxylase activity. *Biology of Reproduction.* **64:**955-964.

KHAN IA, MATHEWS S, OKUZAWA K, KAGAWA H, THOMAS P (2001). Alterations in the GnRH-LH system in relation to gonadal stage and Aroclor 1254 exposure in Atlantic croaker. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology.* **129:**251-259.

KIM IS, ARIYARATNE HBS, MENDIS-HANDAGAMA S (2001). Effects of continuous and intermittent exposure of lactating mothers to Aroclor 1242 on testicular steroidogenic function in the adult male offspring. *Tissue & Cell.* **33:**169-177.

KITAHASHI T, SATO H, SAKUMA Y, PARHAR IS (2005). Cloning and functional analysis of promoters of three GnRH genes in a cichlid. *Biochemical and Biophysical Research Communications.* **336:**536-543.

KOBAYASHI M, AIDA K, HANYU I (1989). Induction of gonadotropin surge by steroid-hormone implantation in ovariectomized and sexually regressed female goldfish. *General and Comparative Endocrinology.* **73:**469-476.

KOVACEVIC R, VOJINOVICMILORADOV M, TEODOROVIC I, ANDRIC S (1995). Effect of PCBs on androgen production by suspension of adult-rat Leydig-cells *in-vitro*. *Journal of Steroid Biochemistry and Molecular Biology.* **52:**595-597.

L

LANGER P (1998). Minireview: Polychlorinated biphenyls and the thyroid gland. *Endocrine Regulations.* **32:**193-203.

LANS MC, KLASSONWEHLER E, WILLEMSEN M, MEUSSEN E, SAFE S, BROUWER A (1993). Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, hydroxy-dibenzo-p-dioxins and hydroxy-dibenzofurans with human transthyretin. *Chemico-Biological Interactions.* **88:**7-21.

LANS MC, SPIERTZ C, BROUWER A, KOEMAN JH (1994). Different competition of thyroxine-binding to transthyretin and thyroxine-binding globulin by hydroxy-PCBs, PCDDs and PCDFs. *European Journal of Pharmacology-Environmental Toxicology and Pharmacology Section.* **270:**129-136.

LARSSON M, PETTERSSON T, CARLSTROM A (1985). Thyroid-hormone binding in serum of 15 vertebrate species - isolation of thyroxine-binding globulin and prealbumin analogs. *General and Comparative Endocrinology.* **58:**360-375.

LAUDER JM, KREBS H (1976). Effects of P-chlorophenylalanine on time of neuronal origin during embryogenesis in rat. *Brain Research.* **107:**638-644.

LEATHERLAND JF, SONSTEGARD RA (1978). Lowering of serum thyroxine and triiodothyronine levels in yearling coho salmon, *Oncorhynchus-kisutch. Journal of the Fisheries Research Board of Canada.* **35:**1285-1289.

LEATHERLAND JF, BARNETT BJ, CHO CY, SLINGER SJ (1980). Effect of dietary cholecalciferol deficiency on plasma thyroid-hormone concentrations in rainbow-trout, *Salmo-gairdneri* (Pisces, Salmonidae). *Environmental Biology of Fishes.* **5:**167-173.

LEATHERLAND JF (1993). Field observations on reproductive and developmental dysfunction in introduced and native salmonids from the Great-Lakes. *Journal of Great Lakes Research.* **19:**737-751.

LEATHERLAND JF (1994). Reflections on the thyroidology of fishes: from molecules to humankind *Guelph Ichthyology Reviews.* **2:**1-67.

LEBLOND VS, BISSON M, HONTELA A (2001). Inhibition of cortisol secretion in dispersed head kidney cells of rainbow trout (*Oncorhynchus mykiss*) by endosulfan, an organochlorine pesticide. *General and Comparative Endocrinology.* **121:**48-56.

LEONARD AW, HYNE RV, LIM RP, PABLO F, VAN DEN BRINK PJ (2000). Riverine endosulfan concentrations in the Namoi River, Australia: Link to cotton field runoff and macroinvertebrate population densities. *Environmental Toxicology and Chemistry.* **19:**1540-1551.

LETHIMONIER C, MADIGOU T, MUNOZ-CUETO JA, LAREYRE JJ, KAH O (2004). Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. *General and Comparative Endocrinology.* **135:**1-16.

LEVAVI-SIVAN B, OFIR M, YARON Z (1995). Possible sites of dopaminergic inhibition of gonadotropin release from the pituitary of a teleost fish, tilapia. *Molecular and Cellular Endocrinology.* **109:**87-95.

LEVAVI-SIVAN B, AVITAN A, KANIAS T (2003). Characterization of the inhibitory dopamine receptor from the pituitary of tilapia. *Fish Physiology and Biochemistry.* **28:**73-75.

LEVAVI-SIVAN B, SAFARIAN H, ROSENFELD H, ELIZUR A, AVITAN A (2004). Regulation of gonadotropin--releasing hormone (GnRH)-receptor gene expression in tilapia: Effect of GnRH and dopamine. *Biology of Reproduction.* **70:**1545-1551.

LI MH, HANSEN LG (1997). Consideration of enzyme and endocrine interactions in the risk assessment of PCBs. *Reviews in Toxicology.* **1:**71-156.

LIN GR, WENG CF, WANG JI, HWANG PP (1999). Effects of cortisol on ion regulation in developing tilapia (*Oreochromis mossambicus*) larvae on seawater adaptation. *Physiological and Biochemical Zoology.* **72:**397-404.

LINARD B, BENNANI S, SALIGAUT C (1995). Involvement of estradiol in a catecholamine inhibitory tone of gonadotropin-release in the rainbow-trout (*Oncorhynchus-mykiss*). *General and Comparative Endocrinology.* **99:**192-196.

M

MACINTOSH DJ, LITTLE DC (1995). Nile tilapia (*Oreochromis niloticus*) In: NR BROMAGE and RJ ROBERTS (Eds.). *Broodstock Management and Egg and Larval Quality.* Blackwell Science, Institute of Aquaculture. 277-320.

MACLATCHY DL, EALES JG (1992). Intracellular and extracellular sources of T3 binding to putative thyroid-hormone receptors in liver, kidney, and gill nuclei of immature rainbow-trout, *Oncorhynchus-mykiss. Journal of Experimental Zoology.* **262:**22-29.

MANANOS EL, ANGLADE I, CHYB J, SALIGAUT C, BRETON B, KAH O (1999). Involvement of gamma--aminobutyric acid in the control of GTH-1 and GTH-2 secretion in male and female rainbow trout. *Neuroendocrinology.* **69:**269-280.

MARIUSSEN E, FONNUM F (2001). The effect of polychlorinated biphenyls on the high affinity uptake of the neurotransmitters, dopamine, serotonin, glutamate and GABA, into rat brain synaptosomes. *Toxicology.* **159:**11-21.

MATSUYAMA T, IIDA T (1999). Degranulation of eosinophilic granular cells with possible involvement in neutrophil migration to site of inflammation in tilapia. *Developmental and Comparative Immunology.* **23:**451-457.

MATTA SLP, VILELA DAR, GODINHO HP, FRANCA LR (2002). The goitrogen 6-n-propyl-2-thiouracil (PTU) given during testis development increases sertoli and germ cell numbers per cyst in fish: The tilapia (*Oreochromis niloticus*) model. *Endocrinology.* **143:**970-978.

MATTHIESSEN P, GIBBS PE (1998). Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. *Environmental Toxicology and Chemistry.* **17:**37-43.

MCALLISTER BG, KIME DE (2003). Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). *Aquatic Toxicology.* **65:**309-316.

MCCLAIN RM (1989). The significance of hepatic-microsomal enzyme-induction and altered thyroid--function in rats - implications for thyroid-gland neoplasia. *Toxicologic Pathology.* **17:**294-306.

MCKINNEY JD, WALLER CL (1994). Polychlorinated-biphenyls as hormonally active structural analogs. *Environmental Health Perspectives.* **102:**290-297.

MELAMED P, GUR G, ELIZUR A, ROSENFELD H, SIVAN B, RENTIERDELRUE F, YARON Z (1996). Differential effects of gonadotropin-releasing hormone, dopamine and somatostatin and their second messengers on the mRNA levels of gonadotropin II beta subunit and growth hormone in the teleost fish, tilapia. *Neuroendocrinology.* **64:**320-328.

MELAMED P, GUR G, ROSENFELD H, ELIZUR A, YARON Z (1997). The mRNA levels of GtH I beta, GtH II beta and GH in relation to testicular development and testosterone treatment in pituitary cells of male tilapia. *Fish Physiology and Biochemistry.* **17:**93-98.

MELAMED P, ROSENFELD H, ELIZUR A, YARON Z (1998). Endocrine regulation of gonadotropin and growth hormone gene transcription in fish. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology.* **119:**325-338.

MELAMED P, GUR G, ROSENFELD H, ELIZUR A, SCHULZ RW, YARON Z (2000). Reproductive development of male and female tilapia hybrids (*Oreochromis niloticus* x *O-aureus*) and changes in mRNA levels of gonadotropin (GH) I beta and II beta subunits. *Journal of Experimental Zoology.* **286:**64-75.

MENDEL CM, WEISIGER RA, JONES AL, CAVALIERI RR (1987). Thyroid hormone-binding proteins in plasma facilitate uniform-distribution of thyroxine within tissues - a perfused-rat-liver study. *Endocrinology.* **120:**1742-1749.

MENDISHANDAGAMA S, SHARMA OP (1994). Effects of neonatal administration of the reversible goitrogen propylthiouracil on the testis interstitium in adult-rats. *Journal of Reproduction and Fertility.* **100:**85-92.

MILLAR RP (2003). GnRH II and type II GnRH receptors. *Trends in Endocrinology and Metabolism.* **14:**35-43.

MOL K, KAPTEIN E, DARRAS VM, DEGREEF WJ, KUHN ER, VISSER TJ (1993). Different thyroid hormone-deiodinating enzymes in tilapia (*Oreochromis-niloticus*) liver and kidney. *Febs Letters.* **321**:140-144.

MOL KA, VAN DER GEYTEN S, DARRAS VM, VISSER TJ, KUHN ER (1997). Characterization of iodothyronine outer ring and inner ring deiodinase activities in the blue tilapia, *Oreochromis aureus*. *Endocrinology.* **138**:1787-1793.

MOL KA, VAN DER GEYTEN S, BUREL C, KUHN ER, BOUJARD T, DARRAS VM (1998). Comparative study of iodothyronine outer ring and inner ring deiodinase activities in five teleostean fishes. *Fish Physiology and Biochemistry.* **18**:253-266.

MOL KA, VAN DER GEYTEN S, KUHN ER, DARRAS VM (1999). Effects of experimental hypo- and hyperthyroidism on iodothyronine deiodinases in Nile tilapia, *Oreochromis niloticus*. *Fish Physiology and Biochemistry.* **20**:201-207.

MOMMSEN TP, VIJAYAN MM, MOON TW (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries.* **9**:211-268.

MONOSSON E, ASHLEY JTF, MCELROY AE, WOLTERING D, ELSKUS AA (2003). PCB congener distributions in muscle, liver and gonad of *Fundulus heteroclitus* from the lower Hudson River Estuary and Newark Bay. *Chemosphere.* **52**:777-787.

MURUGESAN P, KANAGARAJ P, YUVARAJ S, BALASUBRAMANIAN K, ARULDHAS MM, ARUNAKARAN J (2005a). The inhibitory effects of polychlorinated biphenyl Aroclor 1254 on Leydig cell LH receptors, steroidogenic enzymes and antioxidant enzymes in adult rats. *Reproductive Toxicology.* **20**:117-126.

MURUGESAN P, MUTHUSAMY T, BALASUBRAMANIAN K, ARUNAKARAN J (2005b). Studies on the protective role of vitamin C and E against polychlorinated biphenyl (Aroclor 1254) - induced oxidative damage in Leydig cells. *Free Radical Research.* **39**:1259-1272.

MUTHUVEL R, VENKATARAMAN P, KRISHNAMOORTHY G, GUNADHARINI DN, KANAGARAJ P, STANLEY AJ, SRINIVASAN N, BALASUBRAMANIAN K, ARULDHAS MM, ARUNAKARAN J (2006). Antioxidant effect of ascorbic acid on PCB (Aroclor 1254) induced oxidative stress in hypothalamus of albino rats. *Clinica Chimica Acta.* **365**:297-303.

N

NAGAHAMA Y (1987). 17-Alpha, 20-beta-dihydroxy-4-pregnen-3-one - a teleost maturation-inducing hormone. *Development Growth & Differentiation.* **29**:1-12.

NAQVI SM, VAISHNAVI C (1993). Bioaccumulative potential and toxicity of endosulfan insecticide to nontarget animals. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology.* **105**:347-361.

NCBI Taxonomy Browser- http://www.ncbi.nlm.nih.gov/entrez/query.fcgi

NGUYEN LTH, JANSSEN CR (2002). Embryo-larval toxicity tests with the African catfish (*Clarias gariepinus*): Comparative sensitivity of endpoints. *Archives of Environmental Contamination and Toxicology.* **42**:256-262.

NOZAKI M, NAITO N, SWANSON P, DICKHOFF WW, NAKAI Y, SUZUKI K, KAWAUCHI H (1990). Salmonid pituitary gonadotrophs. 2:Ontogeny of GtH-I and GtH-II Cells in the Rainbow-trout (*Salmo-gairdneri-irideus*). *General and Comparative Endocrinology.* **77**:358-367.

NTOW WJ (2001). Organochlorine pesticides in water, sediment, crops, and human fluids in a farming community in Ghana. *Archives of Environmental Contamination and Toxicology.* **40**:557-563.

O

OBERDORSTER E, CHEEK AO (2001). Gender benders at the beach: Endocrine disruption in marine and estuarine organisms. *Environmental Toxicology and Chemistry.* **20**:23-36.

Okimoto DK, Distefano JJ, Kuwaye TT, Ron B, Weber GM, Nguyen TT, Grau EG (1994). On plasma-volume measurement and the effect of experimental stress in the male tilapia, *Oreochromis-mossambicus*, maintained in fresh-water. *Fish Physiology and Biochemistry.* **12:**431-438.

Onitake K, Iwamatsu T (1986). Immunocytochemical demonstration of steroid-hormones in the granulosa-cells of the medaka, *Oryzias latipes*. *Journal of Experimental Zoology.* **239:**97-103.

Orn S, Andersson PL, Forlin L, Tysklind M, Norrgren L (1998). The impact on reproduction of an orally administered mixture of selected PCBs in zebrafish (*Danio rerio*). *Archives of Environmental Contamination and Toxicology.* **35:**52-57.

Orozco A, Silva JE, Valverde C (1997). Rainbow trout liver expresses two iodothyronine phenolic ring deiodinase pathways with the characteristics of mammalian types I and II 5'-deiodinases. *Endocrinology.* **138:**254-258.

Orozco A, Linser P, Valverde-R C (2000). Kinetic characterization of outer-ring deiodinase activity (ORD) in the liver, gill and retina of the killifish *Fundulus heteroclitus*. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology.* **126:**283-290.

Orozco A, Jeziorski MC, Linser PJ, Greenberg RM, Valverde-R C (2002a). Cloning of the gene and complete cDNA encoding a type 2 deiodinase from *Fundulus heteroclitus*. *General and Comparative Endocrinology.* **128:**162-167.

Orozco A, Villalobos P, Valverde C (2002b). Environmental salinity selectively modifies the outer-ring deiodinating activity of liver, kidney and gill in the rainbow trout. *Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology.* **131:**387-395.

Orozco A, Villalobos P, Jeziorski MC, Valverde C (2003). The liver of *Fundulus heteroclitus* expresses deiodinase type 1 mRNA. *General and Comparative Endocrinology.* **130:**84-91.

Orozco A, Valverde-R C (2005). Thyroid hormone deiodination in fish. *Thyroid.* **15:**799-813.

P

Pacheco M, Santos MA (2002). Biotransformation, genotoxic and histopathological effects of environmental contaminants in European eel (*Anguilla Anguilla* L.). Ecotoxicology and Environmental Safety. **53:**331-347.

Palada de Vera MS, Eknath AE (1995). Growth performance of males and females of different strains of Nile tilapia (*Oreochromis niloticus*) in different culture environments. *Aquaculture.* **137:**331.

Palstra A, van Ginneken V, Murk A, van den Thillart G (2006). Are dioxin-like contaminants responsible for the eel (*Anguilla anguilla*) drama? *Naturwissenschaften.* **93:**145-148.

Parhar IS, Soga T, Sakuma Y (2000). Thyroid hormone and estrogen regulate brain region--specific messenger ribonucleic acids encoding three gonadotropin-releasing hormone genes in sexually immature male fish, *Oreochromis niloticus*. *Endocrinology.* **141:**1618-1626.

Peng C, Huang YP, Peter RE (1990). Neuropeptide-Y stimulates growth-hormone and gonadotropin-release from the goldfish pituitary *in vitro*. *Neuroendocrinology.* **52:**28-34.

Peng C, Chang JP, Yu KL, Wong AOL, Vangoor F, Peter RE, Rivier JE (1993a). Neuropeptide-Y stimulates growth-hormone and gonadotropin-II secretion in the goldfish pituitary - involvement of both presynaptic and pituitary cell actions. *Endocrinology.* **132:**1820-1829.

Peng C, Humphries S, Peter RE, Rivier JE, Blomqvist AG, Larhammar D (1993b). Actions of goldfish neuropeptide-Y on the secretion of growth-hormone and gonadotropin-II in female goldfish. *General and Comparative Endocrinology.* **90:**306-317.

Pepels PPLM, Balm PHM (2004). Ontogeny of corticotropin-releasing factor and of hypothalamic--pituitary-interrenal axis responsiveness to stress in tilapia (*Oreochromis mossambicus*; Teleostei). *General and Comparative Endocrinology.* **139:**251-265.

PETER RE, CHANG JP, NAHORNIAK CS, OMELJANIUK RJ, SOKOLOWSKA M, SHIH SH, BILLARD R (1986). Interactions of catecholamines and GnRH in regulation of gonadotropin-secretion in teleost fish. *Recent Progress in Hormone Research.* **42:**513-548.

PETER RE, CHANG JP, NAHORNIAK CS, YU KL, HUANG YP, MARCHANT TA (1990). Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. *Progress in Clinical and Biological Research.* **342:**393-398.

PETER RE, CRIM LW, BILLARD R (1991). A stereotaxic atlas and implantation technique for nuclei of the diencephalon of Atlantic salmon (*Salmo salar*) parr. *Reproduction Nutrition Development.* **31:**167-186.

PETER RE, YU KL (1997). Neuroendocrine regulation of ovulation in fishes: Basic and applied aspects. *Reviews in Fish Biology and Fisheries.* **7:**173-197.

PETRINO TR, GREELEY MS, SELMAN K, LIN YWP, WALLACE RA (1989). Steroidogenesis in *Fundulus heteroclitus*. 2. Production of 17-alpha-hydroxy-20-beta-dihydroprogesterone, testosterone, and 17--beta-estradiol by various components of the ovarian follicle. *General and Comparative Endocrinology.* **76:**230-240.

PHILIPPART JC, RUWETT JC (1982). Ecology and distribution of tilapias In: RSV PULLIN and R LOWE-MCCONNELL (Eds.). *The Biology and Culture of Tilapias. ICLARM Conference Proceedings.* **7:**15-59.

PLOHMAN JC, DICK TA, EALES JG (2002). Thyroid of lake sturgeon, *Acipenser fulvescens* - II. Deiodination properties, distribution, and effects of diet, growth, and a T3 challenge. *General and Comparative Endocrinology.* **125:**56-66.

POCAR P, BREVINI TAL, FISCHER B, GANDOLFI F (2003). The impact of endocrine disruptors on oocyte competence. *Reproduction.* **125:**313-325.

POWER DM, ELIAS NP, RICHARDSON SJ, MENDES J, SOARES CM, SANTOS CRA (2000). Evolution of the thyroid hormone-binding protein, transthyretin. *General and Comparative Endocrinology.* **119:**241-255.

POWER DM, LLEWELLYN L, FAUSTINO M, NOWELL MA, BJORNSSON BT, EINARSDOTTIR IE, CANARIO AVM, SWEENEY GE (2001). Thyroid hormones in growth and development of fish. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology.* **130:**447-459.

Q

QUERAT B (1994). Molecular evolution of the glycoprotein hormones in vertebrates In: KG DAVEY, RE PETER and SS TOBE (Eds.). *Perspectives in Comparative Endocrinology.* 27-35.

QUERAT B (1995). Structural relationships between fish and tetrapod gonadotropins. *Fifth International Symposium on the Reproductive Physiology of Fish. (Austin, TX).* 7-9.

R

REIS-HENRIQUES MA, SILVA L, COIMBRA J (1996). Gill Na+-K+ ATPase, carbonic anhydrase activities and plasma osmotic and ionic variations during smoltification of Atlantic salmon in the north of Portugal. *Aquaculture International.* **4:**117-128.

REIS-HENRIQUES MA, FERREIRA M, SILVA L, DIAS A (2000). Evidence for an involvement of vitellogenin in the steroidogenic activity of rainbow trout (*Oncorhynchus mykiss*) vitellogenic oocytes. *General and Comparative Endocrinology.* **117:**260-267.

RICHARDSON SJ, BRADLEY AJ, DUAN W, WETTENHALL REH, HARMS PJ, BABON JJ, SOUTHWELL BR, NICOL S, DONNELLAN SC, SCHREIBER G (1994). Evolution of marsupial and other vertebrate thyroxine-binding plasma-proteins. *American Journal of Physiology.* **266:**R1359-R1370.

RICHARDSON SJ, MONK JA, SHEPHERDLEY CA, EBBESSON LOE, SIN F, POWER DM, FRAPPELL PB, KOHRLE J, RENFREE MB (2005). Developmentally regulated thyroid hormone distributor proteins in marsupials, a reptile, and fish. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology.* **288:**R1264-R1272.

ROBBINS J, EDELHOCH H (1986). Thyroid hormone transport proteins: Their nature, biosynthesis, and metabolism In: SH INGBAR and LE BRAVERMAN (Eds.). *Werner's The Thyroid.* 116-127.

ROCHA E, MONTEIRO R (1999). Histology and cytology of fish liver: a review In: DN SAKSENA (Eds.). *Icthiology: Recent Research Advances.* Oxford & IBH Publishin Co. Pvt. Ldt. . 321-344.

ROCHA MJ (1996). Estudo da endocrinologia da reprodução em peixes. *Tese de Doutoramento. Instituto de Ciências Biomédicas Abel Salazar. Universidade do Porto.* 134.

ROCHA MJ, REIS-HENRIQUES MA (1996). Plasma and urine levels of C-18, C-19 and C-21 steroids in an asynchronous fish, the tilapia *Oreochromis mossambicus* (Teleostei, cichlidae). *Comparative Biochemistry and Physiology C - Pharmacology Toxicology & Endocrinology.* **115:**257-264.

RODERICK E (2003). Tilapia: a truly global aquaculture industry. *Global Aquaculture Advocate.* 46-47.

RODRIGUES P, REIS-HENRIQUES MA, CAMPOS J, SANTOS MM (2006). Urogenital papilla feminization in male *Pomatoschistus minutus* from two estuaries in northwestern Iberian Peninsula. *Marine Environmental Research.* **62:**S258-S262.

ROLLAND RM (2000). A review of chemically-induced alterations in thyroid and vitamin A status from field studies of wildlife and fish. *Journal of Wildlife Diseases.* **36:**615-635.

ROTHBARD S, PRUGININ Y (1975). Induced spawning and artificial incubation of tilapia. *Aquaculture.* **5:**315-321.

S

SAFE S (1980). Metabolism, uptake, storage and bioaccumulation of halogenated aromatic pollutants In: RD KIMBROUGH (Eds.). *Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products.* Elsevier Science Publishers. 81-107.

SAGER DB (1983). Effect of postnatal exposure to polychlorinated-biphenyls on adult male reproductive function. *Environmental Research.* **31:**76-94.

SALIGAUT C, GARNIER DH, BENNANI S, SALBERT G, BAILHACHE T, JEGO P (1992). Effects of estradiol on brain aminergic turnover of the female rainbow-trout (*Oncorhynchus-mykiss*) at the beginning of vitellogenesis. *General and Comparative Endocrinology.* **88:**209-216.

SANDERS JP, VANDERGEYTEN S, KAPTEIN E, DARRAS VM, KUHN ER, LEONARD JL, VISSER TJ (1997). Characterization of a propylthiouracil-insensitive type I iodothyronine deiodinase. *Endocrinology.* **138:**5153-5160.

SANDERS JP, VAN DER GEYTEN S, KAPTEIN E, DARRAS VM, KUHN ER, LEONARD JL, VISSER TJ (1999). Cloning and characterization of type III iodothyronine deiodinase from the fish *Oreochromis niloticus*. *Endocrinology.* **140:**3666-3673.

SANGALANG GB, FREEMAN HC, CROWELL R (1981). Testicular abnormalities in cod (*Gadus-Morhua*) fed Aroclor-1254. *Archives of Environmental Contamination and Toxicology.* **10:**617-626.

SANTOS CRA, POWER DM (1999). Identification of transthyretin in fish (*Sparus aurata*): cDNA cloning and characterisation. *Endocrinology.* **140:**2430-2433.

SANTOS MM, CASTRO LFC, VIEIRA MN, MICAEL J, MORABITO R, MASSANISSO P, REIS-HENRIQUES MA (2005). New insights into the mechanism of imposex induction in the dogwhelk *Nucella lapillus*. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology.* **141:**101-109.

SAPOZHNIKOVA Y, BAWARDI O, SCHLENK D (2004). Pesticides and PCBs in sediments and fish from the Salton Sea, California, USA. *Chemosphere.* **55:**797-809.

SARAIVA MJM, COSTA PP, GOODMAN DS (1988). Transthyretin (prealbumin) in familial amyloidotic polineuropathy: genetic and functional aspects In: S DIDONATO, A MAMOLI, S DIMAURO and LP ROWLAND (Eds.). *Advances in Neurology.* Ravem Press. **48:**189-200.

SCHREIBER G, RICHARDSON SJ (1997). The evolution of gene expression, structure and function of transthyretin. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology.* **116:**137-160.

SCOTT GI, FULTON MH, MOORE DW, WIRTH EF, CHANDLER GT, KEY PB, DAUGOMAH JW, STROZIER ED, DEVANE J, CLARK JR, LEWIS MA, FINLEY DB, ELLENBERG W, KARNAKY KJ (1999). Assessment of risk reduction strategies for the management of agricultural nonpoint source pesticide runoff in estuarine ecosystems. *Toxicology and Industrial Health.* **15:**200-213.

SHARMA RM (1988). Effect of endosulfan on adenosine-triphosphatase (ATPase) activity in liver, kidney, and muscles of *Channa-gachua*. *Bulletin of Environmental Contamination and Toxicology.* **41:**317-323.

SHEPHERDLEY CA, RICHARDSON SJ, EVANS BK, KUHN ER, DARRAS VM (2002). Characterization of outer ring iodothyronine deiodinases in tissues of the saltwater crocodile (*Crocodylus porosus*). *General and Comparative Endocrinology.* **125:**387-398.

SINHA N, LAL B, SINGH TP (1991a). Effect of endosulfan on thyroid physiology in the fresh-water catfish, *Clarias-batrachus*. *Toxicology.* **67:**187-197.

SINHA N, LAL B, SINGH TP (1991b). Pesticides induced changes in circulating thyroid-hormones in the fresh-water catfish *Clarias-batrachus*. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology.* **100:**107-110.

SINHA N, LAL B, SINGH TP (1992). Thyroid physiology impairment by malathion in the fresh-water catfish *Clarias-batrachus*. *Ecotoxicology and Environmental Safety.* **24:**17-25.

SLOLEY BD, TRUDEAU VL, DULKA JG, PETER RE (1991). Selective depletion of dopamine in the goldfish pituitary caused by domperidone. *Canadian Journal of Physiology and Pharmacology.* **69:**776-781.

SLOLEY BD, KAH O, TRUDEAU VL, DULKA JG, PETER RE (1992a). Amino-acid neurotransmitters and dopamine in brain and pituitary of the goldfish - involvement in the regulation of gonadotropin--secretion. *Journal of Neurochemistry.* **58:**2254-2262.

SLOLEY BD, TRUDEAU VL, PETER RE (1992b). Dopamine catabolism in goldfish (*Carassius-auratus*) brain and pituitary - lack of influence of catecholestrogens on dopamine catabolism and gonadotropin-secretion. *Journal of Experimental Zoology.* **263:**398-405.

SMIALOWICZ RJ, ANDREWS JE, RIDDLE MM, ROGERS RR, LUEBKE RW, COPELAND CB (1989). Evaluation of the immunotoxicity of low-level PCB exposure in the rat. *Toxicology.* **56:**197-211.

SOUSA MM, BERGLUND L, SARAIVA MJ (2000). Transthyretin in high density lipoproteins: association with apolipoprotein A-I. *Journal of Lipid Research.* **41:**58-65.

STEGEMAN JJ, HAHN ME (1994). Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species In: DC MALINS and GK OSTRANDER (Eds.). *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives.* Lewis Publishers.

STGERMAIN DL, GALTON VA (1997). The deiodinase family of selenoproteins. *Thyroid.* **7:**655-668.

STRACHAN WMJ (1988). Toxic contaminants in rainfall in Canada - 1984. *Environmental Toxicology and Chemistry.* **7:**871-877.

SUTIJA M, LONGHURST TJ, JOSS JMP (2003). Deiodinase type II and tissue specific mRNA alternative splicing in the Australian lungfish, *Neoceratodus forsteri*. *General and Comparative Endocrinology.* **132:**409-417.

SUZUKI K, NAGAHAMA Y, KAWAUCHI H (1988). Steroidogenic Activities of 2 Distinct Salmon Gonadotropins. *General and Comparative Endocrinology.* **71:**452-458.

SWANSON P, DICKHOFF WW, GORBMAN A (1987). Pituitary thyrotropin and gonadotropin of coho salmon (*Oncorhynchus-kisutch*) - separation by chromatofocusing. *General and Comparative Endocrinology.* **65:**269-287.

SWANSON P, SUZUKI K, KAWAUCHI H, DICKHOFF WW (1991). Isolation and characterization of 2 coho salmon gonadotropins, GtH-I and GtH-II. *Biology of Reproduction.* **44:**29-38.

SWEETING RM, EALES JG (1992). HPLC analysis of *in vitro* hepatic deiodination products of thyroid-hormones in the rainbow-trout, *Oncorhynchus-mykiss*. *General and Comparative Endocrinology.* **85:**367-375.

T

TAGAWA M, TANAKA M, MATSUMOTO S, HIRANO T (1990). Thyroid-hormones in eggs of various fresh-water, marine and diadromous teleosts and their changes during egg development. *Fish Physiology and Biochemistry.* **8:**515-520.

TAYLOR MR, HARRISON PTC (1999). Ecological effects of endocrine disruption: Current evidence and research priorities. *Chemosphere.* **39:**1237-1248.

TOPPARI J, LARSEN JC, CHRISTIANSEN P, GIWERCMAN A, GRANDJEAN P, GUILLETTE LJ, JEGOU B, JENSEN TK, JOUANNET P, KEIDING N, LEFFERS H, MCLACHLAN JA, MEYER O, MULLER J, RAJPERTDEMEYTS E, SCHEIKE T, SHARPE R, SUMPTER J, SKAKKEBAEK NE (1996). Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives.* **104:**741-803.

TRANT JM, THOMAS P (1989). Isolation of a novel maturation-inducing steroid produced *in vitro* by ovaries of Atlantic croaker. *General and Comparative Endocrinology.* **75:**397-404.

TREWAVAS E (1983). Tilapiine fishes of the genera *Sarotherodon*, *Oreochromis*, and *Danakilia*. *British Museum (Natural History).* **878:**503.

TRUDEAU VL, SLOLEY BD, PETER RE (1993). GABA stimulation of gonadotropin-ii release in goldfish - involvement of GABA(a) receptors, dopamine, and sex steroids. *American Journal of Physiology.* **265:**R348-R355.

TRUDEAU VL (1997). Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Carassius auratus*. *Reviews of Reproduction.* **2:**55-68.

TRUDEAU VL, SPANSWICK D, FRASER EJ, LARIVIERE K, CRUMP D, CHIU S, MACMILLAN M, SCHULZ RW (2000). The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire.* **78:**241-259.

TSAI CL, WANG LH (1997). Effects of estradiol on the serotonin secretion and turnover in the hypothalamus of male tilapia, *Oreochromis mossambicus, in vitro*. *General and Comparative Endocrinology.* **106:**175-180.

TSAI CL, WANG LH (1998). Effects of gonadal steroids on the noradrenergic activity in the early developing tilapia brain. *Biogenic Amines.* **14:**591-598.

TSAI CL, WANG LH (1999). Effects of gonadal steroids on the serotonin synthesis and metabolism in the early developing tilapia brain. *Neuroscience Letters.* **264:**45-48.

TSAI CL, WANG LH (2000). The alteration of gamma-aminobutyric acid and glutamate contents in the discrete brain of female tilapia during certain stages of gonadal cycle. *Neuroscience Letters.* **291:**171-174.

TSAI CL, WANG LH, CHANG CF, KAO CC (2000). Effects of gonadal steroids on brain serotonergic and aromatase activity during the critical period of sexual differentiation in tilapia, *Oreochromis mossambicus*. *Journal of Neuroendocrinology.* **12:**894-898.

TSAI CL, WANG LH, FANG LS (2001a). Estradiol and para-chlorophenylalanine downregulate the expression of brain aromatase and estrogen receptor-alpha mRNA during the critical period of feminization in tilapia (*Oreochromis mossambicus*). *Neuroendocrinology.* **74:**325-334.

TSAI CL, WANG LH, LIN YH (2001b). Effects of estrogen and neurotransmitters on the primary cultures of tilapia brain from different ages. *Developmental Brain Research.* **129:**111-113.

TYLER CR, JOBLING S, SUMPTER JP (1998). Endocrine disruption in wildlife: A critical review of the evidence. *Critical Reviews in Toxicology.* **28:**319-361.

U

UEDA H, HIROI O, HARA A, YAMAUCHI K, NAGAHAMA Y (1984). Changes in serum concentrations of steroid-hormones, thyroxine, and vitellogenin during spawning migration of the chum salmon, *Oncorhynchus-keta*. *General and Comparative Endocrinology.* **53:**203-211.

USAF-US (1989). The Installation Restoration Program Toxicology Guide. Wright-Patterson Air Force Base, Ohio. *AFSC Aerospace Medical Division.* **3**:1-68.

V

VALVERDE-R C, CROTEAU W, LAFLEUR GJ, OROZCO A, STGERMAIN DL (1997). Cloning and expression of a 5'-iodothyronine deiodinase from the liver of *Fundulus heteroclitus. Endocrinology.* **138:**642-648.

VALVERDE-R C, OROZCO A, BECERRA A, JEZIORSKI MC, VILLALOBOS P, SOLIS-S JC (2004). Halometabolites and cellular dehalogenase systems: An evolutionary perspective. *International Review of Cytology - a Survey of Cell Biology.* **234:**143-199.

VAN DEN BERG KJ (1990). Interaction of chlorinated phenols with thyroxine binding sites of human transthyretin, albumin and thyroid binding globulin. *Chemico-Biological Interactions.* **76:**63-75.

VAN DEN BERG KJ, VAN RAAIJ JAGM, BRAGT PC, NOTTEN WRF (1991). Interactions of halogenated industrial chemicals with transthyretin and effects on thyroid hormone levels *in vivo.* Archives in Toxicology **65:**15-19.

VAN DER GEYTEN S, MOL KA, PLUYMERS W, KUHN ER, DARRAS VM (1998). Changes in plasma T3 during fasting/refeeding in tilapia (*Oreochromis niloticus*) are mainly regulated through changes in hepatic type II iodothyronine deiodinase. *Fish Physiology and Biochemistry.* **19:**135-143.

VAN DER GEYTEN S, TOGUYENI A, BAROILLER JF, FAUCONNEAU B, FOSTIER A, SANDERS JP, VISSER TJ, KUHN ER, DARRAS VM (2001). Hypothyroidism induces type I iodothyronine deiodinase expression in tilapia liver. *General and Comparative Endocrinology.* **124:**333-342.

VAN DER KRAAK G (1998). Observations of endocrine effects in wildlife with evidence of their causation. *Pure and Applied Chemistry.* **70:**1785-1794.

VANCE DE (1993). Metabolism of cholesterol In: G ZUBAY (Eds.). *Biochemistry.* William C. Brown Publishers. 635-658.

VANPUTTE CLM, MACKENZIE DS, EALES JG (2001). Characterization of hepatic low-Km outer-ring deiodination in red drum (*Sciaenops ocellatus*). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology.* **128:**413-423.

VIEIRA MM (2005). Vendas de produtos fitofarmacêuticos em Portugal em 2003. *Ministério da Agricultura, do Desenvolvimento Rural e das Pescas. Direcção-Geral de Protecção das Culturas. Direcção de Serviços de Produtos Fitofarmacêuticos.* 1-17.

VILANOVA R, FERNANDEZ P, MARTINEZ C, GRIMALT JO (2001). Organochlorine pollutants in remote mountain lake waters. *Journal of Environmental Quality.* **30:**1286-1295.

VILELA DAR, SILVA SGB, PEIXOTO MTD, GODINHO HP, FRANCA LR (2003). Spermatogenesis in teleost: insights from the Nile tilapia (*Oreochromis niloticus*) model. *Fish Physiology and Biochemistry.* **28:**187-190.

VISSER TJ, DOCTER R, HENNEMANN G (1977). Radioimmunoassay of reverse tri-iodothyronine. *Journal of Endocrinology.* **73:**395-396.

VISSER TJ (1996). Pathways of thyroid hormone metabolism. *Acta Medica Austriaca.* **23:**10-16.

VOS JG, VAN LOVEREN H (1995). Markers for immunotoxic effects in rodents and man. *Toxicology Letters.* **82-83:**385-394.

VOS JG, DYBING E, GREIM HA, LADEFOGED O, LAMBRE C, TARAZONA JV, BRANDT I, VETHAAK AD (2000). Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. *Critical Reviews in Toxicology.* **30:**71-133.

W

WANG LH, TSAI CL (2000). Temperature affects the development of central neurotransmitter systems of tilapia, *Oreochromis mossambicus. Neuroscience Letters.* **285:**95-98.

WEBER GM, POWELL JFF, PARK M, FISCHER WH, CRAIG AG, RIVIER JE, NANAKORN U, PARHAR IS, NGAMVONGCHON S, GRAU EG, SHERWOOD NM (1997). Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules. *Journal of Endocrinology.* **155:**121-132.

WELTZIEN FA, ANDERSSON E, ANDERSEN O, SHALCHIAN-TABRIZI K, NORBERG B (2004). The brain--pituitary-gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology.* **137:**447-477.

WHITE RB, FERNALD RD (1998). Ontogeny of gonadotropin-releasing hormone (GnRH) gene expression reveals a distinct origin for GnRH-Containing neurons in the midbrain. *General and Comparative Endocrinology.* **112:**322-329.

WHITLOCK JP (1994). The aromatic hydrocarbon receptor, dioxin action, and endocrine homeostasis. *Trends in Endocrinology and Metabolism.* **5:**183-188.

WHO (1984). Endosulfan. *Environmental Health Criteria* **40**. World Health Organization Geneva.

WHYTE JJ, JUNG RE, SCHMITT CJ, TILLITT DE (2000). Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Critical Reviews in Toxicology.* **30:**347-570.

WILD SR, JONES KC (1992). Organic-chemicals entering agricultural soils in sewage sludges - screening for their potential to transfer to crop plants and livestock. *Science of the Total Environment.* **119:**85-119.

WOLF CJ, OSTBY JS, GRAY LE (1999). Gestational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) severely alters reproductive function of female hamster offspring. *Toxicological Sciences.* **51:**259-264.

Y

YAMAUCHI K, NAKAJIMA J, HAYASHI H, HARA A, YAMAUCHI K (1999). Purification and characterization of thyroid-hormone-binding protein from masu salmon serum - A homolog of higher-vertebrate transthyretin. *European Journal of Biochemistry.* **265:**944-949.

YARON Z (1966). Demonstration of 3 beta-hydroxysteroid dehydrogenase in testis of tilapia mossambica (Cichlidae Teleostei). *Journal of Endocrinology.* **34:**127-128.

YARON Z, GUR G, MELAMED P, ROSENFELD H, ELIZUR A, LEVAVI-SIVAN B (2003). Regulation of fish gonadotropins. *International Review or Cytology - a Survey of Cell Biology.* **225:**131-185.

YOSHIKUNI M, NAGAHAMA Y (1991). Endocrine regulation of gametogenesis in fish. *Bulletin Institute Zoology,* Academia Sinica (Eds.). **16:**139-172.

YU KL, PETER RE (1991). Changes in brain levels of gonadotropin-releasing-hormone and serum levels of gonadotropin and growth-hormone in goldfish during spawning. *Canadian Journal of Zoology-Revue Canadienne De Zoologie.* **69:**182-188.

YU KL, ROSENBLUM PM, PETER RE (1991). *In vitro* release of gonadotropin-releasing-hormone from the brain preoptic anterior hypothalamic region and pituitary of female goldfish. *General and Comparative Endocrinology.* **81:**256-267.

Z

ZHANG CQ, QIAO HL (2004). Effect of polychlorinated biphenyls on spermatogenesis and testosterone secretion in adult cocks. *Journal Zhejiang University Science.* **5:**193-197.

ZHANG ZL, HUANG J, YU G, HONG HS (2004). Occurrence of PAHs, PCBs and organochlorine pesticides in the Tonghui River of Beijing, China. *Environmental Pollution.* **130:**249-261.

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