

1

2 **Rapid bioconcentration of steroids in the plasma of**

3 **sticklebacks (*Gasterosteus aculeatus*) exposed to water-**

4 **borne testosterone and 17 β -estradiol**

5

6

7 R. J. Maunder^{a, *}, P. Matthiessen^a, J. P. Sumpter^b and T. G.

8 Pottinger^a

9

10

11 ^a*NERC Centre for Ecology and Hydrology, Lancaster Environment Centre,*

12 *Library Avenue, Bailrigg, Lancaster LA1 4AP, U.K.*

13 ^b*Institute for the Environment, Brunel University, Uxbridge Middlesex, UB8*

14 *3PH, U.K.*

15

16

17

18

19

20

21 *Corresponding author. Tel.: +44 1524 595800; fax: +44 1524 61536.

22 *E-mail address: rjmaun@ceh.ac.uk (R. J. Maunder)*

23 **Abstract**

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

41

42

43 *Keywords:* 17β-estradiol; testosterone; stickleback; plasma; uptake;
44 bioconcentration

45 **1. Introduction**

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

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

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

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

79 A static exposure system utilising the three-spined stickleback (*Gasterosteus*
80 *aculeatus*) was adopted. The biology of the stickleback is well described (Wootton,
81 1976; Wootton, 1984; Bell & Foster, 1994) and this species is widely used in
82 behavioural, ecological, physiological and toxicological studies. In the context of
83 endocrine disruption, the stickleback represents a test species with three distinct
84 endpoints of relevance: a range of endocrine-dependent behaviours (Bell, 2001), the
85 estrogen sensitive biomarker vitellogenin (VTG; (Katsiadaki *et al.*, 2002b; Hahlbeck
86 *et al.*, 2004), and a unique androgen sensitive endpoint, the nest building glue,
87 spiggin (Jones *et al.*, 2001; Katsiadaki *et al.*, 2002a). Use of the stickleback in studies
88 of endocrine active substances is increasingly widespread. In this study, the fish
89 were exposed to two natural steroids, testosterone and 17 β -estradiol, both singly and
90 in combination, and concentrations of these steroids were determined in the water,
91 and blood plasma.

92

93

94

95 **2. Materials and methods**

96

97 *2.1 Experimental fish*

98 Sticklebacks (*Gasterosteus aculeatus* L.) were obtained from a commercial
99 supplier (Moore and Moore, Reading) and held in groups of 200 fish in each of four
100 1000 litre circular outdoor tanks supplied with a constant flow of Windermere lake
101 water (10 l min⁻¹) and natural photoperiod and temperature range (4°C – 17°C
102 annually). During the summer of 2002 the fish were sexed by external inspection and
103 then held in single sex groups until the time of study, during December 2002. At this
104 time, water temperature was 11°C, pH was within the range 6.5 to 7.2, hardness 110
105 - 140 mg l⁻¹ CaCO₃, and dissolved O₂ 91-98% saturation. The fish were fed three
106 times weekly on commercial trout feed (Skretting fry crumb 02). At the time of the
107 study the average size of the fish was (mean ± SEM) 3.41 ± 0.05 g, *n* = 144. There
108 was no significant difference in mass between the male and female groups.

109

110 *2.2 Exposure system*

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

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

124

125 *2.3 Dosing*

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

135

136 *2.4 Sampling procedures*

137 Groups of six fish (three males and three females) were sampled at 0, 6, 24,
138 48, 72, & 144 hours from commencement of exposure. The fish were caught in a dip
139 net and rapidly transferred to anaesthetic (2-phenoxyethanol; 1:2000) before being
140 killed by cranial compression using forceps. Blood samples were obtained by
141 severing the caudal peduncle and collecting blood in a heparinised capillary tube.
142 The blood sample was then transferred to a capped 1.5 ml centrifuge tube and held
143 on ice before being centrifuged. Plasma was transferred to a second, individually pre-
144 weighed 1.5 ml tube. The tube and plasma were then weighed together to calculate

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

149

150 *2.5 Extraction of water samples*

151 The water samples, ranging from 497-677ml in volume were thawed overnight
152 at 4°C , filtered under vacuum (Whatman GF/C microfibre filter) and then pumped
153 through a methanol (Analar) primed Sep-Pak C18 (Waters Ltd) solid phase extraction
154 cartridge at $5\ \text{ml min}^{-1}$. Steroids retained on the cartridge were eluted with 5 ml
155 methanol. The methanol eluate was dried under nitrogen at 30°C in a heating block
156 and the sample was redissolved in 1.0 ml of ethyl acetate (Analar), which was stored
157 at -20°C until required for assay. This method consistently provided recoveries of
158 $\approx 85\%$ (86.5 ± 0.63 , $n=8$) when run with radiolabelled steroids and estimates of
159 dissolved steroid concentrations in the exposure tanks are corrected for this level of
160 loss.

161

162 *2.6 Steroid assays*

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

169

170 2.7 Statistical analysis

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

180

181 3. Results

182 3.1 Concentrations of steroid in the exposure water

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

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

196

197 *3.2 Plasma steroid concentrations: E2*

198 Plasma E2 levels are presented in Fig. 1. There was no significant difference
199 between E2 levels in male and female controls (consistently $<0.5 \text{ ng ml}^{-1}$), so these
200 were combined. In fish from both treatment groups (E2 and E2 + T), plasma E2
201 concentrations were significantly greater than those in the control fish ($P<0.001$)
202 throughout. There was no significant difference in the plasma E2 levels between
203 males and females exposed to E2 alone and these data were therefore also
204 combined (Fig. 1a). There was a rapid increase in plasma E2 levels in these fish
205 within 6 h of the start of the exposure period, with mean plasma E2 levels rising from
206 $0.8 \pm 0.2 \text{ ng ml}^{-1}$ at 0 h to $22.3 \pm 5.3 \text{ ng ml}^{-1}$. Plasma levels of E2 were maintained at
207 or around this level throughout the study, remaining significantly elevated at $17.9 \pm$
208 4.2 ng ml^{-1} at 144 h. Plasma levels of E2 in fish from the combined E2 and T
209 exposure tanks showed a similar pattern (Fig. 1b). However, in this case, there was a
210 difference in plasma E2 levels between the sexes, with levels in male fish being
211 significantly greater overall than levels in female fish. Plasma E2 levels in both sexes
212 rose rapidly after the start of the exposure period to reach maximum levels at 6 h
213 (males: $39.9 \pm 10 \text{ ng ml}^{-1}$; females: $31.4 \pm 6 \text{ ng ml}^{-1}$), after which there was a decline
214 to approximately 12 ng ml^{-1} at the end of the study. Mean plasma E2 levels in fish
215 exposed to T alone were significantly ($P<0.001$) higher than control levels (Fig. 1c) at
216 0, 6, 24 and 144 h, although they did not exceed 10 ng ml^{-1} at any time. Male plasma
217 contained significantly more E2 than females only at 24 h.

218

219 3.3 Plasma steroid concentrations: T

220 In fish exposed to T alone, male and female plasma T levels were statistically
221 distinct from each other and are plotted separately. Both sexes however did display
222 the same general trend of a rapid and pronounced increase in plasma T levels within
223 6 h of the start of the exposure (female: 25.1 ± 7.1 ng ml⁻¹; male: 17.8 ± 3.5 ng ml⁻¹),
224 followed by a relatively rapid return to pre-exposure levels within 48h (Fig. 2a). A
225 similar temporal pattern was observed in plasma T levels in fish exposed to a
226 combination of E2 and T (Fig. 2b), although in this case maximum levels achieved
227 were considerably higher, ranging from 1.1 ± 0.4 ng ml⁻¹ at 0 h to 87.7 ± 18 ng ml⁻¹
228 within 6 h of the start of the exposure. T levels in males and females exposed to T
229 alone only differed significantly at 24 and 48 h. Mean plasma T concentrations in
230 male and female fish exposed to E2 only were low, remaining below 4 ng ml⁻¹
231 throughout the study (Fig. 2c).

232

233 4. Discussion

234 The concentrations of steroids in the exposure system changed markedly with time;
235 an effect that was most pronounced in the case of T. Factors likely to have reduced
236 the concentration of dissolved steroids in the static exposure system include the
237 uptake of steroids by the experimental fish, biodegradation by water borne bacteria
238 and adsorption to surfaces of the exposure vessel. These issues have been
239 discussed elsewhere (Nimrod & Benson, 1998; Jurgens *et al.*, 2002; Kiparissis *et al.*,
240 2003). In this instance, the rate of disappearance of T in both single exposure (T
241 only) and dual exposure (T + E2) tanks was much higher than the rate of
242 disappearance of E2. The initial concentrations (at 6 h) of T achieved in both systems
243 were similar to the concentrations of E2 measured in E2-dosed tanks at the same

244 time. However, substantial concentrations of E2 were detected in water throughout
245 the course of the study, suggesting that the rapid loss of T from the tanks in which it
246 was administered was a phenomenon specific to this steroid. Given the broadly
247 similar physicochemical characteristics of the two steroids, and that no evidence to
248 the contrary can be found, differences in the adsorption of T and E2 seem unlikely. It
249 is therefore suspected that either differential metabolism by micro organisms, or
250 differences in the uptake and metabolism of the two steroids by the fish themselves
251 must account for the disparities. While previous reports suggest that differences in
252 the rate of uptake of steroids by fish may exist (Piferrer & Donaldson, 1994), these
253 data are not entirely consistent with the results of the present study. In terms of
254 metabolism, the rapid reduction of plasma T levels compared to plasma E2 levels
255 may in part be attributed to the aromatisation of androgens to estrogens (Borg *et al.*,
256 1987; Andersson *et al.*, 1988; Afonso *et al.*, 1999) but could equally reflect the
257 reduced availability of T in the surrounding water.

258

259 Analysis of T and E2 levels in the blood of the exposed fish revealed that there was a
260 rapid uptake of steroids from the water. The excellent gas transfer properties of fish
261 gills facilitate the movement of chemicals from the water to the blood and the speed
262 at which this occurs depends upon a number of factors such as respiration rate,
263 lamellar recruitment, and the physicochemical properties of the compound (Randall
264 *et al.*, 1996). Steroids are moderately lipophilic molecules ($\log K_{OW}$ of E2 and T are
265 4.01 and 3.32 respectively (Hansch *et al.*, 1995)) which have a high transfer capacity
266 through the gill epithelium and it is therefore unsurprising that blood levels of T and
267 E2 in the exposed sticklebacks were markedly elevated within 6 h of the start of the
268 exposure period. However, the concentrations of steroid measured in the blood of the

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

281
282 For both steroids, mean concentrations in the plasma of exposed sticklebacks were
283 within the range 20 – 100 ng ml⁻¹ within 6 h. This contrasts significantly with the
284 concentrations of each steroid in the water within which the fish were immersed,
285 which were within the range 300 – 900 ng l⁻¹ (= 0.3-0.9 ng ml⁻¹). When matched to
286 their respective water concentration, steroid levels within the blood of the exposed
287 sticklebacks were between 35 and 200-fold greater than those in the water within 6 h
288 of the start of the study. Plasma E2 and T levels in all the control groups were very
289 low throughout the study and all the fish were reproductively inactive, so we assume
290 that the increase in plasma steroid levels can be attributed wholly to uptake from the
291 water. In the case of the increase in plasma E2 during the T exposure (Fig. 1c), this
292 is presumably attributable to partial aromatisation of the absorbed T. It is unclear why
293 the plasma levels of E2 in fish from the combined E2 and T exposure tanks (Fig. 1b)

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

297
298 The exposure concentrations of E2 and T were selected to ensure measurable
299 concentrations in the blood of the exposed fish with no foreknowledge of the outcome
300 of the study and are therefore much higher than those that occur in rivers
301 downstream of sewage treatment works discharges, eg. $E2 < 50 \text{ ng l}^{-1}$ (Desbrow *et al.*, 1998). However, the plasma levels of T achieved in this study were within the
302 range observed in naturally breeding sticklebacks and therefore did not represent a
303 supra-physiological challenge to the fish. Pall *et al.*, (2005) have shown that
304 circulating T levels reach up to 70 ng ml^{-1} at their peak in males during the sexual
305 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
306 the E2 plasma levels in these same fish was not detectable. Further information on
307 plasma E2 levels in wild sticklebacks cannot be found for comparison.

310
311 Rapid uptake of steroids from the water by fish has been reported previously in
312 studies where measurements have been made on whole-body concentrations. For
313 example, whole-body levels of E2 were maximal within 30 mins of the onset of
314 exposure in summer flounder (*Paralichthys dentatus*) exposed to water-borne E2
315 (Specker & Chandlee, 2003). In a more recent study, the plasma bioconcentration of
316 tritiated E2 and T was monitored in tench (*Tinca tinca*). After 6 - 7 hours, the ratio of
317 radioactivity in plasma compared to the surrounding water was similar to those
318 observed for actual steroid in the present study (Scott *et al.*, 2005). The phenomenon

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

321
322 Given the fact that the difference in concentration of steroids across the gill
323 epithelium cannot easily be explained by the hydrophobicity/lipophilicity of the
324 chemicals, other contributing factors must be considered. It might be postulated that
325 active transport of the steroids across the gill epithelium may also account for the
326 maintenance of this concentration gradient but we are unaware of any reports that
327 such a mechanism exists in the fish gill. It is more likely that the maintenance of this
328 high concentration gradient in steroid levels across the gill epithelium is related to the
329 presence in the blood of a sex hormone-binding globulin (SHBG). In seeking to
330 explain similar observations of steroid uptake in *T. tinca*, Scott *et al.* (2005)
331 demonstrated a clear relationship between the rate of uptake of specific steroids and
332 their relative affinity for native tench SHBG. Although it is the case that a SHBG has
333 not yet been identified in the three-spined stickleback, the presence of SHBGs in the
334 blood of other teleost fish is well-documented (Pottinger, 1988; Pottinger & Pickering,
335 1990; Laidley & Thomas, 1997; Hobby *et al.*, 2000; Miguel-Queralt *et al.*, 2004) and it
336 is therefore reasonable to assume that the stickleback also possesses a homologous
337 steroid-binding protein. SHBGs are assumed to perform a transport/protective role for
338 steroids (Rosner, 1990) and in fish are characterised by high affinity for both
339 androgens and estrogens (Ovrevik *et al.*, 2001) and a moderately high binding
340 capacity (Pottinger, 1988). It has been shown that >95% of circulating gonadal
341 steroids in fish is protein bound (Freeman & Idler, 1971).

342

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

357

358 These findings confirm previous speculation concerning the mechanism underlying
359 VTG induction in fish exposed to very low levels of an estrogen, eg significant VTG
360 elevation in several species following exposure to estrone, E2, or 17 α -ethinyl
361 estradiol (Purdom *et al.*, 1994; Panter *et al.*, 1998; Rose *et al.*, 2002; Thorpe *et al.*,
362 2003). In summary the uptake of endocrine active substances from the aquatic
363 environment by three-spined sticklebacks may be bioconcentrated in the blood,
364 resulting in the delivery of higher than predicted levels of such chemicals to target
365 tissues.

366

367

368 **Acknowledgements**

369 R.J.M was supported by a CEH NERC studentship.

370

371 **References**

- 372 Afonso, L. O. B., Iwama, G. K., Smith, J. & Donaldson, E. M. (1999). Effects of the aromatase inhibitor
373 Fadrozole on plasma sex steroid secretion and oocyte maturation in female coho salmon (*Oncorhynchus kisutch*)
374 during vitellogenesis. *Fish Physiology and Biochemistry* **20**, 231-241.
- 375 Andersson, E., Borg, B. & Lambert, J. G. D. (1988). Aromatase activity in brain and pituitary of immature and
376 mature Atlantic salmon (*Salmo salar* L.) parr. *General and Comparative Endocrinology* **72**, 394-401.
- 377 Bell, A. M. (2001). Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined
378 stickleback, *Gasterosteus aculeatus*. *Animal Behaviour* **62**, 775-780.
- 379 Bell, M. A. & Foster, S. A. (1994). *The Evolutionary Biology of the Threespine Stickleback*. Oxford: Oxford
380 University Press.
- 381 Borg, B., Timmers, R. J. M. & Lambert, J. G. D. (1987). Aromatase activity in the brain of the three-spined
382 stickleback, *Gasterosteus aculeatus* . 1. Distribution and effects of season and photoperiod. *Exp. Biol.* **47**, 63-68.
- 383 Borg, B. & Mayer, I. (1995). Androgens and behaviour in the three-spined stickleback. *Behaviour* **132**, 1025-
384 1035.
- 385 Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P. & Waldock, M. (1998). Identification of estrogenic
386 chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environmental Science
387 & Technology* **32**, 1549-1558.
- 388 Fitzsimmons, P. N., Fernandez, J. D., Hoffman, A. D., Butterworth, B. C. & Nichols, J. W. (2001). Branchial
389 elimination of superhydrophobic organic compounds by rainbow trout (*Oncorhynchus mykiss*). *Aquatic
390 Toxicology* **55**, 23-34.
- 391 Freeman, H. & Idler, D. (1971). Binding affinities of blood proteins for sex hormones and corticosteroids in fish.
392 *Steroids* **17**, 233-250.
- 393 Hahlbeck, E., Katsiadaki, L., Mayer, I., Adolfsson-Erici, M., James, J. & Bengtsson, B. E. (2004). The juvenile
394 three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption - II - kidney
395 hypertrophy, vitellogenin and spiggin induction. *Aquatic Toxicology* **70**, 311-326.
- 396 Hansch, C., Leo, A. & Hoekman, D. (1995). *Exploring QSAR - Hydrophobic, Electronic and Steric Constants*.
397 Washington DC: American Chemical Society.
- 398 Harris, C. A., Henttu, P., Parker, M. G. & Sumpter, J. P. (1997). The estrogenic activity of phthalate esters in
399 vitro. *Environmental Health Perspectives* **105**, 802-811.
- 400 Hobby, A. C., Pankhurst, N. W. & Geraghty, D. P. (2000). A comparison of sex steroid binding protein (SBP) in
401 four species of teleost fish. *Fish Physiology and Biochemistry* **23**, 245-256.
- 402 Huggett, D. B., Cook, J. C., Ericson, J. F. & Williams, R. T. (2003). A theoretical model for utilizing
403 mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish.
404 *Human and Ecological Risk Assessment* **9**, 1789-1799.
- 405 Jobling, S., Nolan, M., Tyler, C. R., Brighty, G. & Sumpter, J. P. (1998). Widespread sexual disruption in wild
406 fish. *Environmental Science & Technology* **32**, 2498-2506.
- 407 Jones, I., Lindberg, C., Jakobsson, S., Hellqvist, A., Hellman, U., Borg, B. & Olsson, P. E. (2001). Molecular
408 cloning and characterization of spiggin - An androgen-regulated extraorganismal adhesive with structural
409 similarities to von Willebrand Factor-related proteins. *Journal of Biological Chemistry* **276**, 17857-17863.
- 410 Jurgens, M. D., Holthaus, K. I. E., Johnson, A. C., Smith, J. J. L., Hetheridge, M. & Williams, R. J. (2002). The
411 potential for estradiol and ethinylestradiol degradation in English rivers. *Environmental Toxicology and
412 Chemistry* **21**, 480-488.
- 413 Katsiadaki, I., Scott, A. P., Hurst, M. R., Matthiessen, P. & Mayer, I. (2002a). Detection of environmental
414 androgens: A novel method based on enzyme-linked immunosorbent assay of spiggin, the stickleback
415 (*Gasterosteus aculeatus*) glue protein. *Environmental Toxicology and Chemistry* **21**, 1946-1954.
- 416 Katsiadaki, I., Scott, A. P. & Mayer, I. (2002b). The potential of the three-spined stickleback (*Gasterosteus
417 aculeatus* L.) as a combined biomarker for oestrogens and androgens in European waters. *Marine Environmental
418 Research* **54**, 725-728.

- 419 Kiparissis, Y., Akhtar, P., Hodson, P. V. & Brown, R. S. (2003). Partition-controlled delivery of toxicants: A
 420 novel in vivo approach for embryo toxicity testing. *Environmental Science & Technology* **37**, 2262-2266.
- 421 Kirby, M. F., Allen, Y. T., Dyer, R. A., Feist, S. W., Katsiadaki, I., Matthiessen, P., Scott, A. P., Smith, A.,
 422 Stentiford, G. D., Thain, J. E., Thomas, K. V., Tolhurst, L. & Waldock, M. J. (2004). Surveys of plasma
 423 vitellogenin and intersex in male flounder (*Platichthys flesus*) as measures of endocrine disruption by estrogenic
 424 contamination in United Kingdom estuaries: Temporal trends, 1996 to 2001. *Environmental Toxicology and*
 425 *Chemistry* **23**, 748-758.
- 426 Laidley, C. W. & Thomas, P. (1997). Changes in plasma sex steroid-binding protein levels associated with
 427 ovarian recrudescence in the spotted seatrout (*Cynoscion nebulosus*). *Biology of Reproduction* **56**, 931-937.
- 428 Legler, J., Dennekamp, M., Vethaak, A. D., Brouwer, A., Koeman, J. H., van der Burg, B. & Murk, A. J. (2002).
 429 Detection of estrogenic activity in sediment-associated compounds using in vitro reporter gene assays. *Science of*
 430 *the Total Environment* **293**, 69-83.
- 431 Miguel-Queralt, S., Knowlton, M., Avvakumov, G. V., Al-Nuono, R., Kelly, G. M. & Hammond, G. L. (2004).
 432 Molecular and functional characterization of sex hormone binding globulin in zebrafish. *Endocrinology* **145**,
 433 5221-5230.
- 434 Nimrod, A. C. & Benson, W. H. (1998). Reproduction and development of Japanese medaka following an early
 435 life stage exposure to xenoestrogens. *Aquatic Toxicology* **44**, 141-156.
- 436 Ovrevik, J., Stenersen, J., Nilssen, K. & Tollefsen, K. E. (2001). Partial characterization of a sex steroid-binding
 437 protein in plasma from Arctic charr (*Salvelinus alpinus* L.). *General and Comparative Endocrinology* **122**, 31-
 438 39.
- 439 Pall, M. K., Hellqvist, A., Schmitz, M., Olsson, P.-E., Mayer, I. & Borg, B. (2005). Changes in reproductive
 440 physiology and behaviour over the nesting cycle in male three spined sticklebacks. *Journal of Fish Biology* **66**,
 441 1400-1410.
- 442 Panter, G. H., Thompson, R. S. & Sumpter, J. P. (1998). Adverse reproductive effects in male fathead minnows
 443 (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol
 444 and oestrone. *Aquatic Toxicology* **42**, 243-253.
- 445 Piferrer, F. & Donaldson, E. M. (1994). Uptake and Clearance of Exogenous Estradiol-17-Beta and Testosterone
 446 During the Early Development of Coho Salmon (*Oncorhynchus kisutch*), Including Eggs, Alevins and Fry. *Fish*
 447 *Physiology and Biochemistry* **13**, 219-232.
- 448 Pottinger, T. G. & Pickering, A. D. (1985). The Effects of 11-Ketotestosterone and Testosterone on the Skin
 449 Structure of Brown Trout, *Salmo-trutta-L*. *General and Comparative Endocrinology* **59**, 335-342.
- 450 Pottinger, T. G. (1988). Seasonal-Variation in Specific Plasma-Tissue and Target-Tissue Binding of Androgens,
 451 Relative to Plasma Steroid-Levels, in the Brown Trout, *Salmo-trutta-L*. *General and Comparative*
 452 *Endocrinology* **70**, 334-344.
- 453 Pottinger, T. G. & Pickering, A. D. (1990). The Effect of Cortisol Administration on Hepatic and Plasma
 454 Estradiol-Binding Capacity in Immature Female Rainbow-Trout (*Oncorhynchus-mykiss*). *General and*
 455 *Comparative Endocrinology* **80**, 264-273.
- 456 Pryce-Hobby, A. C., McMaster, M. E., Hewitt, L. M. & Van der Kraak, G. (2003). The effects of pulp mill
 457 effluent on the sex steroid binding protein in white sucker (*Catostomus commersoni*) and longnose sucker (*C.*
 458 *catostomus*). *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* **134**, 241-250.
- 459 Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R. & Sumpter, J. P. (1994). Estrogenic effects
 460 of effluents from sewage treatment works. *Chemistry and Ecology* **8**, 275-285.
- 461 Randall, D. J., Brauner, C. J., Thurston, R. V. & Neuman, J. F. (1996). Water chemistry at the gill surfaces of
 462 fish and uptake of xenobiotics. In *Toxicology of Aquatic Pollution* (Taylor, E. W., ed. pp. 1-16. Cambridge:
 463 Cambridge University Press.
- 464 Rose, J., Holbech, H., Lindholst, C., Norum, U., Povlsen, A., Korsgaard, B. & Bjerregaard, P. (2002).
 465 Vitellogenin induction by 17 beta-estradiol and 17 alpha- ethinylestradiol in male zebrafish (*Danio rerio*).
 466 *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* **131**, 531-539.
- 467 Rosner, W. (1990). The Functions of Corticosteroid-Binding Globulin and Sex Hormone-Binding Globulin -
 468 Recent Advances. *Endocrine Reviews* **11**, 80-91.
- 469 Routledge, E. J. & Sumpter, J. P. (1996). Estrogenic activity of surfactants and some of their degradation
 470 products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry* **15**, 241-248.
- 471 Scott, A. P., Pinillos, M. L. & Huertas, M. (2005). The rate of uptake of sex steroids from water by *Tinca tinca* is
 472 influenced by their affinity for sex steroid binding protein in plasma. *Journal of Fish Biology* **67**, 182-200.
- 473 Specker, J. L. & Chandlee, M. K. (2003). Methodology for estradiol treatment in marine larval and juvenile fish:
 474 uptake and clearance in summer flounder. *Aquaculture* **217**, 663-672.
- 475 Thomas, K. V., Hurst, M. R., Matthiessen, P., McHugh, M., Smith, A. & Waldock, M. J. (2002). An assessment
 476 of in vitro androgenic activity and the identification of environmental androgens in United Kingdom estuaries.
 477 *Environmental Toxicology and Chemistry* **21**, 1456-1461.

- 478 Thorpe, K. L., Cummings, R. I., Hutchinson, T. H., Scholze, M., Brighty, G., Sumpter, J. P. & Tyler, C. R.
479 (2003). Relative potencies and combination effects of steroidal estrogens in fish. *Environmental Science &*
480 *Technology* **37**, 1142-1149.
- 481 Tollefsen, K. E. (2002). Interaction of estrogen mimics, singly and in combination, with plasma sex steroid-
482 binding proteins in rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* **56**, 215-225.
- 483 van Aerle, R., Nolan, M., Jobling, S., Christiansen, L. B., Sumpter, J. P. & Tyler, C. R. (2001). Sexual disruption
484 in a second species of wild cyprinid fish (the Gudgeon, *gobio gobio*) in United Kingdom freshwaters.
485 *Environmental Toxicology and Chemistry* **20**, 2841-2847.
- 486 Williams, R. J., Johnson, A. C., Smith, J. J. L. & Kanda, R. (2003). Steroid estrogens profiles along river
487 stretches arising from sewage treatment works discharges. *Environmental Science & Technology* **37**, 1744-1750.
- 488 Wootton, R. J. (1976). *The Biology of the Sticklebacks*. London: Academic Press.
- 489 Wootton, R. J. (1984). *A functional biology of Sticklebacks*. London: Croom Helm.
- 490
- 491

492 **Tables**

493

494 Table I.

495 The concentration of 17 β -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
6	Male	514.7	737.6	0.6
	Female	682.4	849.4	0.2
72	Male	428.6	400.3	2.5
	Female	445.8	496.7	8.4
144	Male	189.3	139.2	1.9
	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
6	Male	460.2	452.1	5.0
	Female	331.7	542.9	4.8
72	Male	0.1	2.2	3.8
	Female	0.0	0.0	4.8
144	Male	2.8	0.0	3.5
	Female	2.0	3.3	1.8

502 **Figure legends**

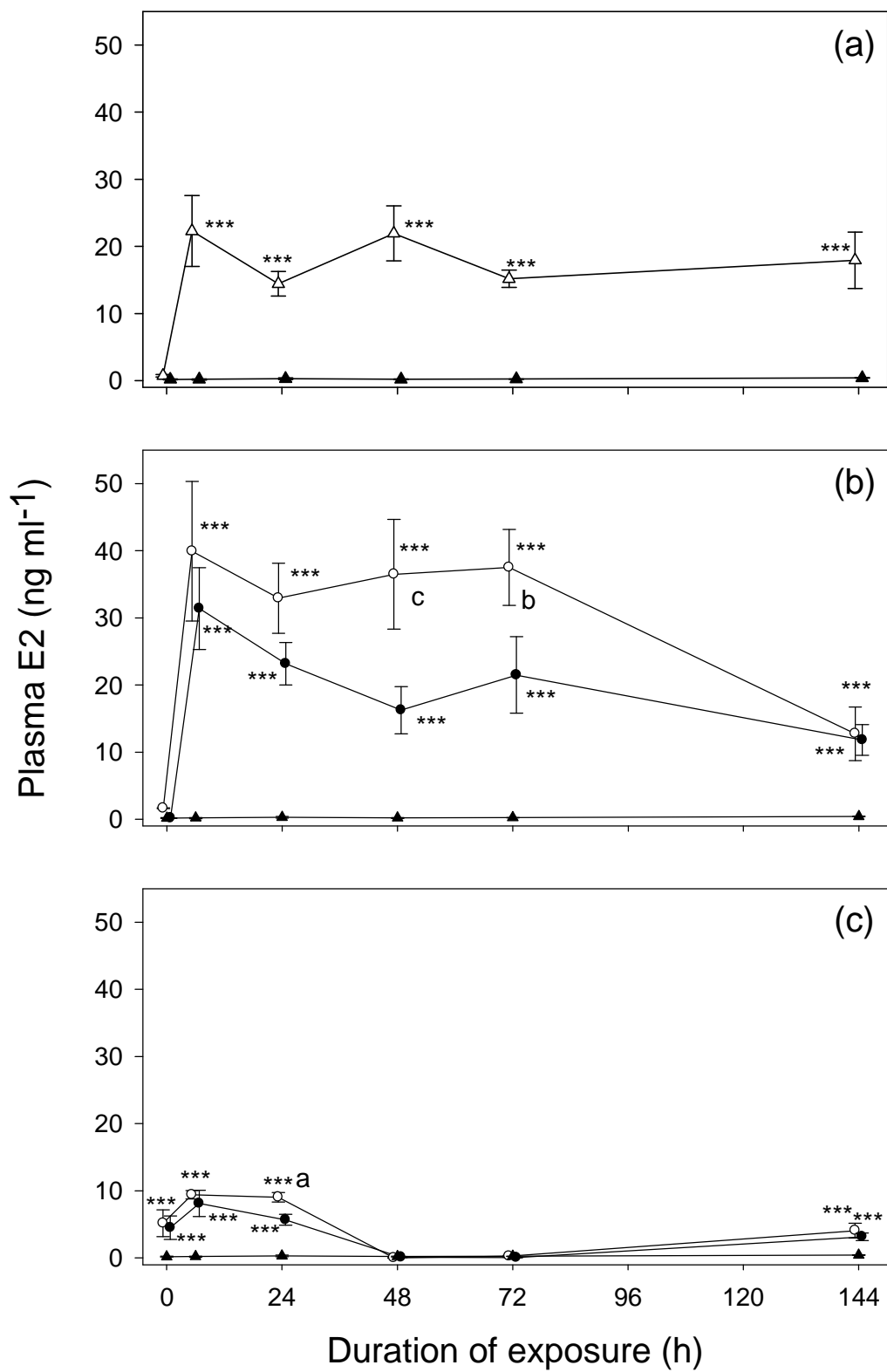
503 Figure 1.

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

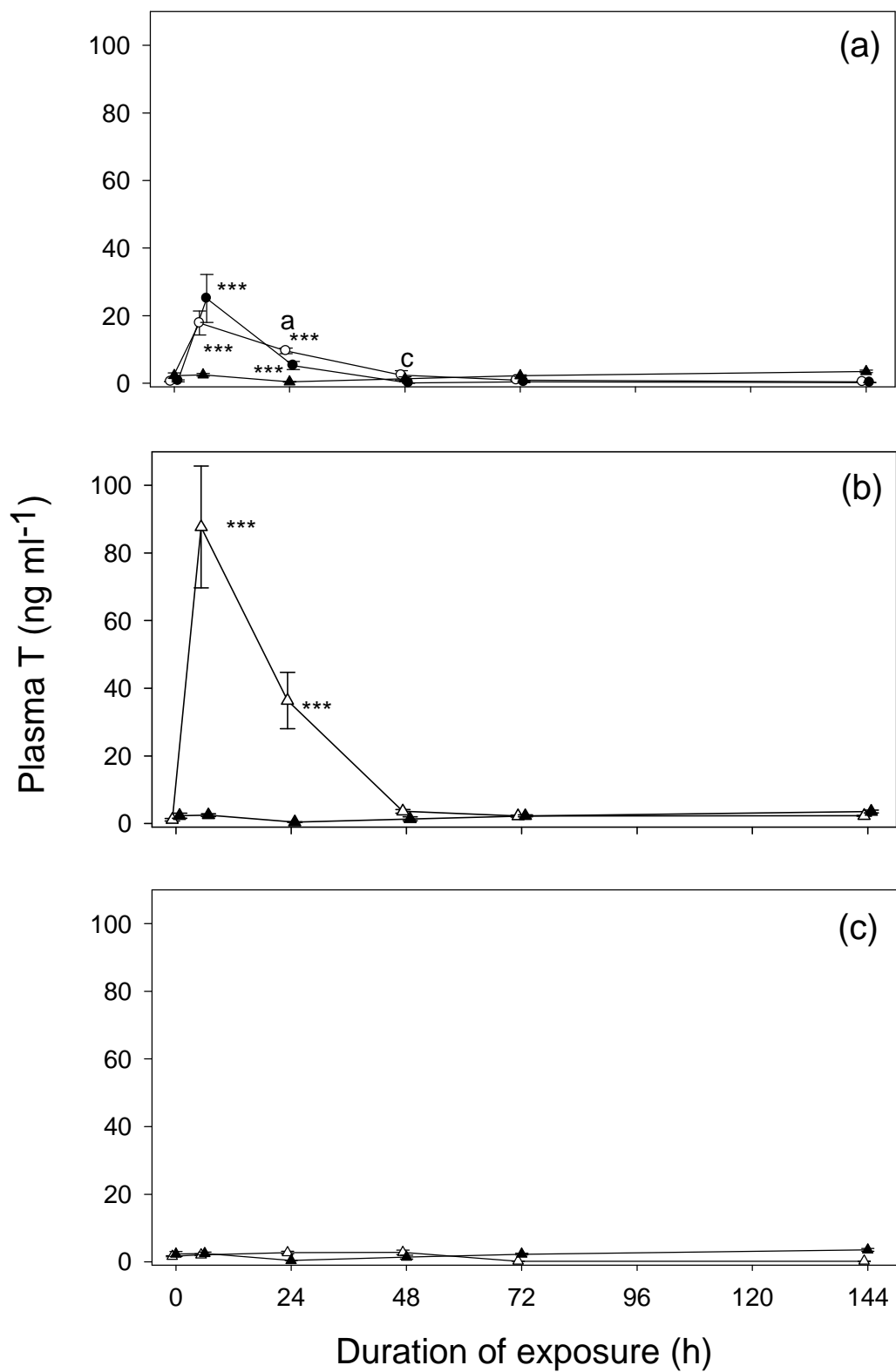
514

515 Figure 2.

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



Maunder, Matthiessen, Sumpter & Pottinger Fig. 1



Maunder, Matthiessen, Sumpter & Pottinger Fig. 2