Hypoxia modifies the response to flutamide and linuron in male three-spined stickleback (*Gasterosteus aculeatus*)

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Abstract

Hypoxia is a major stressor in aquatic environments and it is frequently linked with excess nutrients resulting from sewage effluent discharges and agricultural runoff, which often also contain complex mixtures of chemicals. Despite this, interactions between hypoxia and chemical toxicity are poorly understood. We exposed male three-spined stickleback during the onset of sexual maturation to a model anti-androgen (flutamide; 250μg/L) and a pesticide with anti-androgenic activity (linuron; 250μg/L), under either 97% or 56% air saturation (AS). We assessed the effects of each chemical, alone and in combination with reduced oxygen concentration, by measuring the transcription of spiggin in the kidney, as a marker of androgen signalling, and 11 genes in the liver involved in some of the molecular pathways hypothesised to be affected by the exposures. Spiggin transcription was strongly inhibited by flutamide under both AS conditions. In contrast, for linuron, a strong inhibition of spiggin was observed under 97% AS, but this effect was supressed under reduced air saturation, likely due to interactions between the hypoxia inducible factor and the aryl hydrocarbon receptor (AhR) pathways. In the liver, hypoxia inducible factor 1α was induced following exposure to both flutamide and linuron, however this was independent of the level of air saturation. This work illustrates the potential for interactions between hypoxia and pollutants with endocrine or AhR agonist activity to occur, with implications for risk assessment and management.

Capsule

Hypoxia modifies the toxicity of the anti-androgenic chemical, linuron, potentially via interactions with the aryl hydrocarbon receptor pathway.

Introduction

Hypoxia occurs naturally in both freshwater and marine ecosystems, but in recent decades there has been an exponential increase in its occurrence and severity worldwide (Diaz and Rosenberg, 2008). This has been associated, at least in part, with increases in nutrient load of water bodies, originating from land run-off and sewage discharges (Wu, 2002). Sewage effluents and agricultural runoff both contain a complex cocktail of chemicals with endocrine disrupting activity (Harris, 1995; Harries et al., 1996; Jobling et al., 1998; Jobling et al., 2009; Lange et al. 2012; Purdom et al, 1994; Tyler et al., 1998; Uren Webster et al., 2014). Many of these have been shown to cause reproductive disruption, oxidative stress or interfere with the aryl hydrocarbon receptor pathway among others.

Hypoxia has also been reported to affect reproduction in fish by disrupting endocrine signalling. Disruption of brain reproductive pathways have been reported in the Atlantic croaker (*Micropogonias undulates*), where exposure to hypoxia caused a decrease in serotonin, a regulator of gonadotropin release (Thomas et al., 2007). In addition, studies have shown hypoxia to affect sex steroid concentrations (Wu et al., 2003; Thomas et al., 2006; Thomas et al., 2007) and gonad development (Shang et al.; Thomas et al., 2006; Thomas et al., 2010). At the behavioural level, hypoxia was shown to cause impairment of courtship (Wang et al., 2008), decreased frequency of mating displays (Gotanda et al., 2011), and increased time spent fanning the eggs (Jones and Reynolds., 1999).

To date, only two studies have investigated the effects of endocrine disrupting chemicals (EDCs) in combination with low oxygen conditions. In male fathead minnows (*Pimephales promelas*) hypoxia did not affect the response to a mixture of estrogenic chemicals (Brain et al., 2008). Similarly, experiments testing the effects of hypoxia in combination with oestradiol (E2) in killifish (*Fundulus heteroclitus*) and zebrafish (*Danio rerio*) embryos did not find evidence for interactions between these stressors (McElroy et al., 2012). However, considering the evidence for the effects of hypoxia on reproduction, and the scarcity of data

71 on interactions between hypoxia and reproductive disrupting chemicals, in particular for anti-

72 androgenic compounds, it is important to consider this question. We address this knowledge

73 gap by investigating the hypothesis that interactions between hypoxia and anti-androgenic

74 chemicals are likely to occur, because of the known inhibitory effects of hypoxia on male

75 reproduction, which overlaps with the effects of anti-androgenic chemicals. We selected two

76 chemicals because of their well-established anti-androgenic mechanisms of action (flutamide;

77 linuron) and their potential environmental relevance (linuron).

78 Flutamide is a potent androgenic receptor (AR) antagonist that competes with endogenous

79 androgens at the androgen receptor hormone binding site and prevents AR-DNA binding and

80 transcriptional regulation of androgen-dependent genes (Ankley et al., 2004; Katsiadaki et al.,

81 2006). Although of relatively low environmental concern, the anti-androgenic effects of this

82 chemical have been extensively documented because of its proven clinical efficiency in the

83 treatment of androgen-dependent prostate cancer (Singh et al., 2000), and its extensive use

84 as an anti-androgenic reference chemical in endocrine disruption studies (OECD, 2002).

85 The pesticide, linuron, is a weakly competitive AR antagonist and was shown to induce a

86 positive response for androgenic activity in both the stickleback (Katsiadaki et al., 2006) and

87 the Hershberger assay, via suppression of androgen dependent gene expression (Cook et al.,

88 1993; Lambright et al., 2000). This chemical is used as an herbicide applied to suppress broad

89 leaf and grassy weed growth, resulting in its entering surface waters via agricultural runoff

90 (Patterson, 2004), and has been detected in drinking water and in food samples (Pest

91 Management Regulation Agency, 2012; R.E.D. U.S.E.P.A, 1995). In addition to its anti-

92 androgenic activity, linuron has been shown to activate the aryl hydrocarbon receptor (AhR)

93 signalling pathway (Uren Webster et al., 2015), an important pathway in the cellular

94 metabolism of toxicants that shares a dimerization partner with the hypoxia signalling pathway.

95 These two pathways have previously been shown to interact (Fleming and Di Giulio, 2011),

96 providing an additional hypothesis for the potential interaction between hypoxia and linuron.

97 The three-spined stickleback was used as a model organism in this study because of its useful

98 traits for studying anti-androgens (Katsiadaki et al., 2007). Male sticklebacks produce spiggin,

99 a glue protein expressed in the kidney of mature fish, and used in nest building (Hahlbeck et

100 al., 2004). Spiggin production is under the control of androgens and has been shown to be

101 suppressed upon exposure to anti-androgenic chemicals (Katsiadaki et al., 2006).

102 Suppression of spiggin has been validated as a useful biomarker to detect chemicals able to

103 disrupt androgen signalling (Hahlbeck et al., 2004; Katsiadaki et al., 2006), including in

104 regulatory toxicity testing (OECD, 2011).

105 We exposed male sticklebacks to the anti-androgenic chemicals, linuron and flutamide, under

106 97% or 56% air saturation (AS). The chemical concentrations used for this study were chosen

107 based on their ability to cause inhibition of spiggin production in the stickleback (Katsiadaki et

108 al., 2006) and to facilitate a mechanistic analysis of the interactions between these chemicals

109 and reduced oxygen in the water. An increase in spiggin transcription is expected to occur in

110 adult males as they transition into sexual maturity in preparation of their breeding season

111 starting in spring (Jakobsson et al., 1999). Therefore, we measured the transcription of spiggin

112 in the kidney to determine whether the exposure to the chemicals alone or in combination with

113 reduced air saturation inhibited this expected spiggin increase. We also measured the

114 transcription of 11 genes in the liver, chosen as markers for signalling pathways hypothesised

115 to be regulated by both hypoxia and the chemicals selected. These pathways included

116 androgen and oestrogen signalling, oxidative stress response, hypoxia signalling, cholesterol

117 biosynthesis and insulin growth factor signalling.

118

119 **Material and Methods**

120 *Fish source, culture and husbandry*

121   Freshwater three-spined sticklebacks (originating from the River Erme, Devon, United

122   Kingdom) were kindly provided by the University of Plymouth, and maintained in the Aquatic

123   Resource Centre at the University of Exeter as described in the supplementary information.

124   Males (in total 64 individuals, approximately 9 months old) were selected for this study using

125   a PCR method that identifies a specific sex marker in the stickleback (Peichel et al., 2004),

126   described in the supplementary information.

127   *Exposure to anti-androgens and varying oxygen concentrations*

128   Male sticklebacks were exposed for 7 days to either flutamide (250µg/L; Sigma Aldrich, purity

129   ≥99.99) or linuron (250µg/L; Sigma Aldrich, purity ≥98%), under 100% or 50% air saturation

130   (AS; nominal concentrations), or to appropriate controls. Concentrations for the test chemicals

131   were selected based on their ability to cause anti-androgenic effects in fish (flutamide:

132   Katsiadaki et al., 2006; linuron: Uren Webster et al., 2015). The choice of 50% AS was

133   informed by the critical oxygen level (P_{crit}) for this population of sticklebacks, determined in a

134   separate experiment in our laboratory (P_{crit}= 48.88±2.73 % AS at 15°C; Fitzgerald *et al.*, 2019).

135   We chose a concentration of oxygen just above the P_{crit} for this laboratory population, therefore

136   sufficient to cause physiological acclimation without causing overt adverse effects, and

137   relevant to environments inhabited by this species (Fitzgerald et al., 2017). The oxygen

138   concentration in each tank was controlled by continuously aerating the water with a pre-set

139   gas mixture of O_2, N_2 and CO_2, achieved by controlling the proportional flow rate from cylinders

140   of each gas, using precision gas flow controllers (MC Series Mass Flow Controllers, Qubit

141   Systems Inc., Ontario, Canada), connected to a PC running gas mixture software (C690 Gas

142   Mixing Software, Qubit Systems Inc., Ontario, Canada). The chemical solutions were supplied

143   by a flow-through system to the experimental tanks, using ethanol (0.001%) as a solvent. An

144   absolute control and a solvent control receiving the same concentration of ethanol as the

145   chemical exposures were also included, both under 50% and 100% AS. For each treatment

146   there were 4 tanks, with one individual fish per tank. The experiment was carried out twice

147   over two consecutive weeks, resulting in a replication of n=8 fish per treatment.

148   On day 0 of exposure, tanks were spiked with the appropriate amount of chemical to achieve

149   the desired test concentrations and connected to a flow through system. Flow rates were

150   monitored daily to ensure that the chemical concentrations remained consistent and working

151   stock solutions were replaced every 48h. The level of air saturation was measured twice daily

152   using an OptiOx dissolved oxygen sensor (Mettler Toledo) to confirm that it was maintained

153   as close as possible to the nominal level. Throughout the exposure, daily measurements of

154   pH, conductivity and temperature where also conducted. All fish were fed the same amount of

155   food daily (~4% body weight; *Chironomus sp.*; Tropical Marine Centre, Chorleywood, UK), and

156   starved 24h prior to sampling.

157   To encourage reproductive maturation of the stickleback under the exposure conditions, the

158   photoperiod was raised by 2hr/day, over the first 5 days of exposure. For the final 2 days of

159   exposure, photoperiod was maintained at 18:6 light/dark to mimic summer conditions. The

160   temperature was maintained at 12±1°C during the experimental period. Gravel was placed in

161   a petri dish on one side of the tank to provide environmental enrichment and some substrate

162   to encourage nest building behavior.

163   All fish were sacrificed on day 7 of the exposure period by a lethal dose of benzocaine followed

164   by destruction of the brain, in accordance with the UK Home Office regulations. Wet weight

165   and fork length were recorded and the condition factor (k = (weight (g) x 100) / (fork length

166   (cm^3)) was calculated for each individual fish. Tissues were collected and weighed, and the

167   heaptatosomatic index (HSI = (liver weight (mg) / total weight (mg)) x 100), gonadosomatic

168   index (GSI = (gonad weight (mg) / total weight (mg)) x 100) and the nephrosomatic index (NSI

169   = (kidney weight (mg) / total weight (mg)) x 100) were calculated. The kidney and liver samples

170   were snap frozen in liquid nitrogen and stored at -80°C for molecular analysis. Water samples

171   from each experimental tank were taken on day 0, 1 and 6 of the exposure for chemical

172   analysis. The concentration of each anti-androgen was measured using a LC-MS method, as

173   described in the Supplementary Information.

175   Real-time quantitative PCR (RT-QPCR) was used to quantify the transcription of target genes

176   in the kidney and the liver. In the kidney, spiggin transcription was measured to determine if

177   the treatments (reduced air saturation and/or chemical exposure) inhibited the increase in

178   spiggin expected to occur in males as they transition from winter to summer conditions, a sign

179   of male maturation in preparation to spawn. In the liver, transcription of genes involved in

180   reproductive function (androgen receptors (*ar1* and *ar2*) (Hogan et al., 2008), estrogen

181   receptors (*esr1*, *esr2a* and *esr2b*) (Geoghegan et al., 2008)) were measured to investigate for

182   the effects of both chemicals, alone and in combination with hypoxia, on reproductive

183   signalling pathways. Transcription of the hypoxia inducible factor 1α (*hif1α*) was also

184   measured to serve as a biomarker of hypoxia exposure (Wu, 2002) and a biomarker for

185   activation of the AhR activation (cytochrome P450, family 1, subfamily A (*cyp1a*) (Bucheli et

186   al., 1995)) was also measured. The transcription of two genes that are part of the cholesterol

187   biosynthesis   pathway   (3-hydroxy-3-methylglutaryl-coenzyme   A   synthase   (*hmgcs*),

188   isopentenyl-diphosphate delta isomerase 1 (*idi*)) were measured as they have previously been

189   shown to be downregulated following linuron exposure in mature males (Uren Webster et al.,

190   2015). Finally, we also measured the transcription of catalase (*cat*), as a biomarker for

191   oxidative stress (Lushchak, 2011) and insulin-like growth factor-binding protein 1b (*igfbp1b*),

192   to act as a biomarker for the IGF pathway, which was shown to crosstalk with the androgen

193   signalling pathway and to be affected by exposure to antiandrogens (Filby et al., 2007; Uren

194   Webster et al., 2015). To identify the most appropriate control gene for data normalisation,

195   three control genes were used (ribosomal protein l8 (*rpl8*), ubiquitin (*ubi*) and beta-tubulin

196   (*tubb4*)) and regression analysis was conducted on Ct values to identify those that varied the

197   least between samples and conditions in each tissue. For the kidney and liver, *ubi* and *rpl8*,

198   respectively, were identified to be the most stable genes, and were selected as control genes

199   for the subsequent data analysis. Further details on primer and assay design and validation

200   are provided in the Supplementary Information.

Statistical Analyses

202   Gene expression data was first scrutinised by the Chauvenet's criterion to detect outliers for

203   each gene and these were subsequently removed before analysis (Chauvenet, 1863). Data

204   that did not meet the normality (Sharpo-Wilko test) and equal variance (Bartlett) test was log

205   transformed before analysis, and this transformation resulted in normally distributed data.

206   Comparison of solvent control and water control was performed using a t-test in R (Team R,

207   2014). All remaining data were analysed using an analysis of variance model in R. A separate

208   model was used for each gene, to test for effects on gene expression of the exposure to

209   solvent or chemical, and 100% or 50% AS (categorical variables) and the interaction between

210   these variables. Minimum adequate models were derived by model simplification using F tests

211   based on analysis of deviance (Crawley, 2102). Tests reported refer to the significance of

212   removing terms from the models. When a significant effect of interaction was identified,

213   pairwise comparisons to determine which groups differed were conducted using Tukey's HSD

214   post hoc test. All data were considered statistically significant when $p<0.05$.

215

216   **Results**

217   *Water chemistry measurements*

218   The mean measured chemical concentrations in the tank water, were 102 ± 1.4% (255 µg/l ±

219   3.6) and 109 ± 1.6% (274 µg/l ± 4.0) of the nominal concentrations for flutamide and linuron,

220   respectively (see Table S2 for full details).

221   The mean measured air saturations for the 100% and 50% treatment tanks were 97.1 ± 0.2%

222   and 56.0 ± 0.2%, respectively, and for the remaining of the paper, we refer to these values as

223   the level of air saturation. The average conductivity (323.35 µs ± 2.10) and pH (7.41 ± 0.01)

224   measured in the tanks throughout exposure remained stable over time and were not affected

225   by the treatments.

Morphometric Parameters

227   There was no significant difference between the solvent control and the water control under

228   97% or 56% AS for any of the morphometric parameters measured (Fig. S1), so all chemical

229   exposed groups for the morphometric parameters were compared to the solvent control.

230   Throughout the exposure fish remained in good condition and there were no mortalities. The

231   mean body weight, length and condition factor were 1.40 ± 0.03 g, 5.3 ± 0.03 cm and 0.93

232   ±0.01 g/cm^3, respectively.

233   Exposure to flutamide caused a significant decrease in the GSI (average measured GSI for

234   solvent: 0.81 ± 0.07, and for flutamide: 0.65 ± 0.04; P=0.020; Fig. 1A; Table 1A), but there

235   was no significant interaction between the chemical treatment and air saturation (P=0.612).

236   All other morphometric parameters measured, were not affected by the flutamide treatment

237   and/or reduced air saturation (HSI: P=0.907; NSI: P=0.788; Condition Factor: P=0.858;

238   Weight: P=0.891; Length: P=0.948; Fig. S2; Table S3A).

239   Exposure to linuron also caused a decrease in GSI (average measured GSI for solvent: 0.81

240   ± 0.07 and for linuron: 0.63 ± 0.04; P=0.030; Fig. 1B.; Table 1B), but no significant interaction

241   between treatment and air saturation was observed (P=0.985). Exposure to linuron, either

242   alone or in combination with different air saturations did not affect NSI (P=0.746; Fig. S2G) or

243   HSI (P=0.616; Fig. S2F). Air saturation had a significant effect on the condition factor of fish

244   (average measured condition factor for 97 % AS: 0.90 g/cm^3 ± 0.016, for 56 %: 0.89 g/cm^3 ±

245   0.017; P=0.017; Fig. S2H; Table S3B), but no significant interaction was observed between

246   the treatment and air saturation (P=0.077). In addition, there was a significant effect of

247   exposure to linuron on weight (average measured weight for solvent: 1.27 g ± 0.05, for linuron:

248   1.53 g ± 0.06; P=0.001; Fig. S2I; Table S3B) and length (average measured length for solvent:

249   5.16 cm ± 0.07, for linuron: 5.45 cm ± 0.07; P=0.002; Fig. S2J; Table S3B), but no significant

250   interaction with air saturation was observed (P=0.550 and P=0.787, respectively).

251   *Transcript profiling in the kidney*

252 No differences in gene transcription between the solvent control and the water control under

253 97% or 56% AS were observed for any gene tested (Fig. S3 and Fig. S4, respectively), and

254 all gene transcription data following chemical exposures was compared to the solvent control.

255 Flutamide exposure resulted in significantly lower *spg* transcription (P<0.001, Fig. 2.A.; Table

256 1A), but this transcript was not affected by the level of air saturation and no interaction between

257 flutamide and the level of air saturation were observed (P=0.316).

258 For linuron, a decrease in *spg* transcription under 97% AS compared to the solvent control

259 was observed (P=0.021, Fig. 2.B.; Table 1B), but this effect was absent under 56% air

260 saturation (P=0.992).

261 *Transcript profiling in the liver*

262 Flutamide and linuron, alone and in the presence of reduced air saturation did not affect the

263 transcription profiles of the androgen receptors (flutamide: *ar1*: P=0.713 and *ar2*: P=0.520;

264 Fig.3.; Table 1A; linuron: *ar1*: P=0.171 and *ar2*: P=0.303; Fig. 4.; Table 1B). Similarly, no effect

265 was observed for estrogen receptors (flutamide: *esr1*: P=0.107, *esr2a*: P=0.440 and esr2b:

266 P=0.651; Fig. 3.; Table 1A; linuron: *esr1*: P=0.986, *esr2a*: P= 0.058 and *esr2b*: P=0.324; Fig.

267 4.; Table 1B).

268 Flutamide exposure resulted in significantly lower *cat* transcription in the liver when exposure

269 occurred under 97% AS (P=0.037; Fig. 3.; Table 1A), but not under 56% AS (P=0.970). *cat*

270 transcription was not affected by linuron exposure for both 56% and 97% AS (P=0.405; Fig.

271 4.; Table 1B).

272 Flutamide exposure did not affect *cyp1a* transcription for both 56% and 97% AS (P=0.134;

273 Fig. 3.; Table 1A). However, exposure to linuron significantly up-regulated *cyp1a* transcription

274 in fish exposed under 97% AS (P=0.001; Fig. 4.; Table 1B), but this effect was not evident

275 when fish were exposed under 56% AS (P=0.215).

276   For the genes encoding selected cholesterol biosynthesis enzymes, *hmgcs* and *idi*, there was

277   no effect of treatment for flutamide (P=0.905 and P=0.464, respectively; Fig. 3.; Table 1A) or

278   linuron (P=0.417 and P=0.09, respectively; Fig. 4.; Table 1B). However, there was a significant

279   effect of air saturation on *idi* transcription for flutamide (P=0.011). No interaction was observed

280   for the *igfbp1b* after exposure to flutamide (P=0.362; Fig. 3.; Table 1A) or linuron (P=0.771;

281   Fig. 4.; Table 1B).

282   Exposure to flutamide and linuron resulted in a significant increase in *hif-1α* transcription under

283   both 97% and 56% AS (P<0.001; Fig. 5; Table 1), but these effects were not influenced by the

284   level of air saturation (flutamide: P=0.584; linuron: P=0.509).

285

286   **Discussion**

287   The objective of this study was to determine the influence of reduced oxygen concentrations

288   in the water on the effects of chemicals with an anti-androgenic mode of action in a model fish

289   species. The interactions between low oxygen and each of the anti-androgens was unique to

290   each chemical, and this was illustrated by their contrasting effects on spiggin transcription.

291   The differences in how fish responded to each chemical were also evident in their

292   transcriptional responses in the liver, where low oxygen altered the effects of flutamide and

293   linuron via different gene pathways, reflecting differences in the mode of action of these

294   chemicals.

295   *Flutamide inhibits spiggin transcription independently of the level of air saturation*

296   Spiggin transcription was significantly down regulated in fish exposed to flutamide compared

297   to those maintained under control conditions, and this response was unaffected by low

298   oxygen. The strong androgen antagonistic response is consistent with previous studies

299   reporting that exposure of adult stickleback males to flutamide resulted in reduced spiggin

300   production in the kidney and the number of nests built by males (Sebire et al., 2008). In

301   addition, this response is also consistent with the observed decrease in GSI observed in

302   exposed males, indicating an inhibition of gonadal maturation, a process dependent on

303   androgen signalling. Kidney hypertrophy, which is associated with spiggin production, is

304   controlled by 11-ketotestosterone (11-KT), the physiologically relevant androgen in fish

305   (Jakobsson et al., 1999), in a process induced via an AR-mediated pathway (Jolly et al., 2009).

306   Spiggin induction by 11-KT was shown *in vitro* to be blocked by the androgen receptor agonist,

307   flutamide (Jolly et al., 2006). This chemical acts as a potent AR antagonist via competition

308   with androgens preventing AR-DNA binding and transcription of androgen-dependent genes

309   (Wilson et al., 2007). There was no effect of flutamide exposure on the transcription of

310   androgen receptors, suggesting that auto-regulation of the AR may not have occurred under

311   our experimental conditions.

312   *Reduced oxygen affects catalase transcription following exposure to flutamide*

313   Exposure to flutamide resulted in reduced catalase transcription compared to the solvent

314   control under 97 % AS. Similar results were previously reported in both cell lines and in an *in*

315   *vivo* mice model, where catalase activity was increased under testosterone stimulation but this

316   activity was suppressed with the addition of flutamide (Ahlbom et al., 2001; Lateef et al., 2013).

317   Catalase is an antioxidant enzyme in fish and an important indicator of oxidative stress. When

318   the androgen receptors are blocked, a marked increase ROS generation occurs (Lateef et al.,

319   2013), probably as a result of inhibition of testosterone-induced catalase expression.

320   Therefore, in our experiment, flutamide may have reduced catalase transcription via its anti-

321   androgenic activity. In contrast, exposure to flutamide under 56% AS did not affect catalase

322   transcription. Decreases in oxygen concentration have also been shown to induce oxidative

323   stress in fish (Lushchak, 2011). The mechanisms by which hypoxia induces oxidative stress

324   are not well described, but suggestions have included greater electron leakage in the electron

325   transport chain, forming more ROS (Lushchak, 2011). Exposure to hypoxia was shown to

326   result in inductions of antioxidant response enzymes, including increased superoxide

327   dismutase (SOD) and catalase activities in the liver of the goldfish (*Carassius auratus*)

328   (Lushchak et al., 2001) and the common carp (*Cyprinus carpio*) (Lushchak, 2005). In addition,

329 exposure to hypoxia resulted in increased activities of catalase and glutathione peroxidase in

330 the freshwater clam (*Corbicula fluminea*) (Vidal et al., 2002). Therefore, we hypothesise that

331 the increase in antioxidant defence systems induced by exposure to 56% AS may have

332 masked the suppression of catalase transcription observed when fish were exposed to

333 flutamide under normoxic conditions.

334 *Hypoxia reduces the anti-androgenic effects of linuron likely via inhibition of the AhR pathway*

335 Exposure of male sticklebacks to linuron resulted in a decrease in *spg* transcription under 97%

336 AS. Linuron is a relatively weak competitive AR antagonist *in vitro* and has been shown to

337 suppress androgen-dependent gene expression (Cook et al., 1993; Lambright et al., 2000).

338 Exposure to linuron was shown to inhibit androgen-induced spiggin protein production in the

339 female stickleback model (Jolly et al., 2009; Pottinger et al., 2013) but at the transcription level,

340 *spg* was not shown to decrease under linuron exposure in mature male stickleback (Hogan et

341 al., 2012). This difference in findings is likely to be as a result of different exposure time (Hogan

342 *et al.* exposed males for only 3 days, whereas in our study fish were exposed for 7 days) and

343 experimental conditions (males in this study were stimulated to mature during the experiment

344 by progressively increasing the photoperiod, during the winter to spring transition, whereas

345 the exposure by Hogan *et al.* was carried out under a fixed winter temperature and light

346 regime). Similarly to the study by Hogan *et al.*, exposure to linuron in our study did not result

347 in changes in the transcription of either of the androgen receptors (Hogan et al., 2012).

348 Exposure to linuron under 97% AS resulted in a strong increase in the transcription of *cyp1a*

349 in the liver. The same finding was reported for brown trout (*Salmo trutta*) exposed to linuron

350 over 4 days, where an environmentally relevant concentration of linuron (1.7µg/L) caused a

351 moderate induction of *cyp1a* transcription and exposure to 225.9µg/L dramatically increased

352 the transcription of this gene by 560-fold (Uren Webster et al., 2015). *cyp1a* is involved in the

353 detoxification and/or metabolic activation of several xenobiotics and is primarily regulated via

354 the AhR signalling pathway, which is fundamental for the metabolism of xenobiotics

355   (Monostory et al., 2009). Xenobiotics that have entered the cell bind to the AhR, form an AhR-

356   ligand complex which then translocates to the nucleus. The complex binds to ARNT forming

357   an activated transcription factor which is able to bind to xenobiotic responsive elements,

358   resulting in the transcription of CYP proteins (Fujii-Kuriyama et al., 2005). The induction of

359   *cyp1a* at both the transcript and protein levels has been used extensively as a measure of

360   exposure to a number of xenobiotics with the ability to activate the AhR pathway, including

361   planar aromatic hydrocarbons (PAH), and polychlorinated biphenyls (PCBs) (Bucheli et al.,

362   1995). Linuron has been reported to be one of the most potent activators of the AhR in both

363   fish and mice models (Takeuchi et al., 2008; Uno et al., 2011; Uren Webster et al., 2015). The

364   increase in *cyp1a* transcription observed following exposure to linuron under 97% AS was

365   reduced when exposures occurred in the presence of 56% AS. We hypothesise that, under

366   low air saturation, competition occurs for the dimerization partner (ARNT), which is shared by

367   both the oxygen-sensitive HIF pathway and the AhR pathway (Denison and Dagy, 2003). This

368   competition for ARNT may have resulted in a reduction of AhR-ARNT dimerization and,

369   consequently, a suppression of linuron-induced *cyp1a* transcription.

370   Under reduced oxygen, in addition to the suppression of *cyp1a* induction, the effects of linuron

371   on spiggin were also suppressed. The exact mechanism why this response is observed is

372   currently not known, due to a lack of literature on linuron biotransformation and degradation,

373   and how the parent compounds, as well as its metabolites, interact with androgen receptors

374   and/or other components of the androgen signalling pathway. However, due to the strong

375   effect of linuron on *cyp1a* transcription, we speculate that linuron may be required to undergo

376   bio-activation by CYP1A before acting as an anti-androgen, which in turn would cause the

377   suppression of spiggin. Under hypoxia, the suppression of the activity of the AhR pathway and

378   reduced induction of *cyp1a*, potentially lessening the bio-activation of linuron, may explain why

379   spiggin is no longer affected by the exposure. Currently, there is no information available

380   regarding the metabolism of linuron by the cytochrome P450 enzymes, therefore we can only

381   speculate. However, this mechanism has previously been suggested for the PAH, pyrene; for

382 which a delay in toxicity occurs in CYP1A-morphants, which suggests that a metabolite of

383 pyrene is responsible for its toxicity, and not pyrene itself (Incardona et al., 2005).

384 *Exposure to anti-androgenic chemicals induced the transcription of hypoxia-inducible factor*

385 There was a strong up-regulation of *hif-1α* transcription in fish exposed to linuron and flutamide

386 both under 97% and 56% AS. HIF is a key oxygen sensing molecule and transcription factor

387 regulating the cellular response to hypoxia, which results in the adaptive response of

388 maintaining oxygen homeostasis under low environmental oxygen. This occurs via stimulation

389 of the transcription of genes that promote a series of processes including vasodilation,

390 erythropoiesis and angiogenesis, as well as energy production via anaerobic glycolysis

391 (Majmundar et al., 2010). Under normal intracellular oxygen levels, the HIF-1α subunit is

392 modified by the oxygen sensitive enzymes, PHDs (prolylhydroxlases), allowing the protein-

393 ubiquitin ligase complex to recognise HIF-1α, resulting in its degradation by the proteasome

394 pathway (Bruick, 2003). However, under low oxygen concentrations, HIF-1α is stabilised, due

395 to the inhibition of PHD. There is now accumulating evidence that HIF-1α responds to non-

396 hypoxic stimuli, including hormones such as insulin, growth factors, coagulation factors,

397 vasoactive peptides and cytokines (Fukada et al., 2002; Hellwig-Bürgel et al., 1999; Kietzmann

398 et al., 2003; Richard et al., 2000; Steihl et al., 2002; Triens et al., 2002; Zelzer et al., 1998),

399 but to our knowledge, there is no evidence in the literature for a significant *hif-1α* induction

400 following exposure to organic xenobiotics. In our study *hif-1α* was strongly induced by two

401 chemicals with a known anti-androgen mode of action, however the mechanism by which

402 these chemicals induce *hif1-α* at the transcription level is not known. We hypothesise that

403 intracellular oxygen may have become depleted as a result of the metabolism of these

404 chemicals by the P450 cytochrome monooxygenases. Fish detoxify xenobiotics through the

405 reduction, oxidation or hydrolysis of compounds to more water soluble products, which, in turn,

406 allows their excretion to occur via the bile (Xu et al., 2005). During this process, the xenobiotic

407 substrate will bind to the hydrophobic site on the cytochrome P450 (CYP) enzyme (Meunier

408 et al., 2004), where the iron present in the enzyme is oxidised in a process that uses up cellular

409   oxygen (Meunier et al., 2004; Timbrell, 1998). This process may result in intracellular oxygen

410   depletion in hepatocytes, which in turn could cause the up-regulation in *hif-1α* observed in our

411   study. Further studies are essential to test this hypothesis and to investigate the mechanisms

412   by which these two compounds cause transcriptional changes in *hif-1α*, and whether this is

413   common to other chemicals metabolised by *cyp* enzymes.

414   In many mammals, transcription levels of *hif* isoforms do not correlate with activation of Hif

415   signalling, most likely due to the stabilisation of Hif-1α protein under low cellular oxygen

416   concentration in the absence of transcriptional regulation of *hif* genes (Pelster et al., 2018).

417   However, in fish many studies have reported elevated transcription of *hif* in response to

418   hypoxic conditions. For example, exposure of hypoxia resulted in increased *hif-1α*

419   transcription in the liver, brain and heart muscle of the sea bass (Terova et al., 2008) and the

420   liver, gill and testis of the Wuchang sea bream (Shen et al., 2010). Despite this, little is known

421   regarding the functional significance of these transcriptional changes as a result of hypoxic

422   exposure. In our study, despite significant increases in *hif-1α* transcription observed as a result

423   of exposure to either linuron or flutamide, there was no significant effect of air saturation on

424   the transcription level of this gene. This discrepancy compared to other studies in fish may be

425   explained by a number of factors including differences between the species studied, and/or

426   differences in the duration or in the intensity of the hypoxia exposure.

427   *Conclusions*

428   We have demonstrated that reduced oxygen in the water can suppress the anti-androgenic

429   activity of linuron, and hypothesised that this occurs via competition between the HIF and AhR

430   pathways. In addition, we demonstrated that hypoxia did not modify the effects of flutamide

431   exposure on spiggin transcription, at least for the concentrations and experimental conditions

432   tested. Despite both chemicals being considered to be anti-androgenic, they had a very

433   different molecular response alone and in combination with hypoxia, both in the kidney and in

434   the liver. Given the increased presence and severity of hypoxia in aquatic systems, the data

435   we present supports the need to consider how the concentration of oxygen in the environment

436   affects the toxicity of a wide range of environmental relevant xenobiotics with the potential to

437   adversely impact fish populations.

438

439   **Acknowledgements**
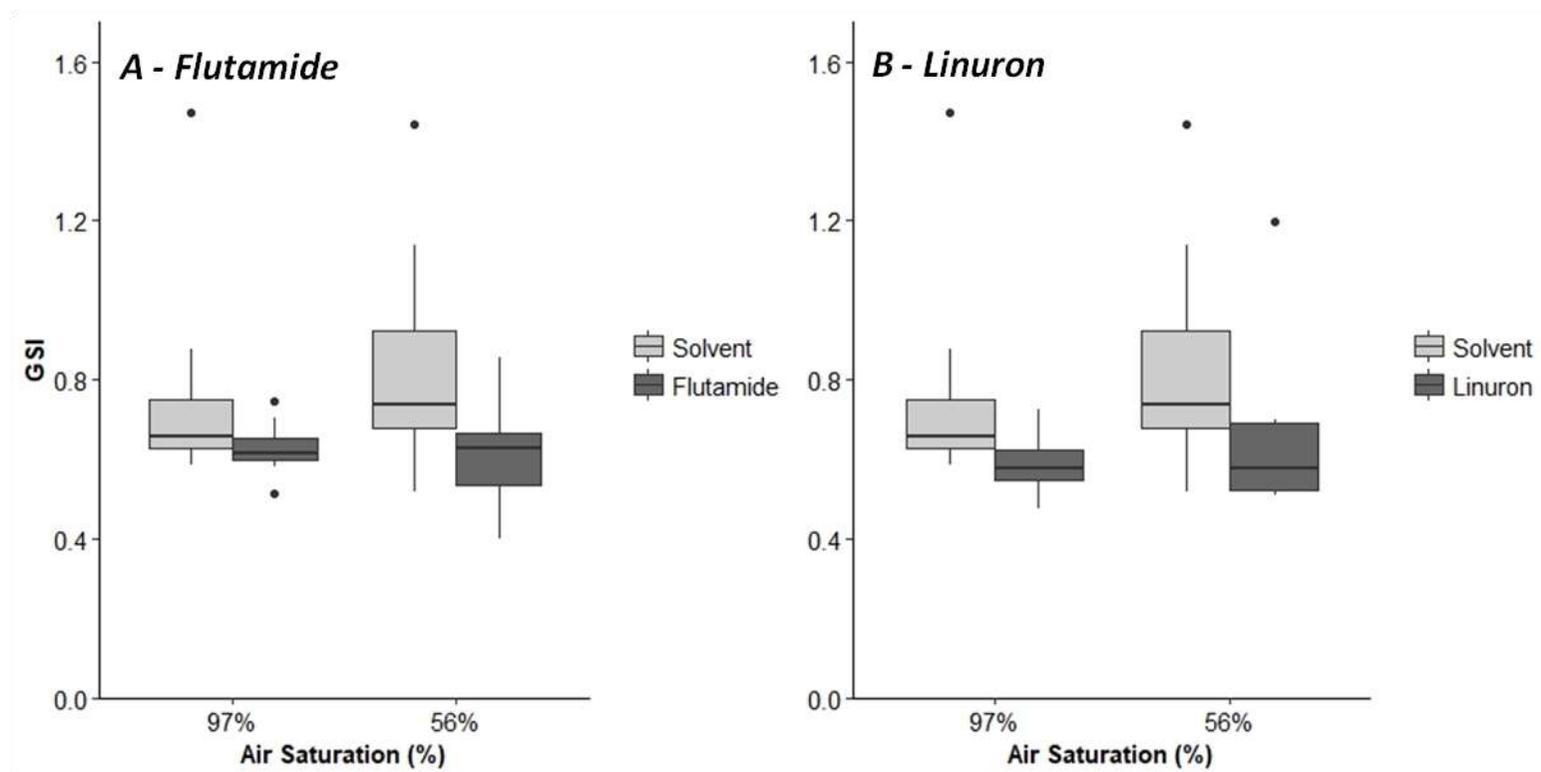
Figures

446 **Figure 1.** Gonadosomatic index (GSI) in fish following exposure to the anti-androgenic chemicals flutamide and linuron under different levels of

447 air saturation. Male sticklebacks were exposed to A: 0 or 250 µg Flutamide/L or B: 0 or 250 µg Linuron/L for 7 days under 97.1 ±0.2% AS and

448 56.0 ±0.2% AS (n=8 individuals per treatment). Statistics were carried out using accepted minimum adequate models (analysis of variance

449 model, R; $P<0.05$) with model details reported in Table 1. There was a significant effect of treatment on GSI for both flutamide and linuron

450 exposure ($P<0.05$).

451
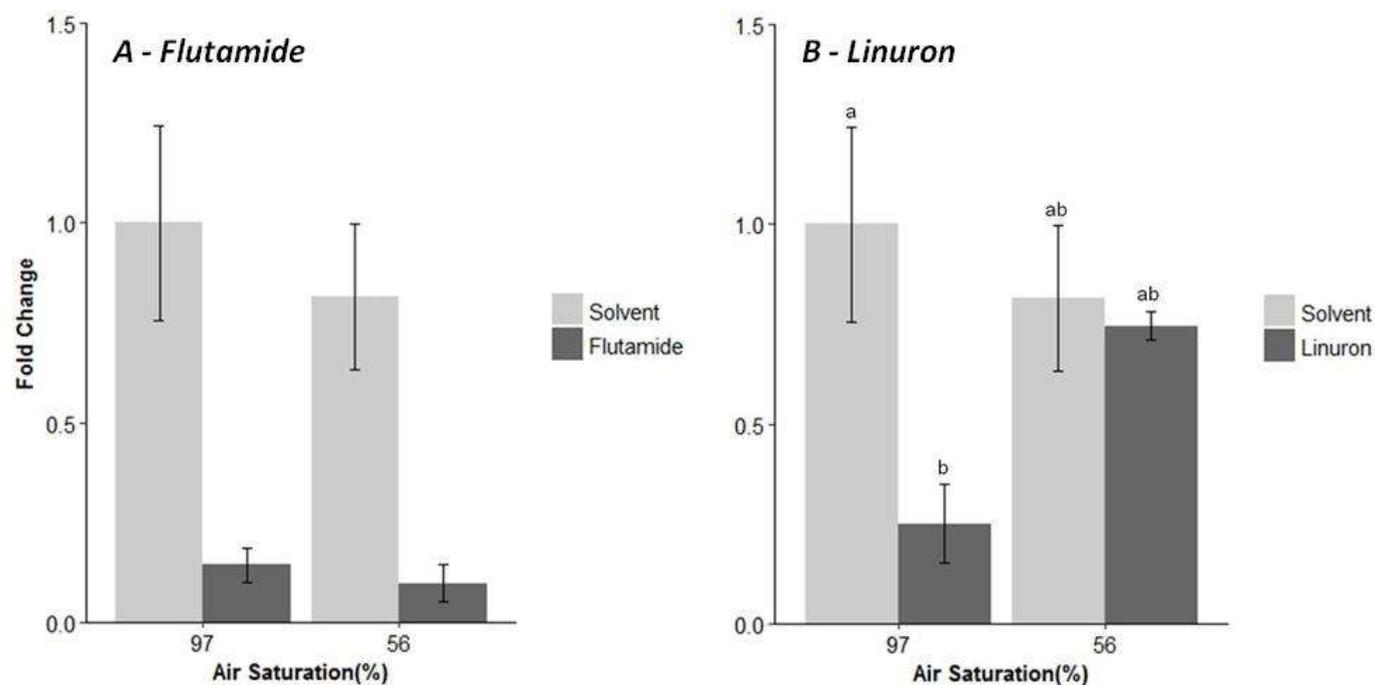
Figure 2. Transcript profiles for spiggin following exposure of fish to the anti-androgenic chemicals flutamide and linuron under different levels of air saturation. Male sticklebacks were exposed to A: 0 or 250 µg Flutamide/L or B: 0 or 250 µg Linuron/L for 7 days under 97.1 ±0.2% AS and 56.0 ±0.2% AS. Plotted data is translated (+1), then LOG10 transformed and presented as fold change (normalised against the expression of the control gene *ubi*, n=6-8 per treatment group). Statistics were carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$) with model details reported in Table 1. For linuron, letters above each bar indicate significant differences between treatment groups when there was a significant interaction from the model (Tukey's HSD post hoc test; $P<0.05$). For flutamide there was no significant interaction from the model but there was a significant effect of treatment ($P<0.001$).

459
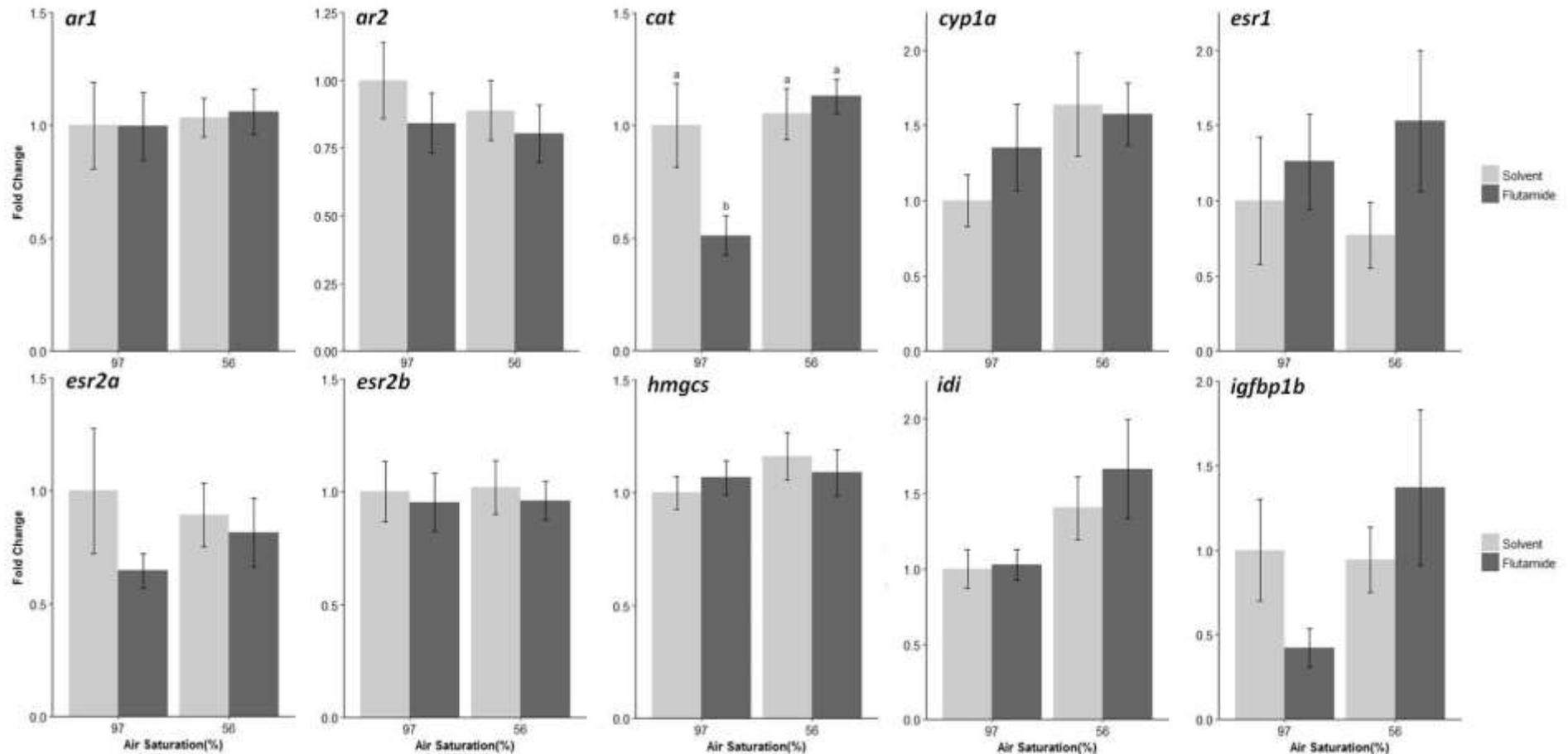
Figure 3. Transcript profiles for selected target genes in the liver of fish following exposure to flutamide under different levels of air saturation.

Male sticklebacks were exposed to 0 or 250 µg flutamide/L for 7 days under 97.1 ±0.2% AS and 56.0 ±0.2% AS. Transcript profiles were

determined using RT-QPCR. Genes analysed included: androgen receptors (*ar1* and *ar2*), catalase (*cat*), cyctochrome P450, family 1, subfamily

463   A (*cyp1a*), estrogen receptors (*esr1*, *esr2a* and *esr2b*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), isopentenyl-diphosphate delta

464   isomerase 1 (*idi*) and insulin-like growth factor-binding protein 1b (*igfbp1b*). Plotted data are presented as fold change (normalised against the

465   expression of the control gene *rpl8*, n=6-8 per treatment group). Statistics were carried out using accepted minimum adequate models (analysis

466   of variance model, R; P<0.05) with model details reported in Table 1A. Letters above each bar indicate significant differences between treatment

467   groups when there was a significant interaction from the model (Tukey's HSD post hoc test; P<0.05). For *idi* and *igfbp1b*, there was a significant

468   effect of air saturation on gene transcription (P=0.011 and P=0.001, respectively).

469
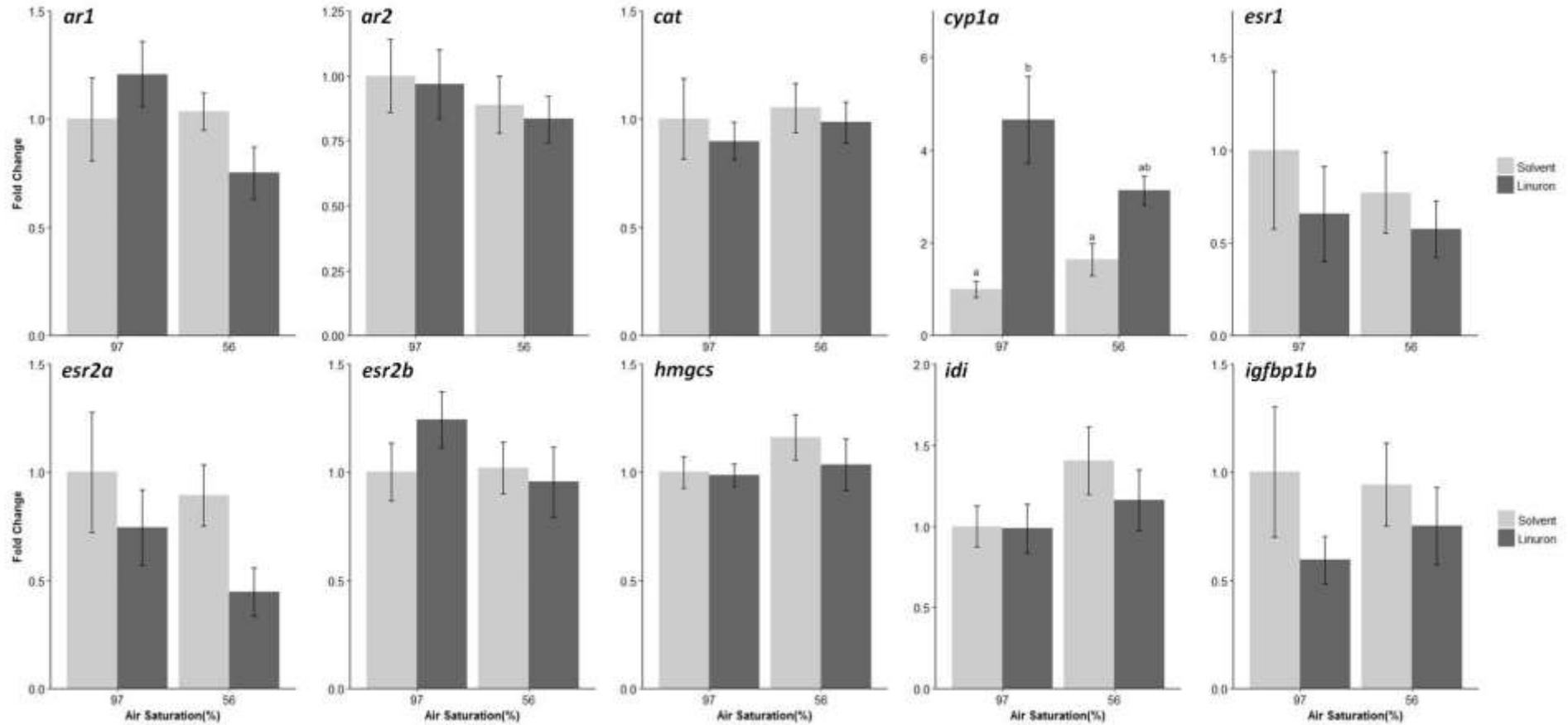
Figure 4. Transcript profiles for selected target genes in the liver of fish following exposure to linuron under different levels of air saturation. Male

sticklebacks were exposed to 0 or 250 µg linuron/L for 7 days under 97.1 ±0.2% AS and 56.0 ±0.2% AS. Transcript profiles were determined

using RT-QPCR. Genes analysed included: androgen receptors (*ar1* and *ar2*), catalase (*cat*), cyctochrome P450, family 1, subfamily A (*cyp1a*),

estrogen receptors (*esr1*, *esr2a* and *esr2b*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), isopentenyl-diphosphate delta isomerase

474   1 (*idi*) and insulin-like growth factor-binding protein 1b (*igfbp1b*). Plotted data are presented as average fold change (normalised against the

475   expression of the control gene *rpl8*, n=6-8 per treatment group). Statistics were carried out using accepted minimum adequate models (analysis

476   of variance model, R; $P<0.05$) with model details reported in Table 1B. Letters above each bar indicate significant differences between treatment

477   groups when there was a significant interaction from the model (Tukey's HSD post hoc test; $P<0.05$)

478
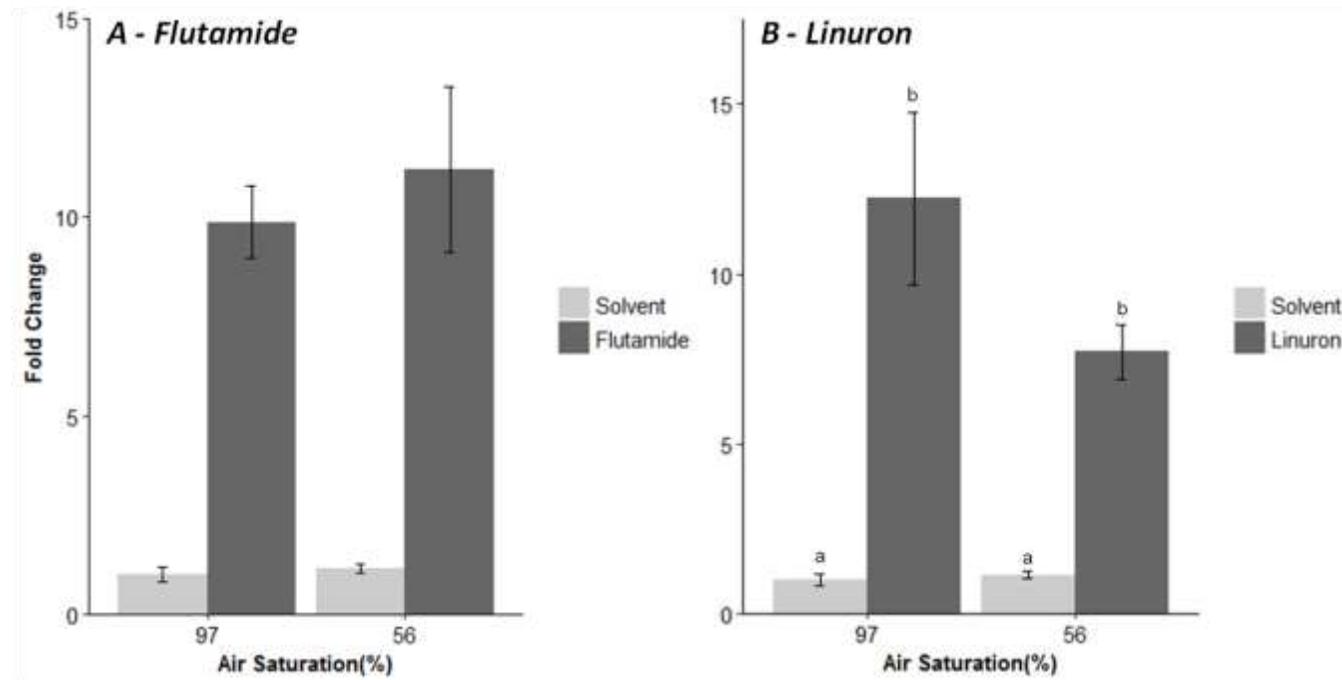
Figure 5. Transcript profiles for *hif-1α* following exposure to two anti-androgenic chemicals under different levels of air saturation. Male sticklebacks were exposed to A: 0 or 250 μg Flutamide/L or B: 0 or 250 μg Linuron/L for 7 days under 97.1 ±0.2% AS and 56.0 ±0.2% AS. Transcript profiles were determined using RT-QPCR and plotted data are presented as average fold change (normalised against the expression of the control gene *rpl8*, n=6-8 per treatment group). Statistics were carried out using accepted minimum adequate models (analysis of variance model, R; P<0.05), with model details reported in Table 1. For linuron, letters above each bar indicate significant differences between treatment groups when there was a significant interaction from the model (Tukey' HSD post hoc test; P<0.05). For flutamide there was no significant interaction from the model, but a significant effect of treatment was observed (P<0.001).

486 **Table 1**

487 Analysis of variance models for the relationships between chemical exposure, air

488 saturation and the chemical/air saturation interaction measured in fish exposed to A)

489 flutamide and B) linuron under 97% or 56% AS. Minimum adequate models (F value)

490 for gonadosomatic index (GSI) and for relative transcription are shown for the genes

491 analysed: androgen receptors (*ar1* and *ar2*), catalase (*cat*), cyctochrome P450, family

492 1, subfamily A (*cyp1a*), estrogen receptors (*esr1*, *esr2a* and *esr2b*), 3-hydroxy-3-

493 methylglutaryl-coenzyme A synthase (*hmgcs*), hypoxia inducible factor 1-α (*hif-1α*),

494 isopentenyl-diphosphate delta isomerase 1 (*idi*), insulin-like growth factor-binding

495 protein 1b (*igfbp1b*) and spiggin (*spg*). The minimum adequate models were selected

496 by model simplification using F tests based on analysis of variance. (Significance

497 codes: * P<0.05, ** P<0.01, *** P<0.001, NS P>0.05; Not Significant).

	df	Minimum Adequate Model		
		Treatment	Air Saturation	Treatment / Air Sat. Interaction
A) Flutamide				
GSI	29	5.39*	NS	NS
ar1	NS	NS	NS	NS
ar2	NS	NS	NS	NS
cat	26	10.38***	2.71	5.80*
cyp1a	NS	NS	NS	NS
esr1	NS	NS	NS	NS
esr2a	NS	NS	NS	NS
esr2b	NS	NS	NS	NS
hif1α	27	294.82***	NS	NS
hmgcs	NS	NS	NS	NS
idi	27	NS	6.39*	NS
igfbp1b	26	NS	4.22	NS
spg	27	22.03***	NS	NS
B) Linuron				
GSI	29	4.76*	NS	NS
ar1	NS	NS	NS	NS
ar2	NS	NS	NS	NS
cat	NS	NS	NS	NS
cyp1a	20	1.53	20.90***	3.98
esr1	NS	NS	NS	NS

esr2a	NS	NS	NS	NS
esr2b	NS	NS	NS	NS
hif1α	26	226.58***	0.45	4.73*
hmgcs	NS	NS	NS	NS
idi	NS	NS	NS	NS
igfbp1b	NS	NS	NS	NS
spg	26	0.63	5.78*	4.02

498

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