Rapid bioconcentration of steroids in the plasma of sticklebacks (*Gasterosteus aculeatus*) exposed to water-borne testosterone and 17ß-estradiol

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Abstract

This study investigated the relationship over time between the concentrations of two steroids, singly and in combination, in a static exposure system and in the blood of sticklebacks, *Gasterosteus aculeatus*, held within the exposure system. Groups of sticklebacks were exposed (nominally) to either 1000 ng l^{-1} 17ß-estradiol (E2), Testosterone (T) or E2 & T in combination at the same concentrations for 6 days. Both water and fish were sampled at intervals and steroid concentrations in both compartments were determined. The plasma steroid time profile revealed a rapid bioconcentration within the first 6 hours of exposure. The plasma steroid levels attained at this time point (20 – 90 ng ml^{-1}) were up to 50-fold (E2) and 200-fold (T) greater than the actual levels of steroid measured in the exposure water, while levels in the blood of control fish did not exceed 4 ng ml^{-1}. The substantial elevation of plasma steroid levels relative to the concentrations of steroid to which the fish were exposed in the ambient water gives scope for delivery of the steroids to target endocrine tissues at levels far in excess of what might be predicted on the basis of passive branchial uptake alone. These results are discussed in relation to endocrine disruption, and in particular the occurrence of effects in fish exposed to levels of endocrine active substances (EAS) that are seemingly physiologically irrelevant.

Keywords: 17ß-estradiol; testosterone; stickleback; plasma; uptake; bioconcentration

1. Introduction

The exposure of fish to chemicals in solution by immersion in either static, semi-static, or flow-through systems is a technique routinely employed in aquatic toxicology. Dose response endpoint data are related to the nominal or actual concentrations of test chemical in the water. However, linking data obtained from *in vivo* exposure systems with data from *in vitro* tests is difficult. This is primarily because we remain largely ignorant of the dynamics of uptake and excretion of the test chemical by the exposed fish other than in those studies where bioaccumulation and depuration are specifically measured (Specker & Chandlee, 2003). This absence of information on the uptake of chemicals by test animals is particularly pertinent to the current interest in EASs.

A range of *in vitro* systems is routinely used for screening chemicals for agonistic or antagonistic endocrine effects, exemplified by the recombinant yeast screen for estrogens and androgens (Routledge & Sumpter, 1996; Harris *et al.*, 1997; Thomas *et al.*, 2002). This system and others (Legler *et al.*, 2002) are extremely responsive to chemicals possessing steroidal activity. However, it is difficult to relate with confidence the dose response data obtained from such a system with the likely sensitivity of an intact animal exposed to the same chemical, or to predict the risk posed by concentrations of the chemical in the environment. One factor contributing to this uncertainty is lack of information relating environmental concentrations to levels in the blood of exposed organisms. In two British rivers in which fish are showing estrogenic effects (Nene and Lea), environmental levels of E2 have been shown to be in the range 0.4 - 4 ng l^{-1} (Williams *et al.*, 2003), though they may be further diluted or concentrated by changeable river flows or inputs downstream of sewage works. Despite these exposure concentrations being at levels which appear

to be physiologically irrelevant, numerous effects linked to the activity of E2 and other estrogenic chemicals have been widely reported from these and other rivers (Jobling *et al.*, 1998; van Aerle *et al.*, 2001; Kirby *et al.*, 2004).

The aim of this study was to provide an insight into the relationship between the concentration of steroid hormone in the water within which the fish is immersed, and the concentration of this hormone in the blood. This relationship was investigated over a 6 day period, making the assumptions for functional purposes that the blood concentration equates to that delivered to the target tissues and that no significant changes in internal steroid metabolism were induced by the hormone exposure.

A static exposure system utilising the three-spined stickleback (*Gasterosteus aculeatus*) was adopted. The biology of the stickleback is well described (Wootton, 1976; Wootton, 1984; Bell & Foster, 1994) and this species is widely used in behavioural, ecological, physiological and toxicological studies. In the context of endocrine disruption, the stickleback represents a test species with three distinct endpoints of relevance: a range of endocrine-dependent behaviours (Bell, 2001), the estrogen sensitive biomarker vitellogenin (VTG; (Katsiadaki *et al.*, 2002b; Hahlbeck *et al.*, 2004), and a unique androgen sensitive endpoint, the nest building glue, spiggin (Jones *et al.*, 2001; Katsiadaki *et al.*, 2002a). Use of the stickleback in studies of endocrine active substances is increasingly widespread. In this study, the fish were exposed to two natural steroids, testosterone and 17ß-estradiol, both singly and in combination, and concentrations of these steroids were determined in the water, and blood plasma.

2. Materials and methods

2.1 Experimental fish

Sticklebacks (*Gasterosteus aculeatus* L.) were obtained from a commercial supplier (Moore and Moore, Reading) and held in groups of 200 fish in each of four 1000 litre circular outdoor tanks supplied with a constant flow of Windermere lake water (10 l min^{-1}) and natural photoperiod and temperature range (4^{o}C – 17^{o}C annually). During the summer of 2002 the fish were sexed by external inspection and then held in single sex groups until the time of study, during December 2002. At this time, water temperature was 11^{o}C, pH was within the range 6.5 to 7.2, hardness 110 - 140 mg l^{-1} $CaCO_3$, and dissolved O_2 91-98% saturation. The fish were fed three times weekly on commercial trout feed (Skretting fry crumb 02). At the time of the study the average size of the fish was (mean ± SEM) 3.41 ± 0.05 g, *n* = 144. There was no significant difference in mass between the male and female groups.

2.2 Exposure system

The experiment was carried out at 11 °C in a temperature controlled laboratory with an 8h light : 16h dark photoperiod. Eight 50 litre glass aquaria were filled with sand-filtered lake water and aerated for 48 h before the introduction of the fish. Four groups of 21 male fish and four groups of 21 female fish were selected at random from the stock ponds and transferred to the exposure aquaria. The fish were allowed to acclimate to these conditions for one week. All fish were adults, but reproductively quiescent, and did not display any secondary sexual characteristics. The fish were fed once daily with commercial trout feed 2 hours into the light period. A 50% water change was carried out twice prior to the start of the exposure period.

The general health of the fish was observed to be good throughout the study, with active, inquisitive behaviours and no visibly evident infections or parasites. Two fish died during the experiment, though the mortalities were not considered to be treatment related.

2.3 Dosing

Stock solutions (50 mg l^{-1}) containing testosterone or 17ß-estradiol (Sigma-Aldrich) were made up in acetone. Four treatments were randomly applied to the four aquaria housing male fish and to the four aquaria housing female fish: (1) E2 at a nominal concentration of 1000 ng l^{-1}; (2) E2 and T in combination, both at nominal concentrations of 1000 ng l^{-1}; (3) T at a nominal concentration of 1000 ng l^{-1}; (4) Control group, solvent carrier only (20 µl l^{-1}). Steroids were introduced into the tanks by removing 1.0 l of tank water in a beaker, adding 1 ml of stock solution containing either E2, T or E2 and T to the beaker, and after mixing, replacing the water in the tank (all tanks contained 0.002% acetone).

2.4 Sampling procedures

Groups of six fish (three males and three females) were sampled at 0, 6, 24, 48, 72, & 144 hours from commencement of exposure. The fish were caught in a dip net and rapidly transferred to anaesthetic (2-phenoxyethanol; 1:2000) before being killed by cranial compression using forceps. Blood samples were obtained by severing the caudal peduncle and collecting blood in a heparinised capillary tube. The blood sample was then transferred to a capped 1.5 ml centrifuge tube and held on ice before being centrifuged. Plasma was transferred to a second, individually pre-weighed 1.5 ml tube. The tube and plasma were then weighed together to calculate

the volume of plasma sample obtained (assuming density of 1g ml^{-1}; mean 26.3 µl). Samples were stored frozen at -20^{o}C until required for analysis. Water samples were taken from the middle of the aquaria by siphon at 0, 72 and 144 hours. These were transferred to stoppered plastic bottles and stored frozen for later analysis.

2.5 Extraction of water samples

The water samples, ranging from 497-677ml in volume were thawed overnight at 4ºC, filtered under vacuum (Whatman GF/C microfibre filter) and then pumped through a methanol (Analar) primed Sep-Pak C18 (Waters Ltd) solid phase extraction cartridge at 5 ml min^{-1}. Steroids retained on the cartridge were eluted with 5 ml methanol. The methanol eluate was dried under nitrogen at 30ºC in a heating block and the sample was redissolved in 1.0 ml of ethyl acetate (Analar), which was stored at -20^{o}C until required for assay. This method consistently provided recoveries of =85% (86.5 ± 0.63, *n*=8) when run with radiolabelled steroids and estimates of dissolved steroid concentrations in the exposure tanks are corrected for this level of loss.

2.6 Steroid assays

Steroids were extracted from plasma samples with ethyl acetate (5:1, ethyl acetate : plasma) and aliquots of plasma extracts from every fish were analysed for both E2 and T using previously validated radioimmunoassay procedures (Pottinger & Pickering, 1985; Pottinger & Pickering, 1990). The assay detection limit is 6 pg steroid per assay tube. All of the extracted water samples were also assayed for both steroids using the same procedures.

2.7 Statistical analysis

Multifactorial analysis of variance (ANOVA, Genstat 5) was employed to assess the significance of changes in steroid levels in steroid-exposed and control groups with time and between sexes. Where mean and variance did not vary independently, as indicated by a plot of residuals against fitted values, a log transformation was applied to improve the homogeneity of variance. Significant differences between treatment groups, times, or sexes were determined using the estimated standard error of the differences between means. Where no overall significant difference was found (P>0.05) between the male and female response, their data were combined.

3. Results

3.1 Concentrations of steroid in the exposure water

The concentrations of E2 in the exposure tanks are depicted in Table I. In control tanks, receiving vehicle only, concentrations of E2 were very low ranging from not detectable to 8.4 ng l^{-1}. In the four tanks receiving E2 only or a combination of E2 and T, maximum levels of E2 (50% to 80% of nominal concentration) were detected at 6 h. In both sets of tanks there was a progressive decline in the concentration of E2 during the course of the study and this was most pronounced in the tanks receiving both E2 and T, such that at 144 h after the start of the study, concentrations had declined to between 2.5% to 34% of the nominal. The concentration of T in the control tanks was also very low (= 5 ng l^{-1}; Table II) but, in contrast to E2, water-borne T concentrations declined from approximately 400 ng l^{-1} (40% to 50% of the nominal value) to control levels within 72 h of the start of the study in both the T-only

and combined T and E2 exposure tanks. Overall, there was good agreement in the water chemistry between the duplicate tanks containing male and female fish.

3.2 Plasma steroid concentrations: E2

Plasma E2 levels are presented in Fig. 1. There was no significant difference between E2 levels in male and female controls (consistently <0.5 ng ml^{-1}), so these were combined. In fish from both treatment groups (E2 and E2 + T), plasma E2 concentrations were significantly greater than those in the control fish (P<0.001) throughout. There was no significant difference in the plasma E2 levels between males and females exposed to E2 alone and these data were therefore also combined (Fig. 1a). There was a rapid increase in plasma E2 levels in these fish within 6 h of the start of the exposure period, with mean plasma E2 levels rising from 0.8 ± 0.2 ng ml^{-1} at 0 h to 22.3 ± 5.3 ng ml^{-1}. Plasma levels of E2 were maintained at or around this level throughout the study, remaining significantly elevated at 17.9 ± 4.2 ng ml^{-1} at 144 h. Plasma levels of E2 in fish from the combined E2 and T exposure tanks showed a similar pattern (Fig. 1b). However, in this case, there was a difference in plasma E2 levels between the sexes, with levels in male fish being significantly greater overall than levels in female fish. Plasma E2 levels in both sexes rose rapidly after the start of the exposure period to reach maximum levels at 6 h (males: 39.9 ± 10 ng ml^{-1}; females: 31.4 ± 6 ng ml^{-1}), after which there was a decline to approximately 12 ng ml^{-1} at the end of the study. Mean plasma E2 levels in fish exposed to T alone were significantly (P<0.001) higher than control levels (Fig. 1c) at 0, 6, 24 and 144 h, although they did not exceed 10 ng ml^{-1} at any time. Male plasma contained significantly more E2 than females only at 24 h.

3.3 Plasma steroid concentrations: T

In fish exposed to T alone, male and female plasma T levels were statistically distinct from each other and are plotted separately. Both sexes however did display the same general trend of a rapid and pronounced increase in plasma T levels within 6 h of the start of the exposure (female: 25.1 ± 7.1 ng ml^{-1}; male: 17.8 ± 3.5 ng ml^{-1}), followed by a relatively rapid return to pre-exposure levels within 48h (Fig. 2a). A similar temporal pattern was observed in plasma T levels in fish exposed to a combination of E2 and T (Fig. 2b), although in this case maximum levels achieved were considerably higher, ranging from 1.1 ± 0.4 ng ml^{-1} at 0 h to 87.7 ± 18 ng ml^{-1} within 6 h of the start of the exposure. T levels in males and females exposed to T alone only differed significantly at 24 and 48 h. Mean plasma T concentrations in male and female fish exposed to E2 only were low, remaining below 4 ng ml^{-1} throughout the study (Fig. 2c).

4. Discussion

The concentrations of steroids in the exposure system changed markedly with time; an effect that was most pronounced in the case of T. Factors likely to have reduced the concentration of dissolved steroids in the static exposure system include the uptake of steroids by the experimental fish, biodegradation by water borne bacteria and adsorption to surfaces of the exposure vessel. These issues have been discussed elsewhere (Nimrod & Benson, 1998; Jurgens *et al.*, 2002; Kiparissis *et al.*, 2003). In this instance, the rate of disappearance of T in both single exposure (T only) and dual exposure (T + E2) tanks was much higher than the rate of disappearance of E2. The initial concentrations (at 6 h) of T achieved in both systems were similar to the concentrations of E2 measured in E2-dosed tanks at the same

time. However, substantial concentrations of E2 were detected in water throughout the course of the study, suggesting that the rapid loss of T from the tanks in which it was administered was a phenomenon specific to this steroid. Given the broadly similar physicochemical characteristics of the two steroids, and that no evidence to the contrary can be found, differences in the adsorption of T and E2 seem unlikely. It is therefore suspected that either differential metabolism by micro organisms, or differences in the uptake and metabolism of the two steroids by the fish themselves must account for the disparities. While previous reports suggest that differences in the rate of uptake of steroids by fish may exist (Piferrer & Donaldson, 1994), these data are not entirely consistent with the results of the present study. In terms of metabolism, the rapid reduction of plasma T levels compared to plasma E2 levels may in part be attributed to the aromatisation of androgens to estrogens (Borg *et al.*, 1987; Andersson *et al.*, 1988; Afonso *et al.*, 1999) but could equally reflect the reduced availability of T in the surrounding water.

Analysis of T and E2 levels in the blood of the exposed fish revealed that there was a rapid uptake of steroids from the water. The excellent gas transfer properties of fish gills facilitate the movement of chemicals from the water to the blood and the speed at which this occurs depends upon a number of factors such as respiration rate, lamellar recruitment, and the physicochemical properties of the compound (Randall *et al.*, 1996). Steroids are moderately lipophilic molecules (log K_{OW} of E2 and T are 4.01 and 3.32 respectively (Hansch *et al.*, 1995)) which have a high transfer capacity through the gill epithelium and it is therefore unsurprising that blood levels of T and E2 in the exposed sticklebacks were markedly elevated within 6 h of the start of the exposure period. However, the concentrations of steroid measured in the blood of the

exposed fish at this time far exceeded levels that might have been predicted on the basis of passive diffusion across the gill epithelium. Others authors have shown that for a system at equilibrium, expected plasma concentrations of a chemical can be calculated from the associated log K_{OW} value and concentration in the exposure water (Huggett *et al.*, 2003). The expected concentration ratio between the water and the blood at equilibrium, known as the blood water partition coefficient (P_{BW}) has been modelled for rainbow trout by the equation: log P_{BW} = 0.73 log K_{OW} – 0.88 (Fitzsimmons *et al.*, 2001), yielding P_{BW} values of 7.74 and 4.68 for E2 and T respectively. As stated, these values are based on a formula constructed from data on trout at steady state, and hence are not directly related to the dynamic uptake observed within the first 6h here. However, they do still significantly underestimate the actual blood concentrations observed.

For both steroids, mean concentrations in the plasma of exposed sticklebacks were within the range 20 – 100 ng ml^{-1} within 6 h. This contrasts significantly with the concentrations of each steroid in the water within which the fish were immersed, which were within the range 300 – 900 ng l^{-1} (= 0.3-0.9 ng ml^{-1}). When matched to their respective water concentration, steroid levels within the blood of the exposed sticklebacks were between 35 and 200-fold greater than those in the water within 6 h of the start of the study. Plasma E2 and T levels in all the control groups were very low throughout the study and all the fish were reproductively inactive, so we assume that the increase in plasma steroid levels can be attributed wholly to uptake from the water. In the case of the increase in plasma E2 during the T exposure (Fig. 1c), this is presumably attributable to partial aromatisation of the absorbed T. It is unclear why the plasma levels of E2 in fish from the combined E2 and T exposure tanks (Fig. 1b)

showed a disparity between the sexes, with male levels significantly higher than female. This may be due to sex-related differences in how the steroids are metabolised.

The exposure concentrations of E2 and T were selected to ensure measurable concentrations in the blood of the exposed fish with no foreknowledge of the outcome of the study and are therefore much higher than those that occur in rivers downstream of sewage treatment works discharges, eg. E2 < 50 ng l^{-1} (Desbrow *et al.*, 1998). However, the plasma levels of T achieved in this study were within the range observed in naturally breeding sticklebacks and therefore did not represent a supra-physiological challenge to the fish. Pall *et al*., (2005) have shown that circulating T levels reach up to 70 ng ml^{-1} at their peak in males during the sexual phase in the breeding season, declining to 8 ng ml^{-1} in the paternal phase. Borg *et al*., (1995) report that mature females have plasma T levels of up to 24 ng ml^{-1}, while the E2 plasma levels in these same fish was not detectable. Further information on plasma E2 levels in wild sticklebacks cannot be found for comparison.

Rapid uptake of steroids from the water by fish has been reported previously in studies where measurements have been made on whole-body concentrations. For example, whole-body levels of E2 were maximal within 30 mins of the onset of exposure in summer flounder (*Paralichthys dentatus*) exposed to water-borne E2 (Specker & Chandlee, 2003). In a more recent study, the plasma bioconcentration of tritiated E2 and T was monitored in tench (*Tinca tinca)*. After 6 - 7 hours, the ratio of radioactivity in plasma compared to the surrounding water was similar to those observed for actual steroid in the present study (Scott *et al.*, 2005). The phenomenon

has also been observed at this laboratory in chub (*Leuciscus cephalus*) exposed to E2 in a flow-through system (T. G. Pottinger & N. Rajapakse, unpublished data).

Given the fact that the difference in concentration of steroids across the gill epithelium cannot easily be explained by the hydrophobicity/lipophilicity of the chemicals, other contributing factors must be considered. It might be postulated that active transport of the steroids across the gill epithelium may also account for the maintenance of this concentration gradient but we are unaware of any reports that such a mechanism exists in the fish gill. It is more likely that the maintenance of this high concentration gradient in steroid levels across the gill epithelium is related to the presence in the blood of a sex hormone-binding globulin (SHBG). In seeking to explain similar observations of steroid uptake in *T. tinca*, Scott *et al.* (2005) demonstrated a clear relationship between the rate of uptake of specific steroids and their relative affinity for native tench SHBG. Although it is the case that a SHBG has not yet been identified in the three-spined stickleback, the presence of SHBGs in the blood of other teleost fish is well-documented (Pottinger, 1988; Pottinger & Pickering, 1990; Laidley & Thomas, 1997; Hobby *et al.*, 2000; Miguel-Queralt *et al.*, 2004) and it is therefore reasonable to assume that the stickleback also possesses a homologous steroid-binding protein. SHBGs are assumed to perform a transport/protective role for steroids (Rosner, 1990) and in fish are characterised by high affinity for both androgens and estrogens (Ovrevik *et al.*, 2001) and a moderately high binding capacity (Pottinger, 1988). It has been shown that >95% of circulating gonadal steroids in fish is protein bound (Freeman & Idler, 1971).

The functional implications of these findings may be of some significance to the interpretation of the mode of action of environmental estrogens and androgens and their mimics. These data suggest that steroidal estrogens and androgens dispersed in the aquatic environment, that are capable of binding to SHBGs, may be delivered to target tissues at a concentration far in excess of that to which the fish is exposed. It has been demonstrated that in addition to the native ligands, T and E2, certain endocrine active substances also bind to teleost SHBG. In rainbow trout plasma, ethynylestradiol, diethylstilbestrol, 4-hydroxytamoxifen, genistein, zearalenone, 4-t-octylphenol, bisphenol A and o,p'-DDT all compete for binding sites with E2, although with varying efficacy (Tollefsen, 2002), and the SHBG may therefore play a role in potentiating the activity of these estrogenic chemicals. It has also been shown that the binding properties of SHBG may be modulated by exposure in the field to constituents of endocrine active effluents (Pryce-Hobby *et al.*, 2003), a factor which may further disrupt normal endocrine function.

These findings confirm previous speculation concerning the mechanism underlying VTG induction in fish exposed to very low levels of an estrogen, eg significant VTG elevation in several species following exposure to estrone, E2, or 17a-ethinyl estradiol (Purdom *et al.*, 1994; Panter *et al.*, 1998; Rose *et al.*, 2002; Thorpe *et al.*, 2003). In summary the uptake of endocrine active substances from the aquatic environment by three-spined sticklebacks may be bioconcentrated in the blood, resulting in the delivery of higher than predicted levels of such chemicals to target tissues.

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491

492 **Tables**

493

494 Table I.

495 The concentration of 17ß-estradiol (E2; ng l^{-1}) in water samples collected from the

496 exposure tanks at intervals during a 6 day static exposure.

497

Table I

		Concentration of E2 (ng l^{-1}) in each treatment group		
Time (h)	Tank (M/F)	E2	E2+T	Control
	Male	514.7	737.6	0.6
6	Female	682.4	849.4	0.2
	Male	428.6	400.3	2.5
72	Female	445.8	496.7	8.4
	Male	189.3	139.2	1.9
144	Female	339.5	25.7	0.0

498

499 Table II.

500 The concentration of testosterone (T; ng l^{-1}) in water samples collected from the

501 exposure tanks at intervals during a 6 day static exposure.

Table II

		Concentration of T (ng l^{-1}) in each treatment group		
Time (h)	Tank (M/F)	T	E2+T	Control
	Male	460.2	452.1	5.0
6	Female	331.7	542.9	4.8
	Male	0.1	2.2	3.8
72	Female	0.0	0.0	4.8
	Male	2.8	0.0	3.5
144	Female	2.0	3.3	1.8

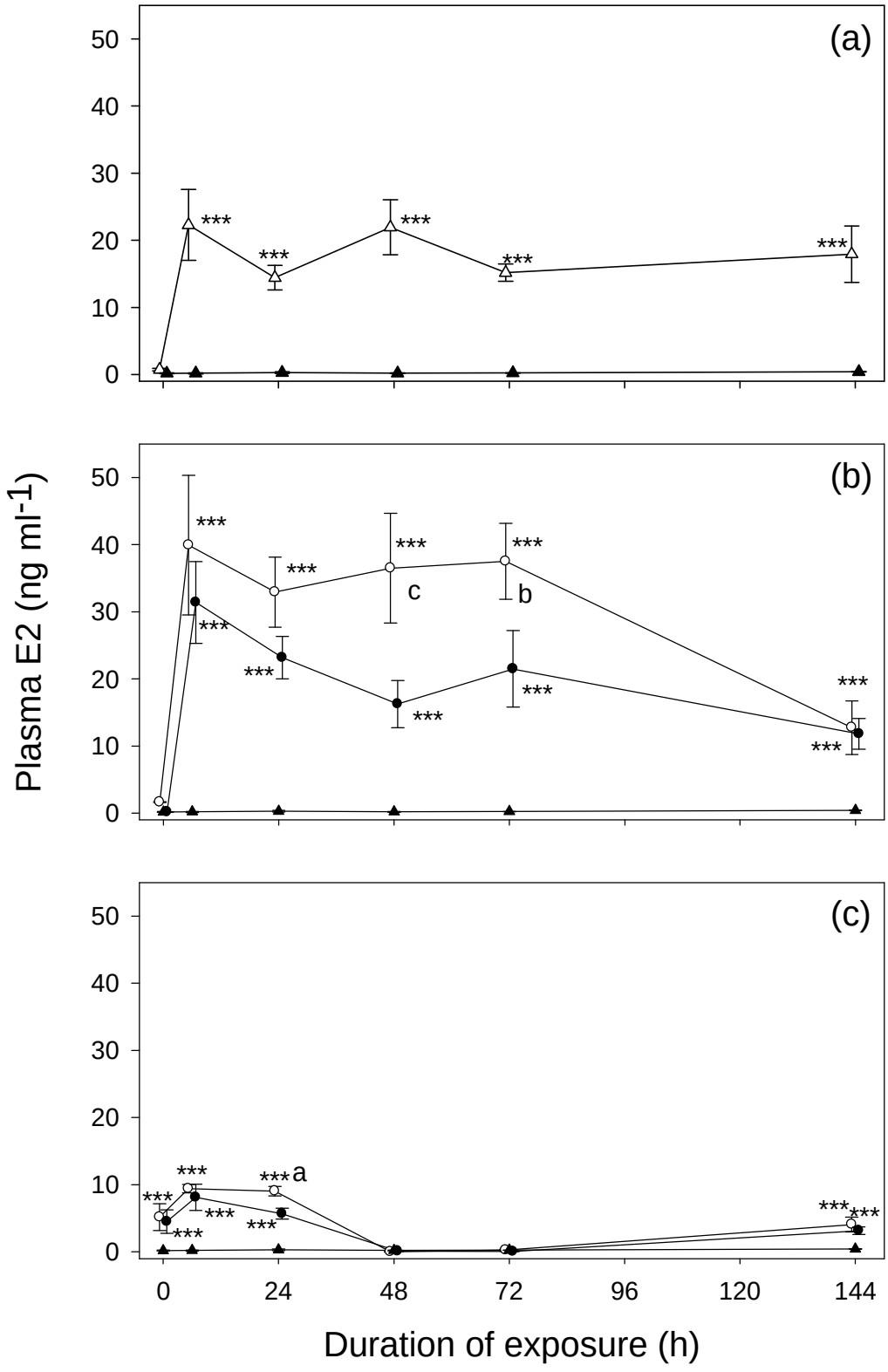
Figure legends

Figure 1.

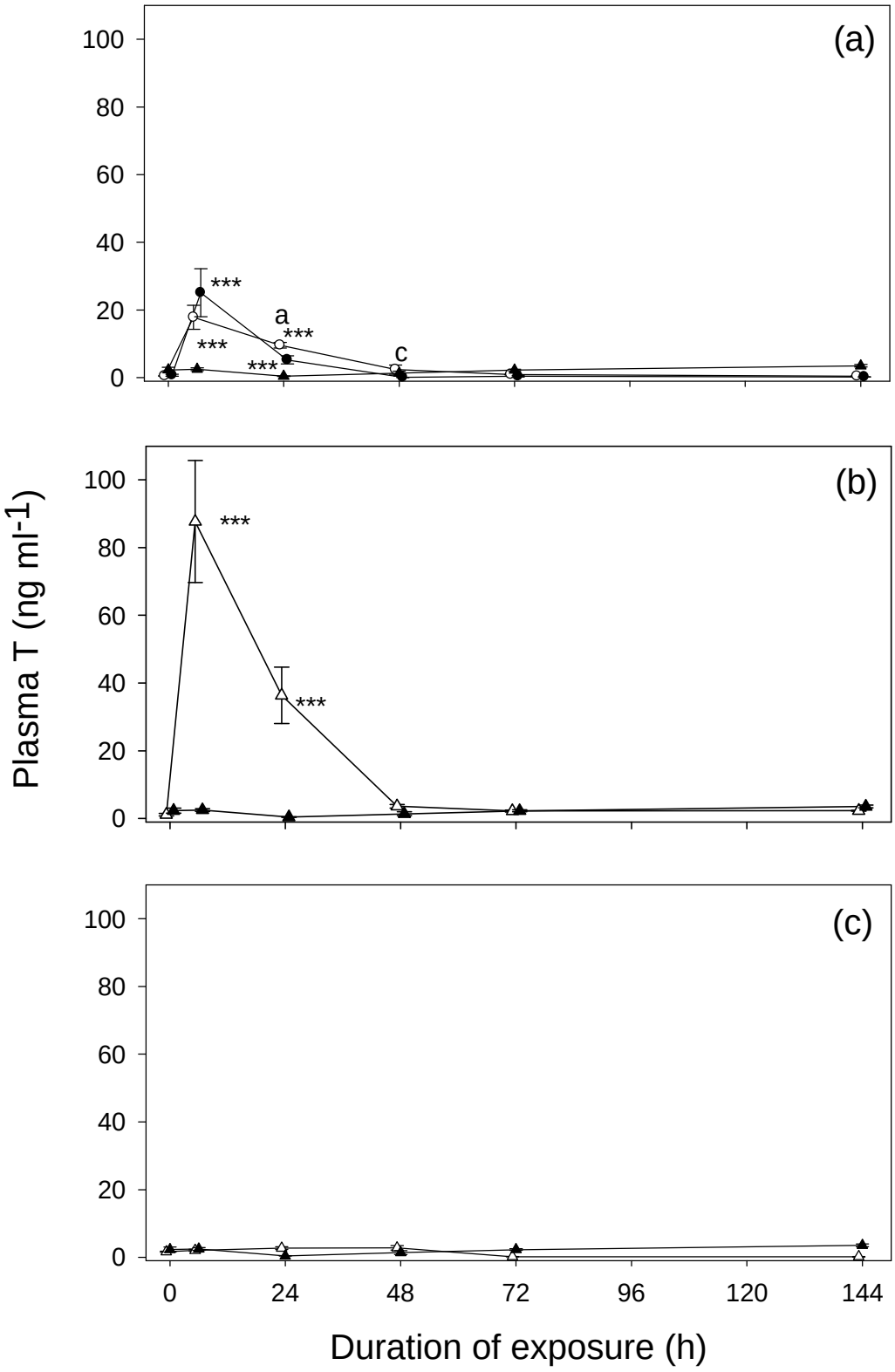
Plasma E2 levels in sticklebacks sampled at intervals during a 6 day static exposure to (a) E2 at a nominal concentration of 1000 ng l^{-1}, (b) E2 and T combined at nominal concentrations of 1000 ng l^{-1}, and (c) T at a nominal concentration of 1000 ng l^{-1}. Symbols denote: ? (solid triangle) combined male and female controls; ? (open triangle) combined male and female steroid-exposed; ? (open circle) male steroid-exposed; ? (solid circle) female steroid-exposed. Each point represents the mean ± SEM (sexes combined, *n*=6; single sex, *n*=3; error bars are obscured by symbols for control values). Significant differences between control and exposed fish are denoted by *** *P*<0.001. Significant differences between exposed male and female fish are denoted by letters: a, *P*<0.05; b, *P*<0.01; c, *P*<0.001.

Figure 2.

Plasma T levels in sticklebacks sampled at intervals during a 6 day static exposure to (a) T at a nominal concentration of 1000 ng l^{-1}, (b) E2 and T together at nominal concentrations of 1000 ng l^{-1}, (c) E2 at a nominal concentration of 1000 ng l^{-1}. Symbols denote: ? (solid triangle) combined male and female controls; ? (open triangle) combined male and female exposed; ? (open circle) male exposed; ? (solid circle) female exposed. Each point represents the mean ± SEM (sexes combined, *n*=6; single sex, *n*=3; error bars are obscured by symbols for control values). Significant differences between control and exposed fish are denoted by *** *P*<0.001. Significant differences between exposed male and female fish are denoted by letter: a, *P*<0.05.



Maunder, Matthiessen, Sumpter & Pottinger Fig. 1



Maunder, Matthiessen, Sumpter & Pottinger Fig. 2