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2 Molecular biomarkers of endocrine disruption in small model fish

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4

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2

3 **Summary**

4 A wide range of environmental contaminants can interfere with the hormonal regulation in
5 vertebrates. These endocrine disrupting chemicals (EDCs) are of high relevance for human
6 and wildlife health, since endocrine signalling controls many essential physiological
7 processes which impact on the individual's health, such as growth and development, stress
8 response, and ultimately reproduction and population development. Small fish represent a
9 cost-effective model for testing potential EDCs allowing the possibility to integrate from
10 molecular to phenotypic and functional effects. We have comprehensively reviewed
11 exposure-effect data from four different small model fish: zebrafish, medaka, fathead
12 minnow, and the three-spined stickleback. The majority of available data refer to EDCs
13 interfering with reproductive hormones. However, we have also included interactions with
14 other hormone systems, particularly the thyroid hormones. We demonstrate that the available
15 data clearly indicates the predictive potential of molecular biomarkers, supporting the
16 development and regulatory application of simple molecular-based screening assays using
17 small model fish for EDC testing.

18

19

20

21 **Keywords**

22 Endocrine disruption, biomarker, hormone receptors, vitellogenin, spiggin, (teleost)

23

24 **1. Introduction**

25 There is an increasing public concern over the adverse effects of anthropogenic
26 contaminants on the health and well-being of both humans and wildlife (WHO, 2002). To

1 date, a wide variety of both developmental and reproductive disorders observed in wildlife
2 species have clearly been linked to the exposure to environmental contaminants which act as
3 endocrine disrupting chemicals (EDCs, Edwards et al., 2006; Tyler et al., 1998; WHO, 2002),
4 that is “exogenous agents that interfere with the production, release, transport, metabolism,
5 binding, action or elimination of natural hormones in the body responsible for the
6 maintenance of homeostasis and the regulation of developmental processes” (Kavlock et al.
7 1996). EDCs are of high environmental relevance, since many essential physiological
8 processes which impact on the individual’s health, such as growth and development, stress
9 response, and ultimately reproduction and population development are controlled by
10 hormones. Regardless of the initial type of interaction, the effects of EDCs with different
11 modes of action are often very similar and resemble changes in endogenous hormone levels.

12 In vertebrates, different types of hormone systems could principally be targets of
13 environmental chemicals: reproductive hormones such as estrogens and androgens, thyroidal
14 hormones, corticosteroids, growth hormone and their associated hypothalamus/pituitary
15 releasing and stimulating hormones. However, research has to date focused nearly entirely on
16 the interference with the steroidal reproductive hormones for obvious reasons. Firstly,
17 reproductive hormones control one of the most important endpoints in the risk assessment of
18 environmental chemicals, that is all aspects of reproductive development from sex
19 differentiation to puberty, aspects of which are critical for population viability. Secondly,
20 reproductive hormones are of vital importance during the early critical stages of embryonic
21 development and sex differentiation. As a result, even though the binding affinity of most
22 EDCs to the ER/AR is low compared to endogenous steroid hormones, exposure to even low
23 concentrations of xenoestrogens/xenoandrogens can disrupt normal
24 developmental/reproductive processes. This is evident for exposure to micropollutants, that is,
25 anthropogenic chemicals continuously detected at low concentrations in the environment. A
26 well known example is ethinylestradiol (EE₂), which is discharged into the aquatic

1 environment via municipal effluents due to its use in contraceptive pills. EE₂ is found in the
2 lower ng/L-range in aquatic surface water, a concentration that leads to sustainable impacts on
3 reproduction and population development in fish (Larsson et al., 1999).

4 Fish are considered as one of the primary risk organisms for EDCs, especially those
5 interacting with the reproductive hormones. Firstly, sex determination in teleost fishes is
6 characterised as being very labile, and can be disturbed or even functionally reversed by the
7 external application of hormones at critical developmental stages (Francis, 1992). Secondly,
8 as the aquatic environment represents the ultimate sink for most anthropogenic contaminants,
9 fish populations are directly exposed to a wide variety of EDCs, originating from industrial,
10 agricultural or municipal effluents. There is now unequivocal evidence showing that EDCs
11 can have long-term effects on reproduction and subsequent population development in natural
12 fish populations (Kidd et al., 2007). However, investigations based on the evaluation of
13 reproductive endpoints are costly, time-consuming and laborious. This has generally resulted
14 in these endpoints not being routinely integrated into most chemical safety assessments.
15 However, since endocrine disruption can be clearly linked to molecular interactions,
16 expression of appropriate biomarkers could be used as predictors for reproductive or other
17 effects.

18

19 **1.1. The relevance of using small fish models for endocrine disruption research**

20 Small fish represent a cost-effective model for large scale chemical testing. They are
21 easy to maintain in small laboratory aquaria, have a short life-cycle, and readily produce
22 many young. In contrast to targeted *in vitro* cellular models such as the yeast screen assay for
23 estrogenic compounds or primary cell cultures, small fish models allow the possibility to
24 integrate alternative modes of action, such as compounds that modify endogenous hormone
25 levels. Furthermore, uptake, metabolism and excretion as important constraints of
26 bioavailability are considered. The attractiveness of using small fish models in chemical

1 testing programmes is further enhanced by recent advances in molecular approaches. DNA
2 markers for molecular sex determination are now available for some small fish models
3 (medaka and stickleback, Griffiths et al., 2000; Matsuda et al., 2002). Moreover, the genome
4 has now been sequenced for three of the species highlighted in this review (medaka, zebrafish
5 and stickleback), resulting in the potential development of new molecular tools.

6 For this review we have selected 4 different model fish that are ideally suited to
7 establish links between molecular biomarkers and phenotypic and functional effects of EDCs:
8 zebrafish, medaka, fathead minnow, and the three-spined stickleback. Each of these
9 organisms is well established for the testing of environmental chemicals providing different
10 advantages and possibilities such as detailed genetic characterisation, sex specific markers,
11 and/or use as an experimental and biomonitoring model. Due to their short life-cycle, the
12 selected species allow for the analysis of reproductive performance, which is one of the most
13 important endpoints for long-term effects with implications on population development. A
14 brief description of the model small fish species is given in Table 1 (detailed information on
15 the different models can be obtained from the review of Ankley and Johnson, 2004 and
16 Katsiadaki et al. 2007).

17

18 **1.2. Scope of the review**

19 In this review, we summarise the available data on endocrine disruption in fish
20 highlighting the links between molecular, phenotypic and physiological effects (in particular
21 reproduction). We focus nearly exclusively on small fish models, since they provide the
22 experimental basis to establish appropriate correlations between the different effect levels
23 including reproductive and transgenerational effects. For the first time, data are presented in a
24 comprehensive manner, providing an overview of the effect levels of different molecular and
25 phenotypic endpoints in the selected model species. The majority of available data refer to
26 EDCs interfering with reproductive hormones. However, we have also reviewed interactions

1 with other hormone systems, indicating further potential applications for small fish models.
2 We show that the available data clearly indicate the predictive potential of molecular
3 biomarkers supporting the development of screening assays for endocrine disruption such as
4 initiated by the OECD (OECD, 2006).

5

6 **2. Data sources and graphical presentation**

7 The Web of Science (<http://portal.isiknowledge.com/>) and references have been
8 searched for publications (up to April 2008) on endocrine disrupting effects in the following
9 four small fish models: fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*),
10 medaka (*Oryzias latipes*), and the three-spined stickleback (*Gasterosteus aculeatus*).
11 Combinations of the terms “endocrine”, “disruption” and partial or entire names of species
12 and hormones following a manual selection of relevant articles have been used for the
13 literature survey. The review has been prepared independently and in parallel to an USEPA
14 report (USEPA, 2007) summarising endocrine disrupting effects on the fathead minnow. Data
15 reported for the fathead minnow largely overlap with the USEPA report, which contains
16 additional data without graphical representation but is partially more detailed.

17 With respect to concentration response levels of experimental studies we established a
18 quality control. Only those studies that used water-borne exposure, tested more than one
19 concentration of a potential EDC (in case several studies with the same endpoint have been
20 available) and provided statistical analysis to extract a LOEC (lowest observed effect
21 concentration) were considered. *Ex vivo* studies (i.e. incubation of oocytes or sperm) have
22 been excluded. In case the lowest tested concentrations caused an effect this concentration
23 was included as the LOEC as well. In the supplemented table S1 these values were marked by
24 “ \leq ”. Furthermore, studies with conflicting results (contradictive effects in repeated
25 experiments) were excluded. Occasionally, effects were reported at one low concentration but
26 not at higher levels. If no mechanistic basis could be ascribed to these findings, these studies

1 were also excluded. Based on these criteria, the data from 147 studies performed with one of
2 the selected small model species were selected for this review. Concentration levels reflect
3 measured or nominal concentrations. Not all studies included nominal concentrations
4 validated by chemical analysis. We decided to represent effect concentration in $\mu\text{g/L}$ or mg/L ,
5 since for the majority of studies concentrations are not given as molar concentration.

6 In order to summarise the large data set on reproductive steroid hormones we chose a
7 graphical representation. Although such a representation cannot provide all the information
8 that might be of interest it is ideal to give a comprehensive and easy to grasp overview of a
9 large number of data. However, it was not possible to include information on exposure
10 conditions into the graphs. Therefore, for the interested reader we have provided tables with
11 detailed data as supplementary information (Table S1). Data were summarised in different
12 categories: effects on molecular biomarkers, secondary sex characteristics (if available for the
13 species), histopathology (gonad histology, sex reversal, gonado-somatic index, thyroid
14 histology) and reproduction (spawning, fecundity, sperm quality and population
15 development). For thyroid interfering compounds, information on hormone levels, teratogenic
16 effects and toxicity have been included as well. The molecular markers were subdivided as
17 vitellogenin expression/level or others (aromatase expression/activity, gene expression of
18 other genes, steroid levels).

19

20 **3. Reproductive hormones**

21 In common with all vertebrates, reproduction in teleost fish is under the control of the
22 brain-pituitary-gonadal (BPG) axis. In seasonally spawning fish external factors such as light
23 and water temperature control the timing of gonad recrudescence and maturation via the
24 brain. Signals from the brain control the hypothalamic secretion of gonadotropin-releasing
25 hormones which stimulate the release of gonadotropins (luteinizing hormone [LH] and
26 follicle-stimulating hormone [FSH]) from the pituitary. The gonadotropins in turn control the

1 production and secretion of steroid hormones from the gonads. These hormones (17- β
2 estradiol and testosterone in females, and testosterone and predominantly 11-ketotestosterone
3 in males, as well as maturation-inducing hormones in both sexes) initiate changes in
4 secondary sex characteristics, behaviour as well as development and maturation of gametes.
5 Via negative or positive feedback mechanisms, depending on the physiological stage,
6 reproductive steroids regulate the secretion of pituitary gonadotropins (Peter & Yu 1997,
7 Schulz & Goos 1999).

8 The BPG axis provides several potential modes of interaction for EDCs ranging from
9 interference with hormone synthesis and secretion, binding to hormone receptors, or altering
10 the availability of endogenous hormones. Eventually, endocrine disrupting effects are
11 mediated via the available level of steroid hormones or its analogs. Thus, biomarkers directly
12 responsive to steroid hormone levels are the most widely used molecular indicators of
13 endocrine disruption. A popular example is the synthesis and secretion of the yolk protein
14 vitellogenin which is induced in the liver by the circulating female steroid hormone, 17 β -
15 estradiol and has been specifically applied to identify xenoestrogens (Tyler et al. 1996).

16

17 **3.1. Estrogenic and anti-estrogenic effects**

18 A wide variety of structurally diverse anthropogenic compounds have been shown to
19 exhibit estrogenic or anti-estrogenic effects (Jobling et al., 1995; Kime, 1998). Estrogenic
20 effects refer to compounds mimicking the action of estrogens via binding to the estrogen
21 receptor (ER) and activation of responsive genes. In teleosts, two receptor isoforms (α and β
22 and subtypes of these) with differential tissue distribution patterns and binding affinities have
23 been identified (e.g., Hawkins & Thomas 2004, Menuet et al. 2002). Furthermore, estrogenic
24 effects may also be mediated by elevation of endogenous free plasma estrogen levels caused
25 by competitive binding of xenobiotics to sex steroid binding globulins (e.g., Danzo 1997,
26 Tollefsen 2002). Anti-estrogenic effects refer to compounds with weak or no estrogenic

1 activity but the ability to competitively bind to the ER. Furthermore, anti-estrogenic action
2 can be mediated by affecting the expression and activity of the cytochrome P450A19 enzyme
3 (aromatase). A number of clinical compounds and pesticides are known to inhibit aromatase,
4 which catalyses the final step in the synthesis of estrogens, i.e. the introduction of an aromatic
5 ring in the androgen hormone precursor. Inhibition of this step leads to a reduction of 17β -
6 estradiol plasma concentration resulting in reduced vitellogenin levels in female fish (see
7 supplemented Table S1). Aromatase inhibitors could also be classified as androgenic
8 compounds due to their associated masculinising effects (e.g. Kwon et al., 2000; Mak et al.,
9 1999). These masculinising effects are presumably provoked by accumulation of androgens
10 since fish treated with the aromatase inhibitor fadrozole show elevated 11-ketotestosterone
11 levels (e.g., Ankley et al. 2002). However, due to their primary involvement in estrogen-
12 synthesis inhibition, we decided to summarise aromatase inhibitors along with
13 (anti)estrogenic and estrogenic compounds. Effect levels from 112 references - in which an
14 effect of an endocrine disruptor using one of the model teleosts has been described for
15 waterborne exposures - were extracted (Fig. 1). The test compounds comprised of known and
16 putative estrogens (17α -ethinylestradiol, 17β -estradiol, estrone, bisphenol A, 4-
17 aminobenzoate, 3-benzylidene camphor, benzophenone, diethylstilbestrol, β -HCH, o,p-DDT,
18 pp-DDE, letrozole, octyl methoxycinnamate, 4-nonylphenol, 4-tert-octylphenol, 4-
19 pentylphenol), anti-estrogens (equol, genistein, tamoxifen, ZM 189,156), aromatase
20 inhibitors (fadrozole, tributyltin) or potential xenoestrogens of unknown mechanisms
21 (atrazine, benzo(a)pyrene, butyl benzyl phthalate, cyproconazole, p-dichlorobenzene, o-
22 dichlorobenzene, m-dichlorobenzene, nianserin, methoxychlor, propylparaben). Some
23 compounds are difficult to classify, since they interfere with the synthesis of steroids and may
24 affect both female and male hormonal regulation (e.g. ketoconazole). Aromatase inhibitors
25 that also inhibit androgen synthesis and bind to the androgen receptor, such as prochloraz and
26 fenimarol, are described in section 3.2 (anti-androgenic and androgenic effects). The majority

1 of studies (43) were performed with the fathead minnow and zebrafish (42), followed by
2 medaka (24) and stickleback (11). Effect levels for individual compounds vary by up to four
3 orders of magnitude (or 5 orders of magnitude if the androgenic marker spiggin is included).
4 However, particularly for the zebrafish and medaka, this can be attributed to the different
5 exposure regimes using either different stages or exposure durations. Most of the studies
6 performed with fathead minnow and stickleback followed OECD recommendations (21 days
7 of exposure of adult fish; OECD, 2006). In these studies, inter-laboratory variation and use of
8 either nominal or measured concentrations to describe the LOEC may represent the major
9 source of variation. Many studies may also have underestimated the effect levels, since the
10 appropriate effects were already detected in the lowest tested concentrations. On the other
11 hand, the ranking of the (anti)estrogenic activity of the different compounds is for most of the
12 compounds highly conserved among the species with average differences in sensitivity below
13 one order of magnitude. Furthermore, despite the variability in experimental parameters, a
14 large overlap in effect concentrations for the endpoint vitellogenin (induction or inhibition),
15 various molecular endpoints (aromatase inhibition, hormone levels, expression of various
16 genes), secondary sex characteristics (tubercles, fins, nuptial coloration), histopathologic
17 effects (gonadal structure, gonadosomatic indices, incidence of intersex and gonadal sex
18 reversal) and reproduction (spawning, fecundity, fertilisation rate in offspring etc.) is apparent
19 (see also supplement Table S1). For those compounds, for which several endpoints have been
20 analysed, the molecular endpoints (VTG levels and others) generally appear to be among the
21 most sensitive endpoints with particular overlap to environmentally relevant endpoints, i.e.
22 reproduction. Exposure periods of 2-3 weeks generally appear to be sensitive enough to affect
23 these molecular endpoints in the same concentration that cause reproductive effects. It may
24 thus be concluded that for screening of chemicals for their (anti)estrogenic capacity, analysis
25 of molecular endpoints in relatively simple screening assays may be sufficient to predict
26 potential adverse effects for higher tier level testing. The inhibition of the androgen marker

1 spiggin (see below) has also been analysed in male sticklebacks exposed to estrogens.
2 Notably, exposure of sticklebacks to relatively high concentrations of estrogens can result in a
3 decrease in spiggin levels probably caused by indirect effects of estrogens on this androgenic
4 marker (Fig. 1 D, Jolly, 2007, Katsiadaki et al., unpublished data). This indicates the need for
5 molecular markers closely linked to the mode-of-action.

7 **3.2. Androgenic and anti-androgenic effects**

8 Androgenic effects are of similar environmental relevance to estrogenic effects, and
9 in fact have historically been detected in wildlife as early as the first reports of estrogenic
10 effects (Howell et al., 1980; Jafri and Ensor, 1979). In these early studies, before the concept
11 of endocrine disruption had been fully recognised, female mosquitofish (*Gambusia affinis*)
12 sampled downstream of a paper mill were found to have developed a gonopodium, a male
13 specific secondary sexual character. Various subsequent studies on fish have confirmed the
14 presence of androgenic ('masculinising') compounds in paper and pulp mill effluent (Cody
15 and Bortone, 1997; Larsson et al., 2000). Anti(androgenic) effects are mediated via direct
16 binding of a xenobiotic to the androgen receptor (AR), but may also be provoked by
17 inhibition of estradiol synthesis and accumulation of endogenous androgen levels (see 3.2).
18 Two AR subtypes were reported for rainbow trout (*Oncorhynchus mykiss*), eel kelp bass
19 (*Paralabrax clathratus*) and Atlantic croaker (*Micropogonias undulatus*), termed either AR1
20 and 2 or AR α and β with distinctive binding properties or transactivation activity for
21 androgens including testosterone and 11-ketotestosterone (Sperry & Thomas 1999, Takeo &
22 Yamashita 2000). For other species only a single AR with homology to either a teleost α or β
23 subtype could be identified so far (Hossain et al. 2007)

24 A number of contaminants interfering with androgen regulation have been
25 identified, such as the pesticides linuron and fenitrothion, and the fungicides vinclozolin and
26 prochloraz (Makynen et al., 2000; Katsiadaki et al., 2006; Vinggaard et al., 2006).

1 Furthermore, there is growing concern over the environmental impact of animal feedlot
2 effluents due to the use of anabolic growth promoters commonly used on livestock in the
3 USA, such as the highly potent synthetic androgen trenbolone (Lange et al., 2002). Two
4 isomers of this androgen, 17 α -trenbolone and 17 β -trenbolone, have recently been detected in
5 animal feedlot effluent in Ohio (Durhan et al., 2006) both of which have been shown to be
6 strongly androgenic in the fathead minnow (Ankley et al., 2003).

7 Detection of (anti)androgenic effects has been hampered by the unavailability of
8 androgen regulated marker genes in fish which may explain the greater focus on
9 (anti)estrogenic effects. However, since the detection, characterisation and cloning of spiggin
10 - a glue protein synthesized in the kidney of male sticklebacks for the purpose of nest building
11 - an appropriate and quantifiable biomarker for screening of androgens both *in vivo* and *in*
12 *vitro* is available in teleosts as well (Jolly et al., 2006; Jones et al., 2001; Nagae et al., 2007).
13 Furthermore, the antiandrogenic properties of test compounds can also be quantitatively
14 evaluated by assessing their ability to suppress the androgen-stimulated induction of spiggin
15 release in male sticklebacks. However, due to the large variability and high levels of spiggin
16 in males the use of females co-exposed with androgens have been preferred (Jolly, 2007;
17 Katsiadaki et al., 2006).
18 Despite the advantages of spiggin measurement, the majority of studies investigating (anti)
19 androgenic effects have been performed using the fathead minnow (19) and zebrafish (6) and
20 only three reports – based on the above listed criteria - are available for the medaka (Figure 2,
21 supplement Table S1). Molecular effects of (anti)androgens were detected indirectly by
22 analysis of vitellogenin induction or suppression, by analysis of various genes/proteins (LH,
23 FSH, aromatase, estrogen receptor a.o.) or testosterone levels. Although less quantitative,
24 (anti)androgenic properties of test compounds could also be identified from secondary sex
25 characters (tubercles), histopathology (gonadal structure, GSI, gonadal sex ratio) and
26 reproductive impairment (spawning, fecundity, fertilisation rate in offspring). Nine

1 publications report (anti)androgens in the stickleback in waterborne exposure experiments.
2 The only endpoints reported were spiggin levels and kidney hypertrophy (kidney epithelial
3 cells are enlarged in male stickleback due to spiggin production, Figure 2, supplement Table
4 S1).

5 In common with the (anti)estrogen studies considered above, data on (anti)androgens
6 exhibited a variability in effect levels by up to four orders of magnitude for the different
7 reproductive endpoints, and up to two orders of magnitude for the same endpoint. The same
8 reasons as discussed for (anti)estrogenic effects may similarly apply for this variability.
9 However, a comparison of those compounds (vinclozolin, flutamide, trenbolone and
10 methyltestosterone) that have been tested on at least three of the selected model species
11 indicate a highly conserved ranking of anti(androgenic) effect levels. Due to the lower
12 number of available studies in general and particularly those analysing reproductive
13 endpoints, it is difficult to judge whether molecular endpoints may be used as a predictor of
14 adverse reproductive effects in screening assays. Although vitellogenin is among the most
15 sensitive endpoints, a molecular marker that is directly linked to the mode of action may be
16 more desirable and may provide a closer link to higher tier effect levels. However,
17 impairment of reproduction has not been investigated in parallel to the androgenic marker
18 spiggin.

19 Identification of androgen-sensitive genes may help to establish sensitive markers for
20 fathead minnow, zebrafish and medaka. A current microarray study by Leon et al. (2008) in
21 medaka exposed to the teleost-specific androgen 11-ketotestosterone, the antiandrogen
22 flutamide, and the antiandrogenic fungicide vinclozolin identified 578 androgen-sensitive
23 genes, of which 50 were able to discriminate between the test compounds. These genes
24 include gonadotropin-releasing hormones and their receptors, and genes involved in
25 steroidogenesis. Detailed analysis of the affected genes may reveal marker genes directly

1 regulated by androgens and would allow comparing higher tier levels with molecular
2 responses in medaka, fathead minnow and zebrafish as well.

3

4 **3.3. Other hormones of the BPG-axis**

5 The analysis of endocrine disrupting effects has to date focussed mostly on estrogens
6 and androgens and their target organs, the gonads or liver. However, the hormonal control of
7 reproduction via the BPG-axis provides a number of additional potential targets for EDCs
8 such as the synthesis and binding of releasing and trophic hormones or the control of final
9 maturation by "maturation-inducing steroids" (C21-progestins). Interferences with releasing or
10 trophic hormones are likely to be reflected in alterations in steroid hormones levels, and cause
11 effects similar to direct interference (receptor binding or modulation of synthesis) with
12 estrogens or androgens (Katti and Sathyanesan, 1986). Vice versa, changes in expression or
13 circulating levels of gonadotropin by environmental contaminants may originate from
14 exposure to (anti)estrogens or (anti)androgens and subsequent feedback adaptations via the
15 hypothalamus and pituitary (Singh et al., 1994; Villeneuve et al., 2007b). Thus, *in vivo*, any
16 type of interference with hypothalamus or pituitary hormones may be detected by the same
17 endpoints demonstrating interactions with reproductive steroids, such as vitellogenin
18 expression. Cloning and sequencing of genes for releasing and stimulating hormones in small
19 model teleosts allows for a detailed analysis of interactions between hormones of the BPG
20 axis (Gopinath et al., 2004; Hellqvist et al., 2004; Kwok et al., 2005; Okubo et al., 2001;
21 Villeneuve et al., 2007b). A good example of this mode of action was demonstrated by
22 Villeneuve et al. (2007b), who showed that exposure to the chemical ketoconazole, an
23 inhibitor of steroid synthesis, led to an elevation of FSH β mRNA expression in male fathead
24 minnows. No effect on LH and FSH expression could be observed in zebrafish exposed to
25 benzo(a)pyren, albeit both aromatase and VTG expression were induced (Hoffmann and Oris,
26 2006).

1 Another important target of EDCs may involve maturation of oocytes and sperm,
2 which is triggered by gonadotropins. Specifically, maturation of germ cells is triggered by
3 gonadotropins but mediated by specific testicular and ovarian C21-steroid hormones
4 (maturation-inducing hormone, MIH; $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one, $17\alpha,20\beta$ -DP in
5 salmonid fishes; $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one, 20β -S in sciaenid fishes)
6 synthesised from the ovarian follicular layer or sperm (Nagahama, 1994; Nagahama et al.,
7 1993). Effects on maturation by EDCs have been studied for oocytes *in vivo* and *in vitro* in
8 zebrafish. Dithylstilbestrol (DES) induced the maturation of oocytes, while pentachlorophenol
9 caused an inhibition (Hachisuka et al., 2005; Tokumoto et al., 2004). The time and change in
10 germinal vesicle breakdown and synthesis of cyclin B indicated that there was no
11 distinguishable difference between exposure to either DES or the natural maturation-inducing
12 hormone $17\alpha,20\beta$ -DP. Binding of DES to the membrane progesterin receptor- α suggests a
13 specific interaction with oocyte maturation via a non-genomic pathway as well (Tokumoto et
14 al., 2007). The analysis of target genes or proteins of the maturation hormones would allow
15 the quantification of the strength of interaction, and to estimate the specificity of EDC
16 interference with these hormones. However, these target genes – with the exception of the
17 receptor itself - have yet not been identified for teleosts.

18 The previous reported examples of EDC action on the BPG-axis demonstrate the
19 strength of *in vivo* testing systems in identifying potential adverse effects. On the other hand,
20 it may be difficult to detect the underlying mode of action. This may be facilitated by the
21 analysis of molecular targets used in combination with simple *in vitro* systems, in particular
22 those allowing for the determination of binding to steroid hormone receptors (Coldham et al.,
23 1997).

24

25 **4. Interaction with thyroid hormones**

1 The thyroid hormones play a crucial role in the maintenance of a normal physiological
2 state in all vertebrates, notably in the control of development, growth and behaviour. In
3 addition, in teleost fishes, the thyroid hormones also play a pivotal role in the regulation of
4 metamorphosis, either the transition from larval to juvenile stages in marine flat fishes and
5 eels, the transformation from adult yellow to silver eels, or smoltification in salmonids
6 (Brown et al., 2004; Power et al., 2001). Seasonal variations of thyroid hormones are
7 associated with the reproductive status in teleost fishes. These changes indicate a potential
8 function in the control of both sexual differentiation and reproductive development (Cyr and
9 Eales, 1996). The latter is also supported by a recent report on the masculinisation of
10 sticklebacks by perchlorate, an inhibitor of thyroid hormone synthesis (Bernhardt et al.,
11 2006). Furthermore, thyroids are also involved in the regulation of the immune system, as
12 indicated by the suppression of immune function by hyperthyroidism (Yada and Nakanishi,
13 2002).

14 In common with the reproductive steroid hormones, the synthesis and release of the
15 thyroid hormones is under the control of a central brain-pituitary-thyroid (BPT) axis.
16 Releasing and stimulating hormones trigger the production and release of the thyroid
17 hormones. Thyroid-disrupting compounds can interact at the various levels of this regulatory
18 pathway, in particular with the synthesis (via thyroglobulin iodination, or deiodination of
19 thyroxine [T_4] to triiodothyronin [T_3]) and metabolism (conjugation) of thyroid hormones,
20 interference with the plasma binding proteins, or by binding to the thyroid receptor (TR,
21 Brown et al., 2004). Upon binding to its ligand, corepressors are replaced by activator
22 proteins and TR homodimers or heterodimers with the retinoic X receptors induce the
23 expression of target genes containing thyroid responsive elements - such as the
24 phosphoenolpyruvate carboxykinase (Park et al., 1995) or 5'diodinase (Jakobs et al., 1997).
25 Similar to what has been observed in mammals, two TR isoforms (α and β) and splicing
26 variants of these have been identified for many teleosts (Marchand et al. 2001). All the

1 subtypes tested so far have exhibited transactivation activity for thyroid hormones.
2 (Kawakami et al. 2006, Marchand et al. 2001)

3 Environmental chemicals mimicking the thyroid hormone have not been described nor
4 have TR-binding or transactivation properties been included in current screening strategies. In
5 contrast, a wide variety of structurally diverse chemicals has been shown to interfere with
6 thyroid hormone levels in fish (Brown et al., 2004). The majority of these effects, however,
7 are mediated apparently indirectly through a general and/or complex interference of the
8 contaminant with the physiology of the fish rather than specific interaction with thyroid
9 regulation. Examples of environmental compounds that have been shown to specifically
10 disrupt thyroid hormone synthesis include perchlorate, PCBs (Kime, 1998) and arsenic
11 (Davey et al., 2008). In order to identify thyroid-disrupting compounds which can interfere
12 with the synthesis of thyroid hormones or act as agonists/antagonists of the thyroid hormones,
13 it is necessary to define the typical effect patterns of model compounds. This has been
14 performed in zebrafish, fathead minnow and stickleback for inhibition of the thyroid action
15 using compounds that interfere with thyroid hormone synthesis via thyroidal iodine uptake,
16 thyroperoxidase and/or 5-deiodinase (Table 2). These model compounds derive from medical
17 applications in the treatment of thyroidal disease. One of these model compounds,
18 perchlorate, is also an environmentally relevant contaminant due to its application in solid
19 fuel rockets and in the manufacture of flares, fireworks and automobile airbags (Greer et al.,
20 2002). Typical effects observed in fish are lowering of endogenous T4 and/or T3 levels and
21 thyroidal hypertrophy (Table 2). Frequently, a reduction of growth and developmental
22 retardations has also been reported. Maternal effects on oocyte size, hatching and – in one
23 case reported for the stickleback – sexual differentiation and spawning also suggest an
24 interference with reproduction (Bernhardt et al., 2006). However, the effects occur generally
25 in the mg/L range close to concentrations that elicit morphologically visible toxic effects.
26 Interestingly, the effective concentrations overlap with those for compounds known to inhibit

1 metamorphosis in amphibians (Opitz et al., 2005). The potential long-term effects of lower
2 dose treatments have not been studied in detail. Relevant long-term effects could include
3 reduced reproductive performance, immune suppression (e.g. Lam et al., 2005) and growth
4 effects. These types of effects would indicate a reduced individual fitness and performance
5 with implications at the population level. It is obvious that such subtle effects may be difficult
6 and laborious to detect. Therefore, molecular markers that are clearly linked to adverse effects
7 could serve as indicators or early warning signals in short-term experiments. Genes (or their
8 corresponding gene products) containing TRE elements such as phosphoenolpyruvate
9 carboxy kinase (Park et al., 1995) could be – similar to estrogen regulated genes such as
10 vitellogenin – potential sensitive markers for the interference with the thyroid axis. However,
11 the differential regulation of fish genes with TRE elements, e.g. growth hormone (Farchi-
12 Pisanty et al., 1997), has not yet been studied in response to potential endocrine disrupting
13 chemicals.

14

15

16 **5. Corticosteroids**

17 Corticosteroids (glucocorticoids and mineralcorticoids) are involved in the regulation
18 of various important physiological processes of environment adaptation and the stress
19 response (changes in salinity, low oxygen concentrations, capture, presence of predators,
20 pollutants etc., Barton, 2002). Like thyroid and reproductive steroid hormones, they are
21 controlled by hypothalamus and pituitary hormones and exert their action through binding to
22 intracellular receptors. Some species of fish possess two receptor isoforms of glucocorticoids
23 resulting from a genome duplication (Schaaf et al., 2008, Alsop & Vijayan 2008, Bury et al.
24 2003). Although the mineral corticoid receptor has been detected, its function is not clear,
25 since teleosts lack the capacity to synthesise the mineralcorticoid aldosterone. However,
26 corticosteroids with high binding affinity that are not inactivated in MR-specific tissues, such

1 as 11-deoxycorticosterone, have been identified. It has therefore been suggested that these
2 glucocorticoids control osmoregulatory functions associated with mineralcorticosteroids in
3 higher vertebrates (Bury & Sturm 2007, Prunet et al. 2006, Sturm et al. 2005). Piscine
4 corticosteroids are synthesised in the interrenal cells of the head kidney (Chester Jones &
5 Mosley 1980) in response to various stress or environmental stimuli, and mediated via the
6 hypothalamus-pituitary-interrenal (HPI) axis. Corticosteroids can have many diverse
7 physiologic roles, such as, for instance, osmogulatory adaptations in migrating euryhaline
8 species or the redirection of energy to the central nervous system and suppression of immune
9 function during a stress response (McCormick 2001, Barton, 2002, Charmandari et al., 2005).
10 Thus, corticosteroid hormone-disrupting compounds could have severe impacts on adaptive
11 responses with obvious implications for individual health and population development even
12 though these effects may be subtle and difficult to detect.

13 Various anthropogenic compounds, such as PCBs, can suppress corticosteroid levels
14 and/or attenuate the corticosteroid response (Pottinger, 2003; Quabius et al., 1997). However,
15 an elevation of cortisol levels has also been reported in fishes following exposure to
16 estrogenic compounds such as estradiol and 4-nonylphenol (Lerner et al., 2007; Pottinger,
17 2003). While suppression of the corticosteroid response could impair the organism's ability to
18 adapt to changing environments or stress, a chronic or frequent hyperactivation may more
19 seriously lead to growth suppression, reproductive dysfunction, and immunosuppression
20 (Pottinger, 2003). Similar to that reported for thyroid-disrupting compounds, many of the
21 compounds interfering with corticosteroid regulation may act via an unspecific, complex
22 interaction. These compounds may also interfere with the early steps of steroid synthesis and
23 thus effect reproductive steroid hormones as well (Aluru and Vijayan, 2006). Finally, there
24 are strong links between corticosteroids and reproductive hormones explaining the suppressed
25 immune response during sexual maturation (Yada and Nakanishi, 2002) and by elevated
26 levels of estradiol and 11-ketotestosterone (Kurtz et al., 2007; Pottinger, 2003).

1 Molecular markers could assist in both identifying mechanisms of action, and in determining
2 the specificity of compounds interfering with corticosteroid regulation. The genes directly
3 under control of the corticosteroid receptors are largely unknown, but early studies in
4 mammals have revealed various genes with glucocorticoid receptor promoter elements that
5 might be useful markers to study the effects of contaminants on glucocorticoid response also in
6 fish as well (Phuc Le et al., 2005; Wang et al., 2004). An example of differential gene
7 regulation in fish has already been reported, i.e. the upregulation of the glucocorticoid
8 receptor and interleukin-1-beta by PCBs in rainbow trout anterior kidney cells (Quabius et al.,
9 2005). Current knowledge on the stress response – and particularly its interference by
10 environmental contaminants - of the species covered in this review is scarce. Cortisol has
11 been shown to be involved in the adaptation to crowding stress, salinity and acidity in
12 zebrafish and medaka (Ramsay et al., 2006; Sakamoto et al., 2001; Yada and Ito, 1999). Thus,
13 the stress response in these model species is likely to resemble those of other fish species.
14 With regards to their extensive use as laboratory fish (including endocrine disruption
15 research) and the extensive knowledge on their molecular genetics, these species would also
16 be ideally suited for a molecular-based model for screening the ability of environmental
17 compounds to interfere with the glucocorticoid stress response.

18

19 **6. Other hormones**

20 Studies on endocrine disruption in fish have focused almost exclusively on the reproductive
21 steroids, thyroidal hormones and glucocorticoids. However, these hormones regulate only a
22 portion of the vital physiological processes under endocrine control. In principle, a number of
23 different hormones and their corresponding physiological processes could be disrupted by
24 environmental contaminants, provoking adverse effects. Potential targets could be those
25 processes regulated by growth hormones, somatostatin, vasotocin, isotocin, melanin-
26 concentrating hormones, endorphin, somatolactin, prolactin, atrial natriuretic peptide and

1 parathyroid hormones among others. In a few cases, potential target genes have been
2 identified in the zebrafish (Hoshijima and Hirose, 2007). However, it will be difficult to
3 include this array of potential endocrine targets into present testing schemes. Hence, model
4 species with extensive genetic characterisation could be used for a comprehensive approach
5 using modern techniques for screening whole genomes or proteoms. This type of screening
6 may indicate relevant hormone regulated processes and pathways which could be integrated
7 in targeted testing approaches.

8

9

10 **7. Conclusions**

11 A diverse array of important physiological functions are controlled by secreted hormones. As
12 these processes are susceptible to interference by EDCs, the identification of these chemicals
13 is highly relevant to the health and well-being of both wildlife and humans. Therefore, the
14 inclusion of endocrine interference in the regulation of chemical use has been demanded
15 (Lyons, 2006). In fish – which are directly exposed to discharged industrial, agricultural and
16 municipal effluents - endocrine disrupting effects have been reported for a diverse array of
17 anthropogenic chemicals which can mimic/interfere with reproductive steroids, thyroidal
18 hormones and glucocorticoids. Molecular biomarkers for EDC effects have to date only been
19 investigated for (anti)estrogenic and (anti)androgenic effects. For these EDCs, the existing
20 data in small model fish indicate a high correlation between the upregulation of endocrine
21 target genes and long-term adverse effects such as reproduction. This finding is not elusive,
22 since many compounds – modifying endogenous hormone levels via binding to nuclear
23 receptors – directly interfere with the transcription of target genes. The potential of molecular
24 screening approaches has already been recognised as a valuable tool in regulatory
25 applications, and has led to the development of fish screening assays for compounds
26 interfering with reproductive hormones (Hutchinson et al., 2006; OECD, 2006; USEPA,

1 2007) The strong correlation between xenoestrogen exposure and reproductive impairment
2 allows for the establishment of simple but integrative screening assays for regulatory
3 purposes. Such screening assays may be established additionally for other hormones and
4 corresponding physiologic processes, once appropriate target genes/proteins have been
5 identified. Thus, screening assays could contain a battery of molecular targets allowing a
6 more comprehensive approach in the identification of endocrine disrupting compounds in fish
7 and vertebrates in general.

8

9

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12 graphically.

13

14

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1 **Figure legends**

2

3 **Table 1:** Brief description of the model organisms covered in this review

4

5 **Table 2:** Summary of lowest observed effect concentrations (mg/L) of thyroid disrupting
6 compounds in zebrafish, fathead minnow and stickleback. Perchlorate is an inhibitor of
7 thyroidal iodine uptake. Propylthiouracil, phenylthiourea, and thiourea inhibit
8 thyroperoxidase and 5'-deiodinase. Methimazole is an inhibitor of thyroperoxidase. The
9 mechanism of arsenite interference is not known but is discussed as a general interference
10 with nuclear hormone receptors (Davey et al., 2008).

11

12 **Fig. 1:** Estrogenic and anti-estrogenic chemicals and their effect levels (lowest observed
13 effect concentrations, LOEC) for vitellogenin/choriogenin expression, various molecular
14 endpoints, secondary sex characters, histopathology and reproduction in males and or females
15 of the small model species fathead minnow (A), zebrafish (B), medaka (C) and three-spined
16 stickleback (D). The data were extracted from Allen et al., 2008; Andersen et al., 2004;
17 Andersson et al., 2007a,b; Ankley et al., 2001; Ankley et al., 2002; Ankley et al., 2007;
18 Bell, 2001; Bell, 2004; Biales et al., 2007a,b; Bistodeau et al., 2006; Bogers et al., 2006;
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23 Jobling et al., 2003; Jolly, 2007; Katsiadaki, Pottinger and Mayer, unpublished data;
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15 Wester et al., 2003; Williams et al., 2007; Yang et al., 2006; Yokota et al., 2000; Zerulla et
16 al., 2002; Zhang et al., 2008. Detailed information on exposure conditions (stage, period) and
17 description of effects can be found in the supplement available online (Tables S1). Criteria for
18 the selection of references are outlined in the paragraph 2 (Data sources and graphical
19 presentation). Please note different scale of the x-axis for medaka (C).

20

21

22 **Fig. 2:** Androgenic and anti-androgenic chemicals and their effect levels (lowest observed
23 effect concentrations) for vitellogenin, spiggin, various molecular endpoints, secondary sex
24 characters, histopathology and reproduction in males and or females of the small model
25 species fathead minnow (A), zebrafish (B), medaka and three-spined stickleback (C). The data
26 were extracted from Allen et al., 2008; Andersen et al., 2006; Ankley et al., 2001; Ankley et

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6 al., 2008; Seki et al., 2006; Thorpe et al., 2007; Villeneuve et al., 2007; Wester et al., 2003;
7 Wester et al., 2003; Zerulla et al., 2002. Detailed information on exposure conditions (stage,
8 period) and description of effects can be found in the supplement available online (Tables
9 S1). Criteria for the selection of references are outlined in the paragraph 2 (Data sources and
10 graphical presentation).

11

12

1 **Tables and figures**

2

3

4

Table 1

The **fathead minnow** (*Pimephales promelas*) is a cyprinid fresh water fish with broad distribution across North America. It is used for a number of different regulatory tests for the risk assessment of chemicals. Appropriate testing guidelines for EDCs including reproductive endpoints have been established and genes involved in sexual development have been identified. With respect to endocrine disruption, secondary sex characteristics – androgen-dependent formation of tubercles - are of interests (Ankley and Villeneuve, 2006; Panter et al., 2004) (photo: Matthew Winter, AstraZeneca UK).



The **zebrafish** (*Danio rerio*), a small tropical fish native to the rivers of India and South Asia (Eaton and Farley, 1974), is one of the most popular model organisms in developmental genetics and (eco)toxicology (e.g. Coverdale et al., 2004; Goldsmith, 2004; Hill et al., 2005; Nagel, 2002; Parg et al., 2002). Its rapid development, easy maintenance in the laboratory and large number of offspring provides advantages for experimental studies. The extensive genetic characterisation and corresponding functional data are in particular useful for molecular studies in endocrine disruption (photo: Andre Künzelmann, UFZ).



The **medaka** (*Oryzias latipes*) native to Japan, Korea, China and Taiwan, is the only fish species of which the sex determination locus has been identified. Furthermore, sex specific markers (chromatophores, PCR-marker) and secondary sex characters (shape of the anal and dorsal fin) have lead to increasing attraction of the medaka as a model for endocrine disruption (Matsuda et al., 2002; Nanda et al., 2002; Yamamoto, 1975) (photo: Andre Künzelmann, UFZ).



The **stickleback** (*Gasterosteus aculeatus*) is unique among the small model fish due to the synthesis of an androgen-regulated protein called spiggin, a glue protein produced in the male kidney and used for nest building. Furthermore, the stickleback's quantifiable courtship behaviour (Bell, 2001) and male breeding colour (Maunder et al., 2007) have been used to analyse endocrine disruption. DNA markers for sex determination are available for the stickleback (Griffiths et al., 2000; Peichel et al., 2004). The stickleback is a euryhaline species and the only model indicator species that can be used for biomonitoring in marine and freshwaters in the Northern hemisphere (photo: Ian Mayer, UIB).



5

Table 2

Compound	Species	Hormone levels, molecular effects	Histology	Teratogenicity	Toxicity	Reproduction	Exposure period	Reference
Perchlorate	ZF		≤ 0.011 colloidal follicular structure, angiogenesis				Adults, 12 w + 12 w recovery	(Mukhi et al., 2005)
	ZF		≤ 6 Follicle structure, angiogenesis			≤ 6 PEV reduced	Adults, 8 w	(Patino et al., 2003)
	ZF	≤ 10 T4 reduced in females and embryos	≤ 10 thyroidal hypertrophy, jar malformation		≤ 10 reduced weight in female	≤ 10 PEV reduced, egg and larval offspring size increased	Adults, 10 w + 6 w recovery	(Mukhi and Patino, 2007)
	ZF	≤ 10 Collidal area, epithelial cell height					Adult, 90 d	(Liu et al., 2006)
	ZF	250 T4 reduced		250 (2500) Delayed hatch (retarded, less motile)	25000 Cell death		6 hpf – 6 dpf	(Elsalini and Rohr, 2003)
	FM		≤ 1 Thyroid hyperplasia	≤ 10 Retardation, reduced pigmentation			0-28 dph	(Crane et al., 2005)
	SB ^a		≤ 30 (100) Male nuptial coloration (intersexual gonads)			≤ 30 Reduced survival	≤ 30 Reduced spawning success and courtship behaviour	Adult, 350 d
Propylthiouracil	ZF	10	≤ 1	100	100	10-100 ^b	Adult, 21 d	(van der Ven

		T3, T4 decreased	thyroid follicle synthetic activity	Body weight F1 generation	Reduced body weight	Increased egg number and egg size	Juvenile, 42 d	et al., 2006)
Phenylthiourea	ZF	100 T4 reduced		100 Retarded, curved body	1000 Reduced survival		6 hpf – 6 dpf	(Elsalini and Rohr, 2003)
	ZF	30 T4 reduced		30 (300) no pigmentation (retarded growth, curved body)	300 Reduced survival		6 hpf – 6 dpf	(Elsalini and Rohr, 2003)
Thiourea	ZF	≤ 300 T3, T4 decreased					0 hpf – 16 dpf	(Tagawa and Hirano, 1991)
Methimazole	ZF	117 T4 reduced		117 (234) Delayed hatch (retarded, less motile, curved body shape)	1170 Reduced survival		6 hpf – 6 dpf	(Elsalini and Rohr, 2003)
	ZF			34 mg/L Various morphometric parameters reduced	≤ 34,000 Reduced length		5 hpf-5dpf	(Liu and Chan, 2002)
	ZF	≤ 34 RagI, IgL-C-expression,			≤ 34 Reduced length		5-32 dpf	(Lam et al., 2005)
	FM	0.032 T3 (28 d inhibition, 56 induction)		0.032 Delayed hatching (not at 0.32 mg/L)			0-48 dpf	(Crane et al., 2004)
Arsenite	ZF		≤ 10 Colloid area and height (not in all concentrations and time points)				Adults, 90 d	(Liu et al., 2006)
Thyroxine (T4)	ZF	≤ 0.008		0.023	0.023		5 hpf-5dpf	(Liu and Chan,

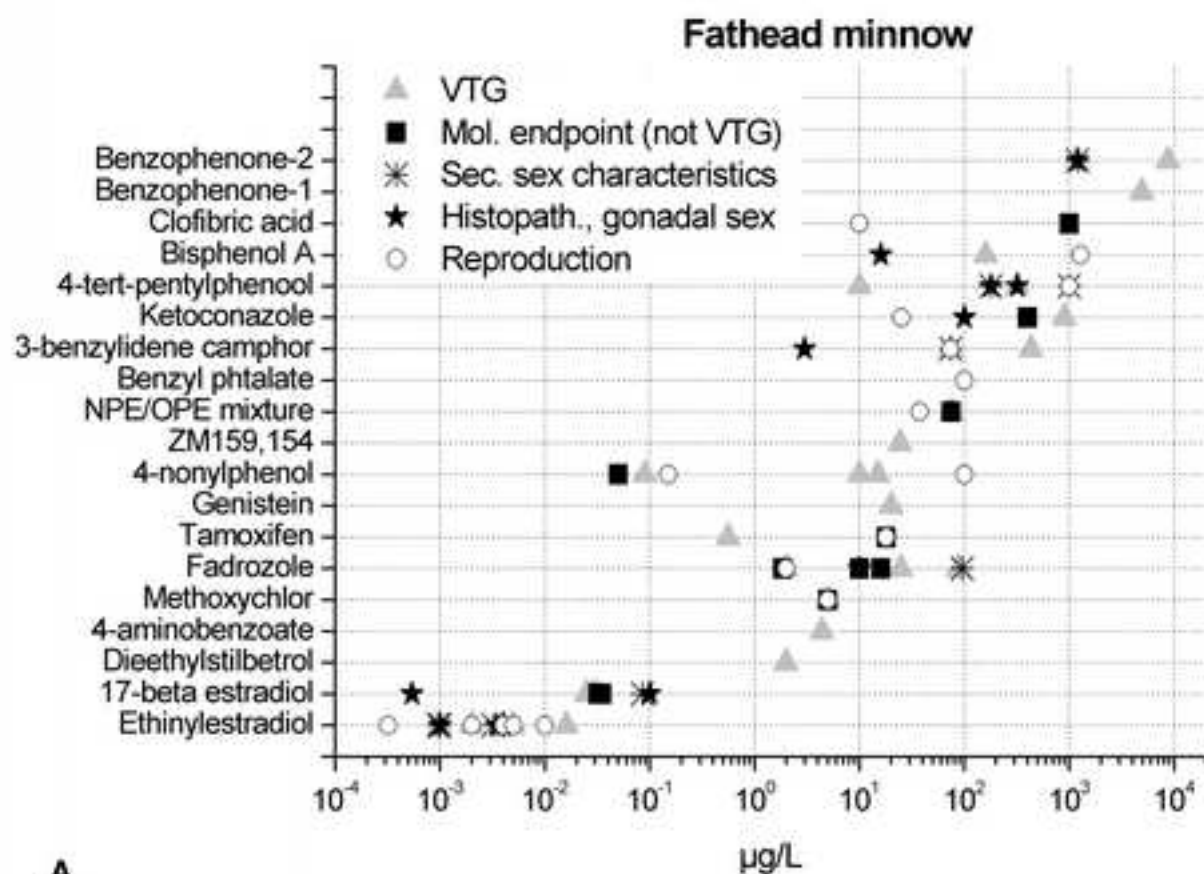
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ZF	TR α , TR β gene expression increased	≤ 23.3	Deflation of swim bladder	Reduced length	5-32 dpf	2002) (Lam et al., 2005)
	RagI-expression	≤ 23.3	Thymus structure			

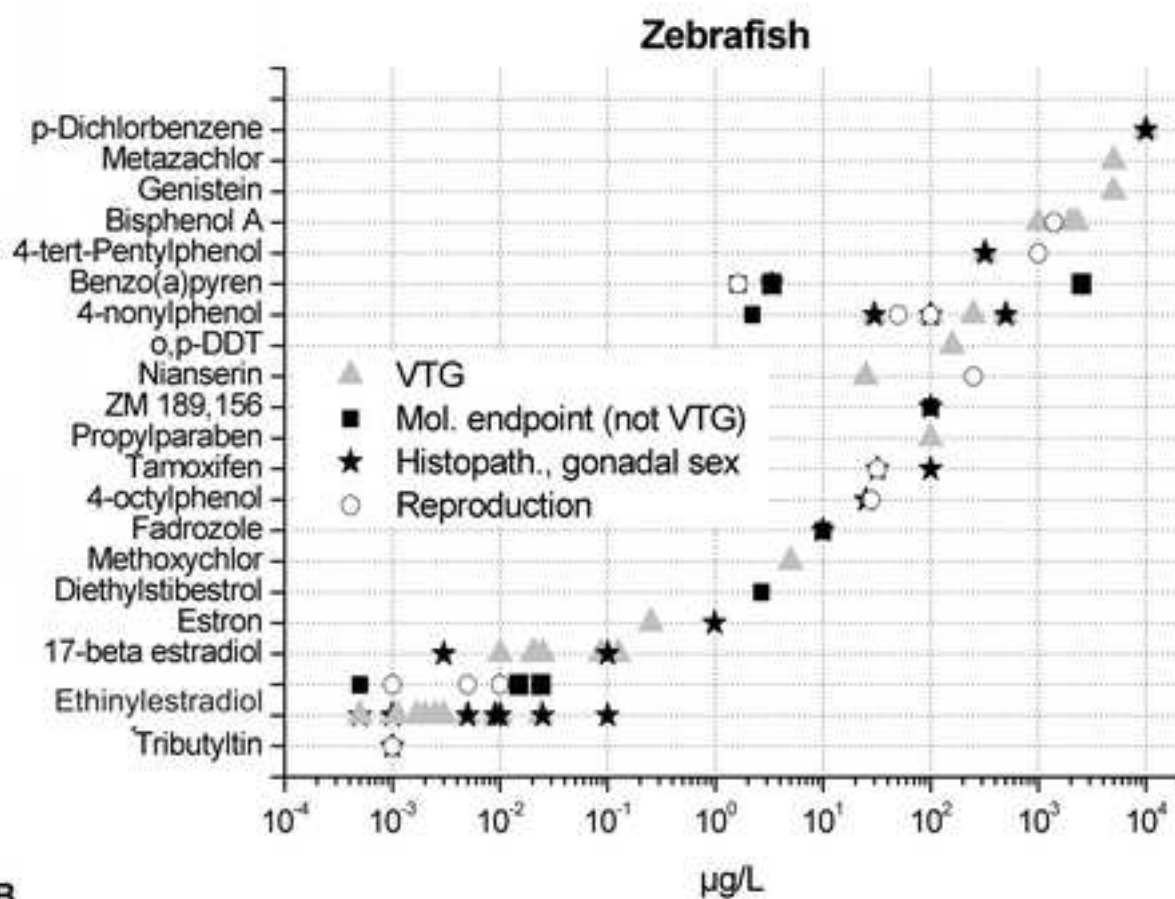
^a Data on statistic significance not presented in the paper.

^b No LOEC calculated, p-value significant for regression analysis

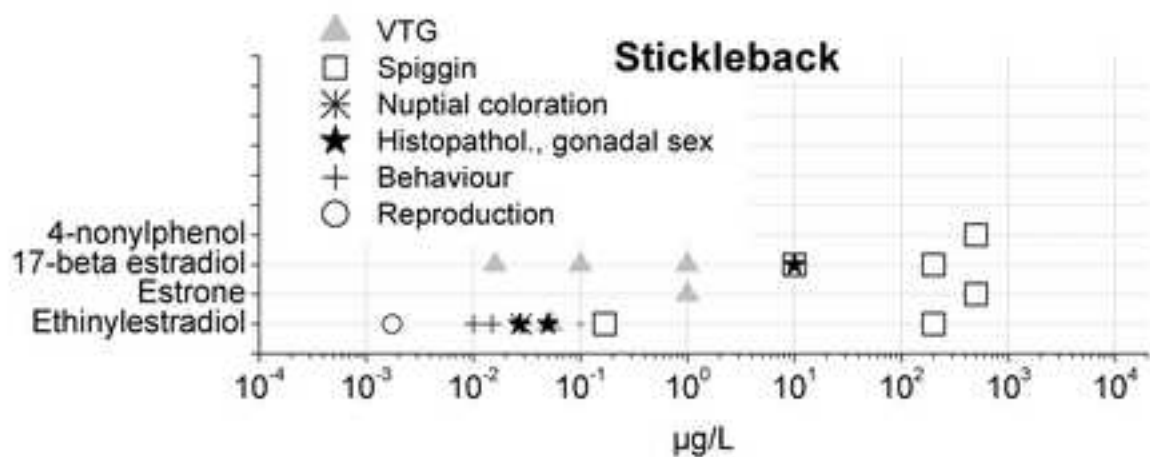
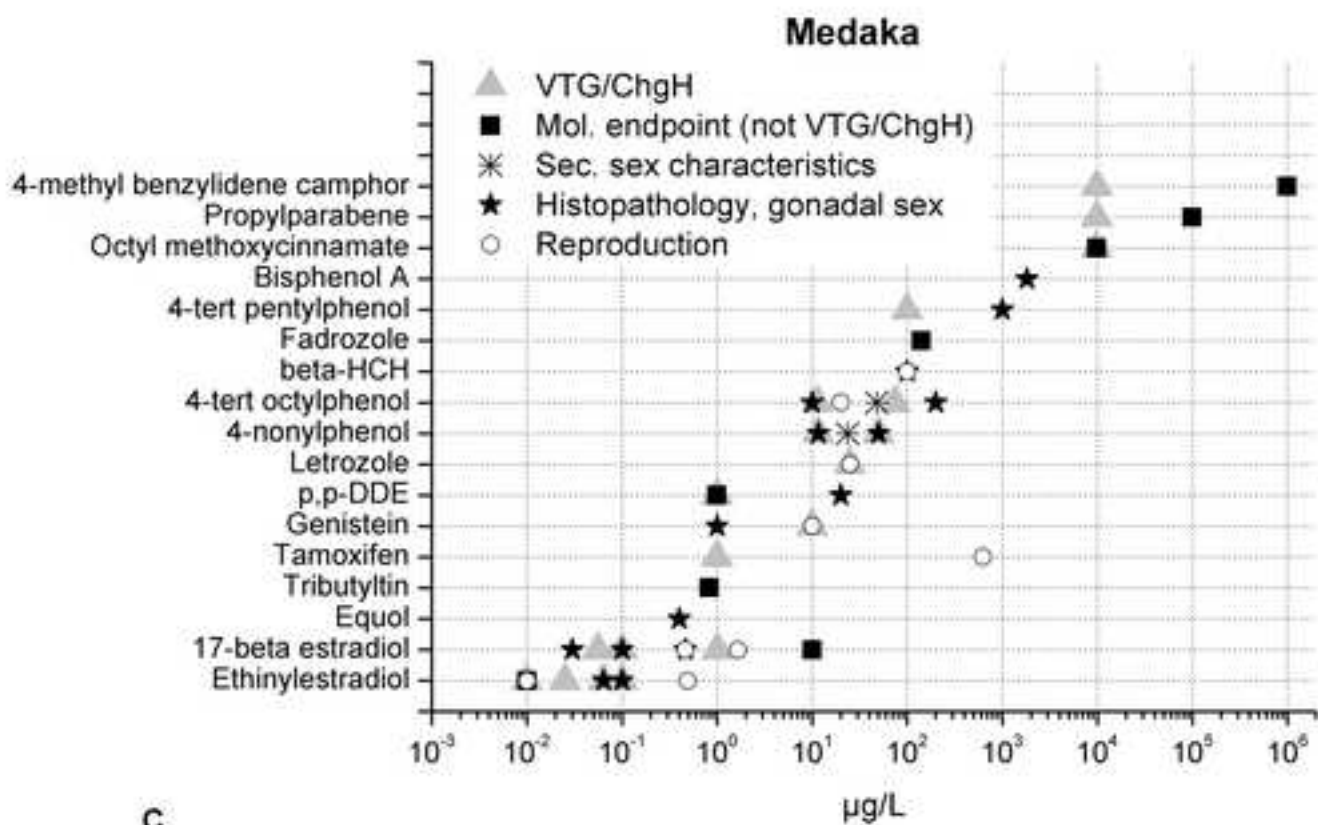
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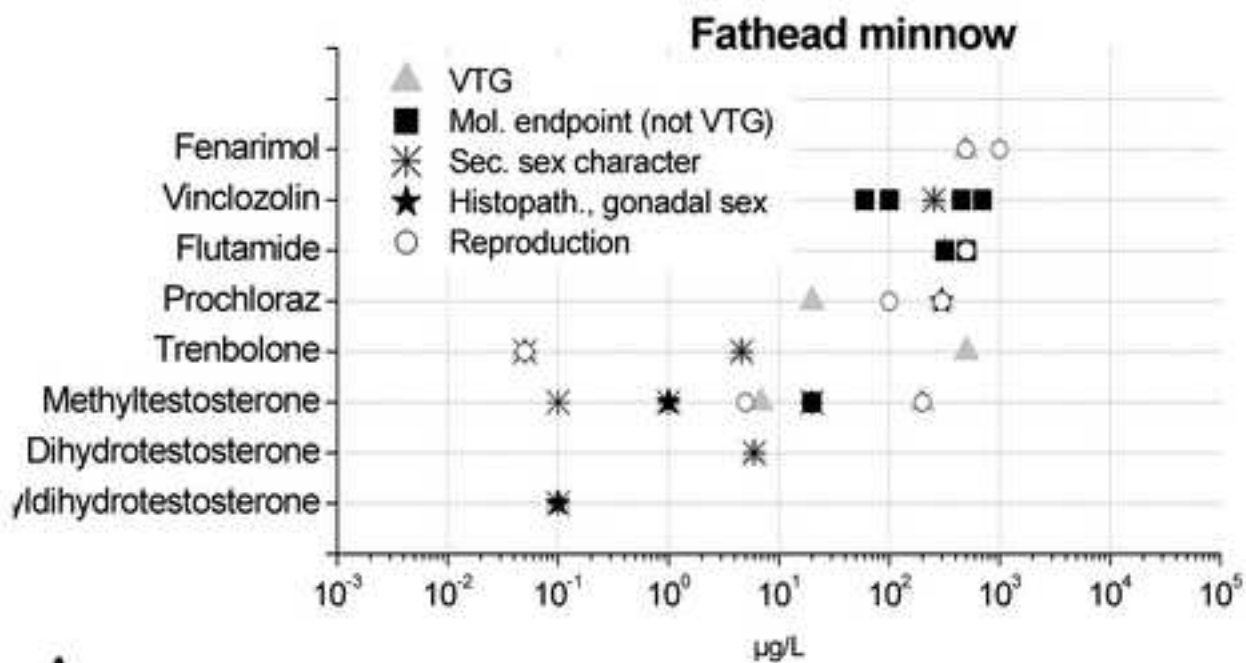


A

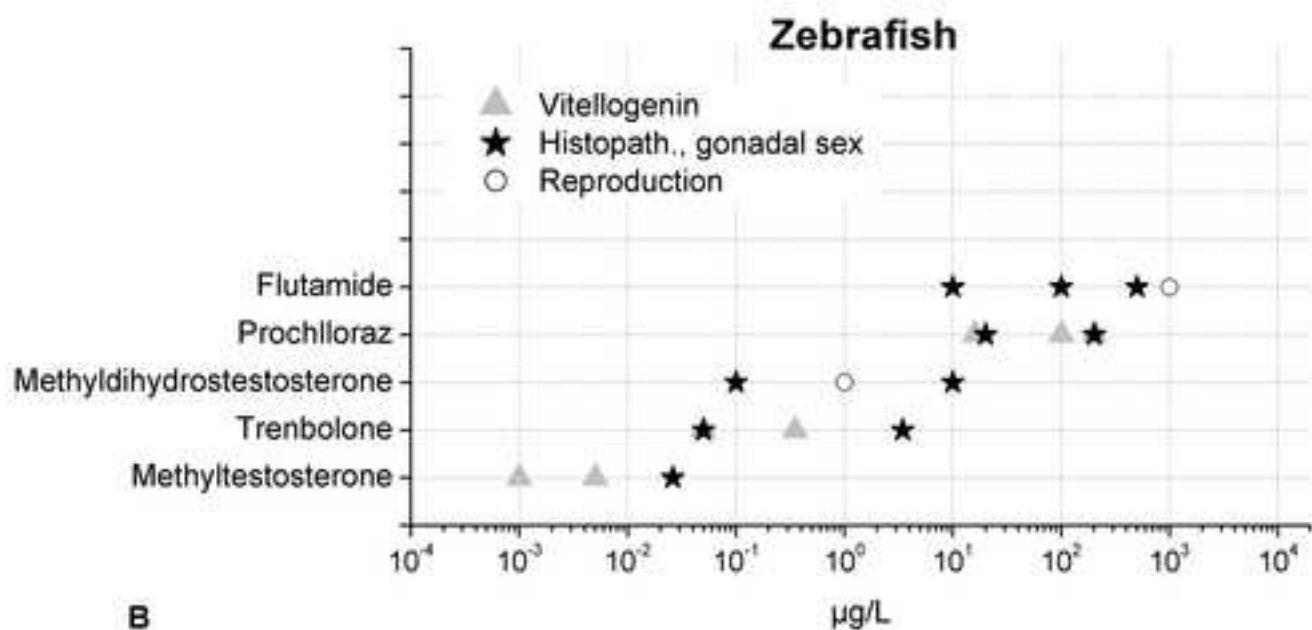


B

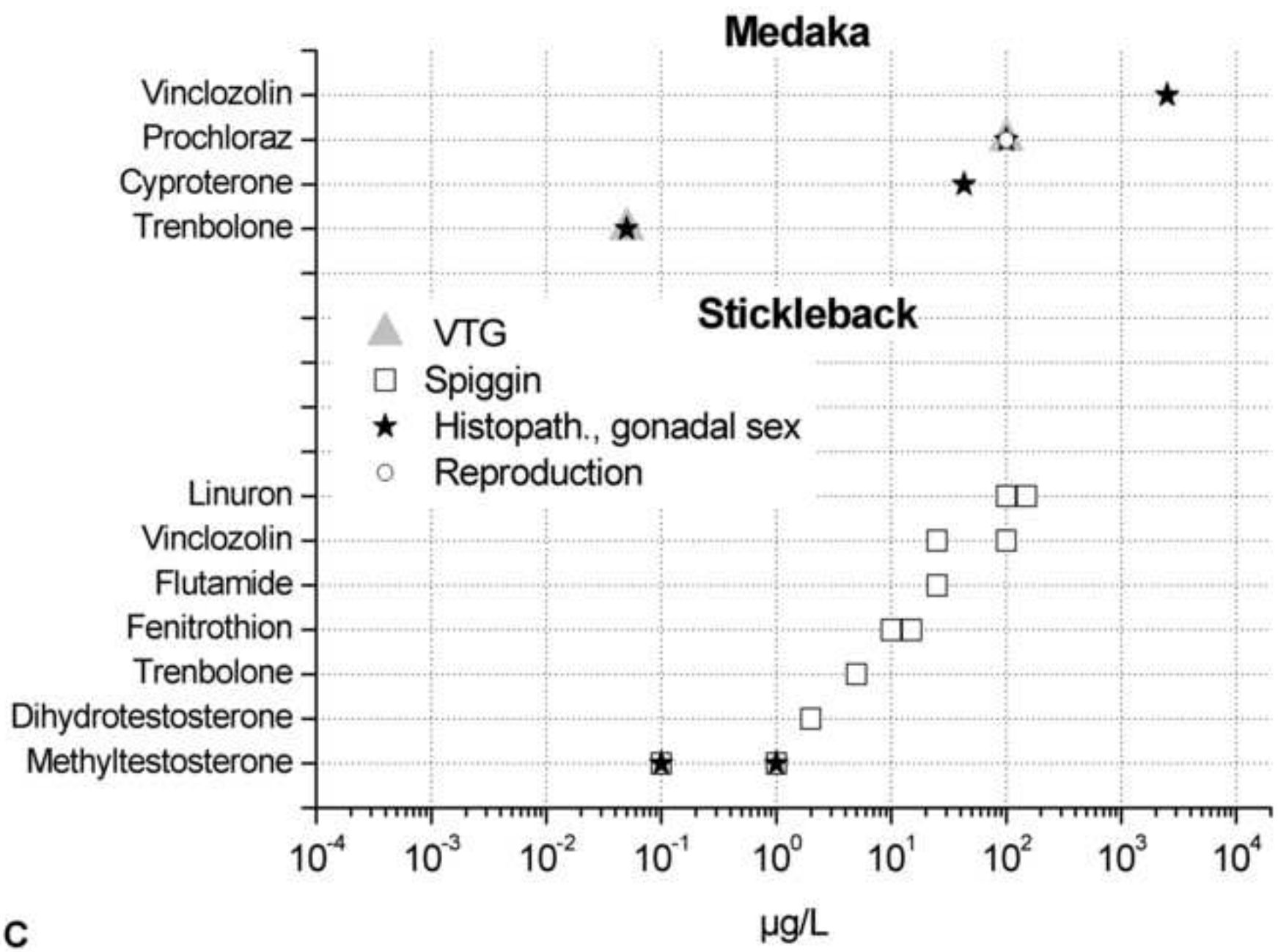




A



B



C