

# Adrenal and sex steroid receptor evolution: environmental implications

**M E Baker**

Department of Medicine, 0823, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0823, USA

(Requests for offprints should be addressed to M E Baker; Email: mbaker@ucsd.edu)

## ABSTRACT

The nuclear receptor family responds to a diverse group of ligands, including steroids, retinoids, thyroid hormone, prostaglandins and fatty acids. Previous sequence analyses of adrenal and sex steroid receptors indicate that they form a clade separate from other nuclear receptors. However, the relationships of adrenal and sex steroid receptors to each other and to their ancestors are not fully understood. We have used new information from androgen, estrogen, mineralocorticoid and progesterone receptors in fish to better resolve the phylogeny of adrenal and sex steroid receptors. Sequence divergence between fish and mammalian steroid receptors correlates with differences in steroid specificity, suggesting that phylogeny needs to be considered in evaluating the endocrine effects of xenobiotics. Among the vertebrate steroid receptors, the most ancient is the estrogen receptor.

The phylogeny indicates that adrenal and sex steroid receptors arose in a jawless fish or a protochordate and that changes in the sequence of the hormone-binding domain have slowed considerably in land vertebrates. The retinoid X receptor clade is closest to the adrenal and sex steroid receptor clade. Retinoid X receptor is noteworthy for its ability to form dimers with other nuclear receptors, an important mechanism for regulating the action of retinoid X receptor and its dimerization partners. In contrast, the adrenal and sex steroid receptors bind to DNA as homodimers. Moreover, unliganded adrenal and sex steroid receptors form complexes with heat shock protein 90. Thus, the evolution of adrenal and sex steroid receptors involved changes in protein-protein interactions as well as ligand recognition.

*Journal of Molecular Endocrinology* (2001) **26**, 119–125

## INTRODUCTION

The adrenal and sex steroids: cortisol, aldosterone, estrogen, testosterone and progesterone, have a central role in development, reproduction and homeostasis in humans and other vertebrates (Evans 1988, DeGroot 1995, Mangelsdorf *et al.* 1995). These steroids act through nuclear receptors, which are a family of transcription factors that also includes receptors for retinoids, thyroid hormone, prostaglandins and fatty acids, as well as receptors without known ligand: the orphan receptors (Evans 1988, Mangelsdorf *et al.* 1995, Chambon 1996, Enmark & Gustafsson 1996, Giguere 1999).

Sequence analyses show that the adrenal and sex steroid receptors are a distinct clade in the nuclear receptor family (Baker 1997, Escriva *et al.* 1997, 2000, Laudet 1997, Thornton & Kelley 1998). This clade arose at the time that coincides with the origins of vertebrates and, indeed, steroid hormones

may have had an important role in the early evolution of vertebrates and their subsequent survival of global catastrophes (Baker 1997). Phylogenetic analyses of the ligand-binding domain (Baker 1997) indicate that the estrogen receptor (ER) is the most ancient of the adrenal and sex steroid receptors, the progesterone receptor (PR) and androgen receptor (AR) cluster on one branch, and the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) cluster on another branch. However, the early events in steroid receptor evolution were poorly defined because there were few 'molecular fossils' from fish and because strong sequence conservation of steroid receptors from amphibia to mammals (Baker 1997, Laudet 1997, Thornton & Kelley 1998) reduced the resolution at the base of the steroid receptor tree. This obstacle to a more complete phylogeny was removed with the recent reports of the sequences of fish AR (Todo *et al.* 1999, Takeo & Yamashita 1999, Ikeuchi *et al.*

1999), fish PR (Todo *et al.* 2000) and fish MR (Colombe *et al.* 2000).

We constructed a phylogeny of adrenal and sex steroid receptors that includes these newly determined fish steroid receptor sequences. We have found that the sequences of the hormone-binding domain in fish steroid receptors have diverged more from their orthologs in land vertebrates than amphibian and mammalian orthologs have from each other. This sequence divergence correlates with recently reported differences in ligand specificity between fish and land vertebrate steroid receptors. An important implication of our analysis is that phylogeny needs to be considered in evaluating the effects of environmental chemicals on steroid-responsive processes; that is, some chemicals will have different endocrine effects in mammals and fish.

We have also traced the steroid receptor clade to an ancestral retinoid X receptor (RXR), which forms heterodimers with a variety of nuclear receptors, but not with adrenal and sex steroid receptors (Glass 1994, Mangelsdorf *et al.* 1995, Chambon 1996, Enmark & Gustafsson 1996, Giguere 1999). In contrast, adrenal and sex steroid receptors are active as homodimers in regulating gene transcription and, in the unliganded state, these steroid receptors bind heat shock protein 90 (HSP90) (Pratt & Toft 1997). Thus, protein-protein interactions as well as changes in ligand recognition were important in the evolution of adrenal and sex steroid receptors.

## MATERIALS AND METHODS

### PSI-BLAST analysis

Position-specific iterated basic local alignment research tool (PSI-BLAST) allows a search of GenBank on the National Library of Medicine website ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) with a probe that contains information about sequence conservation in several homologous proteins (Altshul *et al.* 1997). The first search is identical to a BLAST analysis. PSI-BLAST output has an option for choosing sequences that are collected and aligned for the next search of the database. For this search, PSI-BLAST constructs a scoring matrix that is based on the frequency of occurrence of amino acids at each position in the 'collection' of sequences. A search of the database with this position-specific matrix will uncover protein sequences that most resemble that of the protein collection. To determine the closeness of other nuclear receptors to the ER, we searched GenBank with the ligand-binding domain of eel ER- $\beta$ . Then

TABLE 1. PSI-BLAST analysis of the estrogen-binding domain of the ER (database: nr 517 973 sequences)

Accession number	Protein	e-value
BAA19851.1	Eel ER- $\beta$	e-145
P03372	Human ER- $\alpha$	e-127
Q92731	Human ER- $\beta$	e-123
AAD13610.2	Trout ER- $\alpha$	e-122
AAC99409.1	Human ERR-2	7e-37
P11474	Human ERR-1	7e-35
P28701	Chick RXR- $\gamma$	4e-23
P51129	<i>Xenopus</i> RXR- $\gamma$	6e-23
P06186	Rabbit PR	6e-22

we added the sequences of trout ER- $\alpha$  and human ER- $\alpha$  and human ER- $\beta$  for the first iteration. These receptors were chosen after pairwise comparisons revealed that their estrogen-binding domains have from 55% to 65% sequence identity with each other.

### Phylogenetic analysis

The Feng & Doolittle (1990) algorithm was used to construct a multiple alignment of the ligand-binding domain of adrenal and sex steroid receptors, estrogen-related receptor (ERR) and RXR. In this method, protein sequences are progressively aligned using the Dayhoff PAM-250 scoring matrix to assess pairwise similarity of each sequence with the others. The pairwise similarity scores are assembled into a distance matrix to construct a phylogenetic tree. The method of Fitch & Margoliash (1967) is then used to obtain the branching order for the sequences. Branch lengths are calculated by linear regression analysis of the best fit of the pairwise distances and the branching order. Branch lengths are proportional to distances between proteins.

## RESULTS AND DISCUSSION

### PSI-BLAST analysis

Preliminary BLAST searches (data not shown) confirmed that among the adrenal and sex steroid receptors, the ER is the closest to other nuclear receptors. Thus, we constructed a PSI-BLAST probe of eel ER- $\beta$ , human ER- $\alpha$ , human ER- $\beta$  and trout ER- $\alpha$ . The results of this analysis are shown in Table 1. As expected, ERR is closest to ER because, as the name ERR indicates, when this gene was cloned comparisons of its DNA-binding and putative ligand-binding domains with that of the ER indicated a close relationship (Enmark & Gustafsson 1996, Giguere 1999).

Interestingly, the next closest nuclear receptor is RXR. We used RXR as an outgroup for the phylogenetic analysis of adrenal and sex steroid receptors and ERR.

### Phylogenetic analysis

Figure 1 shows a phylogenetic tree of adrenal and sex steroid receptors, ERR1, ERR2 and RXR- $\alpha$  constructed using the program of Feng & Doolittle (1990). The phylogeny in Fig. 1 reveals some interesting features regarding relationships among vertebrate steroid receptors. The PR and AR cluster on one branch and the GR and MR cluster on another branch. The ER separated from the other steroid receptors at node IV. The ER is closer to node IV than the AR, PR, GR and MR. The slower change in the ER sequence indicates that it is under functional constraints, which is consistent with the estrogen response being the most ancient of the adrenal and sex steroid responses (Baker 1997). The phylogeny indicates that ER- $\alpha$  and ER- $\beta$  converge in an ancestral fish and that the estrogen response arose in jawless fish (hagfish or lamprey) or possibly earlier in amphioxus or in a tunicate. Escriva *et al.* (1997) used PCR to investigate the presence in chordates of genes with sequences that are homologous to DNA-binding domains of the ER and other steroid receptors. They found evidence for the AR, ER and GR in sharks and the PR in hagfish. The presence of PR in hagfish places the origin of adrenal and sex steroid receptors the the early Cambrian (Shu *et al.* 1999).

Figure 1 also indicates that the duplications at nodes I and II leading to the GR/MR and PR/AR clades respectively occurred in jawless fish or earlier, which is consistent with the PCR data of Escriva *et al.* (1997). The ancestral GR/MR, PR/AR and ER are likely to lack functions that their orthologs have in land animals (Baker 1997). For example, the reproductive actions of ER in the placenta would not be found in fish. However, as discussed later, some ancient functions of the ER and other steroid receptors are likely to be important in modern animals and be influenced by xenobiotics.

Figure 1 shows that the molecular clock for the hormone-binding domain is not uniform among the various vertebrate steroid receptors. Considering that human and amphibian lines diverged about 350 million years ago and teleosts arose about 425 million years ago, the branch lengths between the hormone-binding domain in steroid receptors in land animals are much shorter than those between fish and amphibia. This indicates that there was a reduction in the rate of change in the steroid-

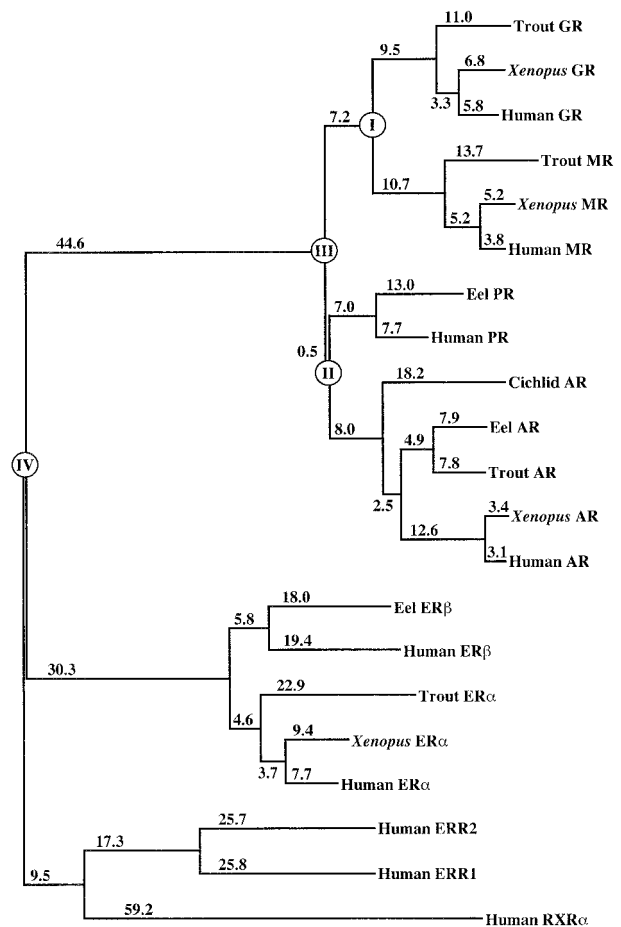


FIGURE 1. Phylogenetic analysis of adrenal and sex steroid receptors, ERRs and RXR. The Feng & Doolittle (1990) algorithm was used to construct a multiple alignment of the ligand-binding domain of adrenal and sex steroid receptors, ERR and RXR. Branch lengths are proportional to distances between proteins. GenBank accession numbers for each receptor are: trout GR (P49843), *Xenopus* GR (P49844), human GR (P04150), trout MR (AF209873.1), *Xenopus* MR (Q91573), human MR (P08235), eel PR (BAA89539.1), human PR (P06401), cichlid AR (AAD25074.1), eel AR (BAA75464.1), trout AR (BAA32784.1), *Xenopus* AR (AAC97386.1), human AR (P10275), eel ER- $\beta$  (BAA19851.1), human ER- $\beta$  (Q92731), human ER- $\alpha$  (P03372), trout ER- $\alpha$  (AAD13610.2), *Xenopus* ER- $\alpha$  (P81559), human ERR1 (P11474), human ERR2 (AAC99409.1) and human RXR- $\alpha$  (P19793).

binding domain in land animals compared with fish. Among the adrenal and sex steroid receptors, the hormone-binding domain in the AR has been most conserved and the hormone-binding domain in the GR has been least conserved since the separation of amphibia and humans from a common ancestor.

The length of the branches in the GR/MR clade indicates that the duplication at node I leading to the MR and GR arose in jawless fish or earlier. In land vertebrates, the MR regulates electrolyte balance under the influence of aldosterone. Aldosterone is not found in most fish. Aldosterone regulates electrolyte transport in amphibia. The MR has been found in tissues, in which aldosterone is not likely to regulate electrolyte balance. For example, in the brain the MR functions with the GR in the response to glucocorticoids (Ruel & De Kloet 1985, Arriza *et al.* 1988, Funder 1996). This had led to an alternative name of GR-type 1 for the MR, to show that it responds to glucocorticoids (Arriza *et al.* 1987, 1988, Funder 1997) and to distinguish it from GR-type 2, the classical glucocorticoid receptor.

The ancient evolution of the GR-type 1/type 2 response to glucocorticoids can explain the presence of MR in tissues that are not involved in electrolyte balance. It also explains the slower changes in the GR-type 1 (MR) in amphibia and mammals compared with GR-type 2 in these animals as shown by the shorter branches in Fig. 1. The requirement that MR respond to aldosterone in kidney and gut, and to corticosterone and cortisol in brain and other tissues with GR-type I/type II physiology puts constraints on changes in the MR sequence.

ERR and ER arose by a duplication of a gene that was close to the RXR branch of the nuclear receptor superfamily. ERRs have functional similarities with the ER, such as binding to an estrogen response element and cross-talk between the ERR and ER responses (Luo *et al.* 1997, Vanacker *et al.* 1999a,b, Xie *et al.* 1999). Although ERR is thought to be a ligand-independent regulator of gene transcription (Enmark & Gustafsson 1996, Luo *et al.* 1997, Giguere 1999, Xie *et al.* 1999), Vanacker *et al.* (1999b) recently presented indirect evidence that ERR is activated by a hydrophobic molecule. Identification of this molecule may provide clues to the ancestral ligand(s) that regulated ERR and ER.

The descent of the ER from RXR is interesting because it marks a change in both ligand-binding and protein-protein interactions. RXR is an unusual nuclear receptor because it acts as a partner for numerous other receptors including the thyroid hormone, retinoic acid and peroxisome proliferator-activated receptors (Glass 1994, Mangelsdorf *et al.* 1995, Chambon 1996). In contrast, adrenal and sex steroid receptors bind to DNA as homodimers. Also, unliganded adrenal and sex steroid receptors are stabilized in complexes with HSP90 (Pratt & Toft 1997) unlike RXR. Thus, changes in protein-protein interactions as well as changes in the ligand

led to the clade of receptors that responds to adrenal and sex steroids.

The genomes of *Caenorhabditis elegans* and *Drosophila melanogaster* contain nuclear receptors (Escriva *et al.* 2000). One of these, the ecdysone receptor in *D. melanogaster* recognizes a steroid hormone. Also *C. elegans* contains a nuclear receptor that has sequence similarity to the vitamin D receptor. However, proteins with sequence similarity to adrenal and sex steroid receptors have not been found in invertebrates. This is consistent with phylogenetic analyses, which indicate that the ecdysone and vitamin D receptors belong to nuclear receptor clades that branched off from the ancestral receptor before the origin of the adrenal and sex steroid receptors (Baker 1997, Laudet 1997, Escriva *et al.* 2000). The phylogeny shown in Fig. 1 and other analyses (Baker 1997, Escriva *et al.* 2000) indicate that the adrenal and sex steroid receptors arose in an ancient deuterostome.

### Fuzzy recognition of steroids in fish

5 $\alpha$ -Dihydrotestosterone (DHT) (Fig. 2), testosterone, 11 $\beta$ -hydroxytestosterone and 11-ketotestosterone (11-ketoT) bind to eel AR and activate gene transcription in cell culture (Ikeuchi *et al.* 1999, Takeo & Yamashita 1999). Despite the broad recognition of androgens by eel AR, it is 11-ketoT that is the major circulating androgen in most fishes and the steroid that mediates the androgen response (Miura *et al.* 1991, Borg 1994). In contrast, DHT and testosterone are the principle androgens in amphibia and mammals. 11-ketoT is not an important androgen in amphibia and mammals. This indicates that steroid specificity for the AR changed during the transition from fish to land animals.

Corresponding to the change in the biologically active androgen during the transition from fish to land animals was a change in function of 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD). In fish, 11 $\beta$ -HSD catalyzes the synthesis of 11-ketoT from 11 $\beta$ -hydroxytestosterone (Miura *et al.* 1991, Borg 1994). In amphibia and mammals, 11 $\beta$ -HSD regulates the interconversion of cortisol, the biological active glucocorticoid, and cortisone, an inactive glucocorticoid (Monder 1991, Edwards *et al.* 1996, Funder 1997). By inactivating cortisol in the kidney, 11 $\beta$ -HSD-type 2 prevents unwanted occupancy of the MR by cortisol, which allows aldosterone to regulate mineralocorticoid responsive genes (Monder 1991, Edwards *et al.* 1996, Funder 1997). Similarly, 11 $\beta$ -HSD-type 2 prevents cortisol from inhibiting testosterone synthesis in testes. Thus, during the evolution from fish to amphibia,

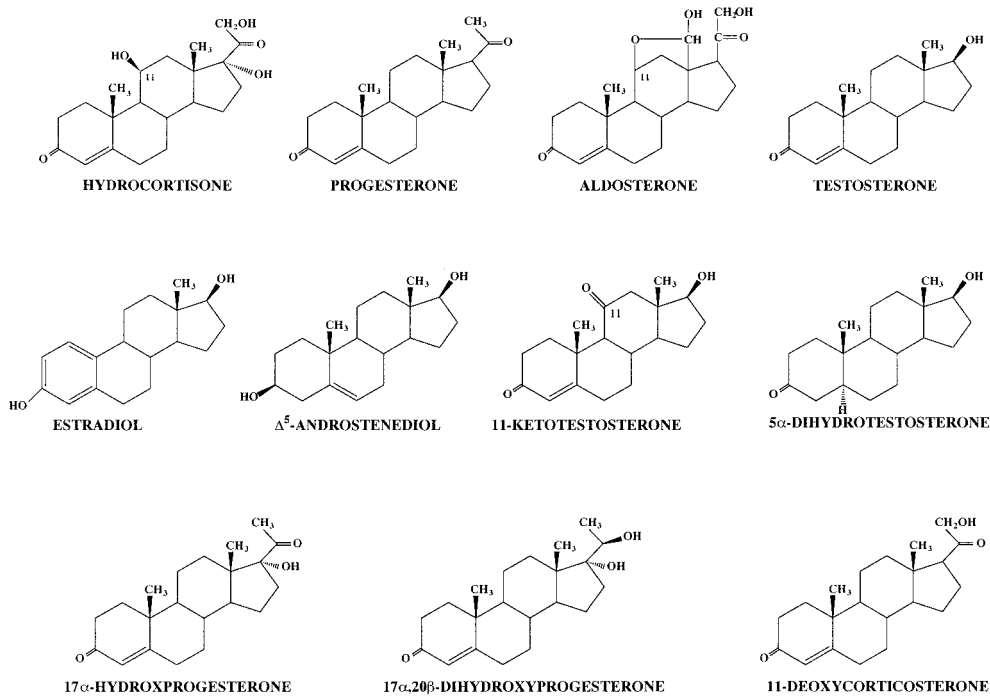


FIGURE 2. Diverse steroids are active in vertebrates. Testosterone, 5 $\alpha$ -DHT, 11 $\beta$ -hydroxytestosterone and 11-ketoT have high affinity for fish AR. However, in fish, the biologically active androgen is 11-ketoT. In mammals, the biologically active androgens are 5 $\alpha$ -DHT and testosterone. However, in mammals,  $\Delta^5$ -androstenediol has both androgenic and estrogenic activity. In fish, 17 $\alpha$ ,20 $\beta$ -dihydroxyprogesterone, 17 $\alpha$ ,20 $\beta$ ,21-trihydroxyprogesterone, progesterone, 11-deoxycorticosterone (21-hydroxyprogesterone), and 17 $\alpha$ -hydroxyprogesterone have high affinity for the PR. The biologically active progestin in fish is 17 $\alpha$ ,20 $\beta$ -dihydroxyprogesterone, which induces the final stages of spermatogenesis in the testis. In birds and mammals, steroids with 17 $\alpha$ -hydroxy substituents have less than 1% of the affinity for the progesterone receptor than that of progesterone. In amphibia, birds and mammals, 11-deoxycorticosterone is a mineralocorticoid.

11 $\beta$ -HSD changed from regulating the synthesis of the active androgen to regulating cellular glucocorticoid levels, which allows aldosterone to regulate the mineralocorticoid response in the kidney and is permissive for androgen synthesis in the testis.

17 $\alpha$ ,20 $\beta$ -Dihydroxyprogesterone (Fig. 2), 17 $\alpha$ ,20 $\beta$ ,21-trihydroxyprogesterone, progesterone, 17 $\alpha$ -hydroxyprogesterone and 11-deoxycorticosterone (21-hydroxyprogesterone) have high affinity for a nuclear receptor in eel (Todo *et al.* 1999), which has closest sequence similarity to the PR (Fig. 1 and Todo *et al.* (1999)). However, of these steroids, it is 17 $\alpha$ ,20 $\beta$ -dihydroxyprogesterone that induces the final stages of spermatogenesis in the testis. In birds and mammals, steroids with 17 $\alpha$ -hydroxy substituents have less than 1% of the affinity for the progesterone receptor than that of progesterone (Smith *et al.* 1974). Thus, like androgens, there is a

change in the biologically active progestin during the transition from fish to land animals.

Trout GR-type 1 (Colombe *et al.* 2000) which is orthologous to the aldosterone receptor does not have high affinity for aldosterone, consistent with evidence that aldosterone is not an active steroid in fish. Trout GR-type 1 binds the potent mammalian glucocorticoids, cortisol and corticosterone, with high affinity. However, trout GR-type 1 also binds 11-deoxycortisol, 21-deoxycortisol, 17-hydroxyprogesterone and 11 $\beta$ -hydroxyprogesterone with high affinity, indicating that trout GR-type 1 has lower steroid selectivity than mammalian GR-type 1 (Arriza *et al.* 1987, Rupprecht *et al.* 1993).

Only limited studies of steroid specificity for trout GR-type 2 have been reported (Ducouret *et al.* 1995). An important difference between trout and mammalian GRs is that cortisol has about 50% of cortisol's affinity for trout GR and less than 1% of

cortisol's affinity for mammalian GR (Arriza *et al.* 1987, 1988).

The differences in steroids that activate receptors in fish and land animals have important implications for mammalian physiology. Steroids that are active in fish or were active in ancestral vertebrates may still have activity in mammals, but in a different context. Recognition of androgens and estrogens by an ancestral AR and ER may explain recent findings for cross-recognition of steroids by the mammalian AR and ER. Chang's laboratory found that estradiol regulates androgen-responsive genes by binding to the AR (Yeh *et al.* 1998), and  $\Delta 5$ -androstenediol can regulate estrogen-responsive genes by binding to the ER (Miyamoto *et al.* 1998). Thus, a steroid with a saturated A-ring that contains a C-3 alcohol can activate the ER.  $\Delta 5$ -Androstenediol or other  $\Delta 5$ -steroids may have regulated steroid-responsive transcription early in vertebrate evolution. It will be interesting to determine if  $\Delta 5$ -androstenediol and other  $\Delta 5$ -steroids activate the AR, ER, GR or PR in fish.

### Environmental implications

The evidence that fish respond to steroids that are not active in mammals implies that there will be differences between fish and other animals in responses to some xenobiotics, which has important environmental implications. For example, a chemical may bind to more than one hormone receptor and/or enzyme in fish and, thus, have multiple effects on the endocrine system.

Compounds that inhibit steroid-metabolizing enzymes can have different effects on steroid-mediated gene expression in fish and mammals. For example, in mammals, carbenoxolone has glucocorticoid and mineralocorticoid activity due to inhibition of  $11\beta$ -HSD (Baker & Fanestil 1991, Monder 1991, Baker 1995, Edwards *et al.* 1996, Funder 1997). In fish, inhibition of  $11\beta$ -HSD by carbenoxolone would lower the concentration of 11-ketoT, and carbenoxolone would appear to be an anti-androgen.

The aqueous environment of fish would increase their exposure to xenobiotics, compared with that of land animals. Binding of these chemicals to fish ER, other nuclear receptors and other classes of hormone receptors and enzymes (Baker 1996, 2001) was likely to be important in their evolution. Considering the complexity of the steroid response in animals, including cross-talk in the transcriptional response among steroids and other hormones, one needs to be cautious in extrapolating data from studies on the effects of xenobiotics in mammals to fish and other phylogenetically distant animals.

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RECEIVED IN FINAL FORM 11 November 2000

ACCEPTED 22 November 2000