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

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SI – MATERIAL AND METHODS

Fish culture and husbandry

Fish were maintained in mixed sex stock tanks (112 L), at a temperature of 12±1°C and a photoperiod of 8:16 light/dark (winter conditions; with a 30-minute transitional dawn/dusk period). Tanks were supplied with mains tap water which was filtered by reverse osmosis (Environmental Water Systems (UK) Ltd) and reconstituted with Analar-grade mineral salts to standardized synthetic freshwater (final concentrations to give a conductivity of 300 mS: 122 mg/L $CaCl_2 2H_2O$, 9.4 mg/L $NaHCO_3$, 50 mg/L $MgSO_4 7H_2O$, 2.5 mg/L KCl, 50 mg/L), aerated, and maintained at 12±1 °C in a reservoir (Paull et al., 2008).

Sex identification

Males were identified using PCR for an allozyme of isocitrate dehydrogenase (*idh*), which was found to be sexually dimorphic in the three-spined stickleback (Peichel et al., 2004). Initially, fish were pre-selected as males based on morphological parameters as described by De Kermoysan et al. (De Kermonysan et al., 2013). Spine clips (second dorsal spine) from fish perceived to be males were collected, DNA was extracted using the HotSHOT method (Truett et al., 2000), and then amplified in PCR reactions using the *idh* primers (5′-GGGACGAGCAAGATTTATTG-3′ and 5′-TTATCGTTAGCCAGGAGATGG-3′). The amplified products were then visualised on a 2% agarose gel, as described in Peichel et al (Peichel et al., 2004). The primers amplified a single 302bp fragment in females and two (302bp and 271bp) fragments in males, allowing the confirmation of genetic males. After identification (100% accurate), males were maintained in high-density under winter conditions to prevent sexual maturation prior to the experimental exposures.

Water chemistry analysis

Initially water samples containing flutamide were mixed with methanol (HPLC grade, Fischer chemical) and 0.2% formic acid (Sigma Aldrich), and linuron samples were mixed with methanol (HPLC gradient, Fischer), all in a 1:1 ratio. For each solvent and water control tanks, 3 water samples were taken and each treated in the same way as samples containing each of these chemicals. Samples were stored at -20˚C until analysis with LC-MS was carried out.

Analyses were performed using Surveyor MS Pump Plus HPLC pump with HTC PAL autosampler coupled to TSQ Vantage triple quadrupole mass spectrometer equipped with heated electrospray (HESI II) source (ThermoFisher Scientific, Hemel Hempstead, UK). Chromatographic separation was achieved using reversed-phase, 3 µm particle size, C18 Hypersil GOLD column 50 mm × 2.1 mm i.d. (Thermo Scientific, San Jose CA, USA). Analytes were separated using a linear gradient of (A) water and (B) methanol, both containing 0.1% of formic acid. Solvent B (methanol) increased from 20% to 100% in 1.5 min and was maintained for 1.5 min before returning to the initial conditions for 2 min. The flow rate was 500 µL/min and the temperature of the autosampler was set at 8˚C, while the column was kept at room temperature. The HESI probe was operating in both negative and positive mode; an ion-spray voltage of -2.75 kV was applied for analysis of flutamide and +3.75 kV for linuron. The heated capillary temperature was set at 270 °C and the vaporizer temperature was 350 °C. Nitrogen was employed as sheath and auxiliary gas at a pressure of 60 and 2 arbitrary units, respectively. The argon CID gas was used at a pressure of 1.5 mTorr and the optimum collision energy (CE) for each transition was selected. Quantification of the target compounds was performed by monitoring two characteristic multiple reaction monitoring transitions using an external standard method (Table S4).

Transcript Profiling

Spiggin primers were designed over a conserved generic region from all annotated *spg* genes in order to measure the transcription of all isoforms, simultaneously. Beacon Designer 3.0 software (Premier Biosoft International, Paulo Alto, CA) was used to design the primers for all other genes. Assays were optimised and standard curves were generated for each transcript as previously described (Uren Webster et al. 2014). Single amplification of products, at the expected melting temperature, confirmed primer specificity. For each case, the linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was >0.99, and efficiency values were between 1.8 and 2.1. The primer sequences, annealing temperatures, efficiencies and PCR product sizes for each primer pair are shown in Table S1.

RNA was extracted from the kidneys and livers of 8 male fish from each treatment group using the TRI reagent method (Sigma-Aldrich, UK), according to the manufacturer's instructions. After extraction, a NanoDrop-1000 Spectrophotometer (NanoDrop technologies, Wilmington, USA) was used to assess the concentration and purity of the resulting RNA. DNase treatment (RQ1 DNase, Promega, Southampton, UK) was carried out on one µg of RNA to remove potential DNA contamination, prior to conversion to cDNA using M-MLV reverse transcriptase (Promega, UK) and primed with random hexamers (MWG-Biotech), according to the manufacturer's instructions (MJ Research PTC200 Thermal Cycle). The resulting cDNA was diluted (1:2) before RT-QPCR was performed. RT-QPCR was carried out using an iCylcer iQ Real-time Detection System (BioRad Laboratories, Hercules, USA) and SYBR green chemistry (BioRad Laboratories, Hercules, USA). Each sample was run in duplicate in 96-well optical plates (BioRad Laboratories, Hercules, USA) in 15µl reaction volumes. Efficiency-corrected relative expression levels were determined prior to normalisation to a control gene, using the $2^{-\Delta\Delta}$CT method (Livak et al., 2001). For *esr1*, due to low expression levels, neat cDNA (instead of 1:2 diluted) was used, with the control gene also run in neat samples for

appropriate normalisation. Efficiency values for the standard curves starting from neat cDNA were 2.13 and 2.11 for *esr1* and *rpl8*, respectively.

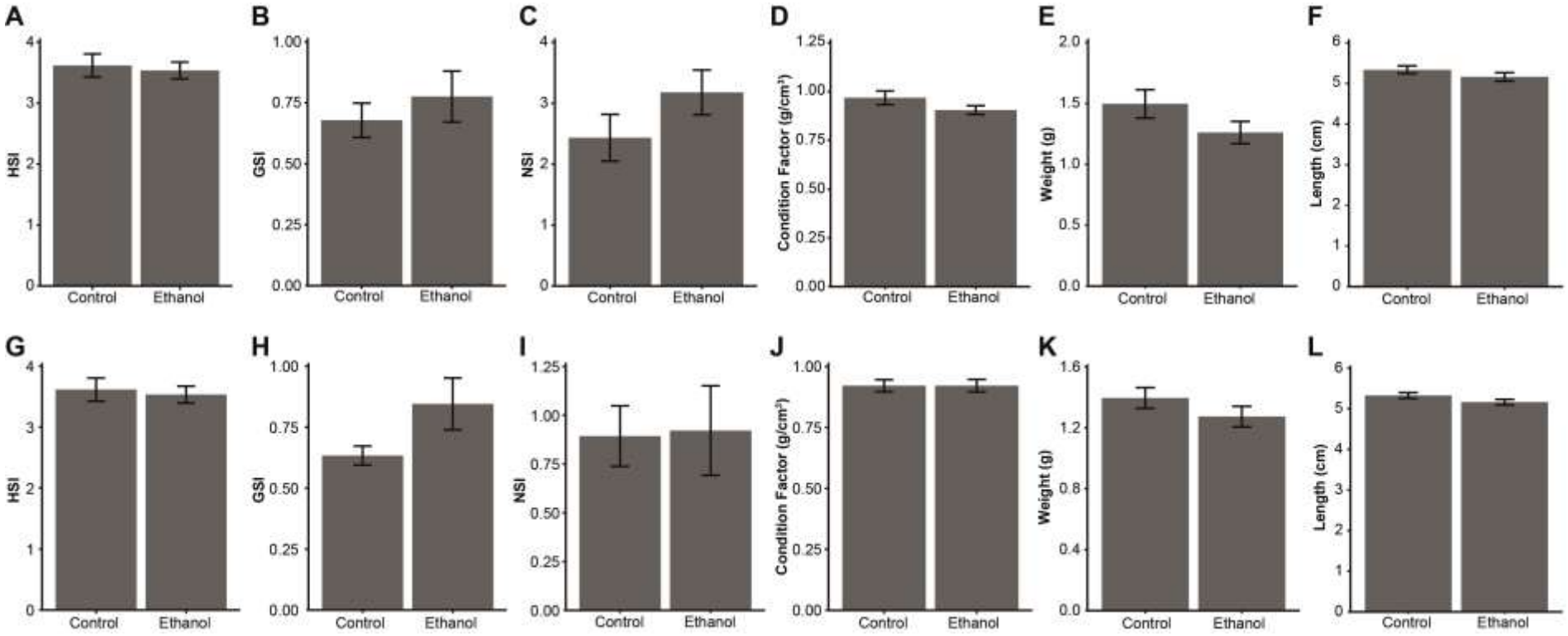


Figure S1. Comparison between control and solvent control (0.001% ethanol) treatments for the different morphometric parameters under 97.1 ±0.2% air saturation (AS; A - F) and 56.0 ±0.2% AS (G - H). Morphometric parameters measured included: heaptatosomatic index (HSI; A and G), gonadosomatic index (GSI; B and H), nephrosomatic index (NSI; C and I), condition factor (g/cm^3; D and J), wet weight (g; E and K) and fork length (cm; F and L). There was no significant difference between the water control and the solvent control for any of the parameters under 97% AS tested (HSI: P=0.734, GSI: P=0.422, NSI: P=0.184, Condition Factor: P=0.160, Weight: P=0.137, Length: P=0.240) and 56% AS (HSI: P=0.734, GSI: P=0.081, NSI: P=0.929, Condition Factor: P=0.994, Weight: P=0.224, Length: P=0.240).

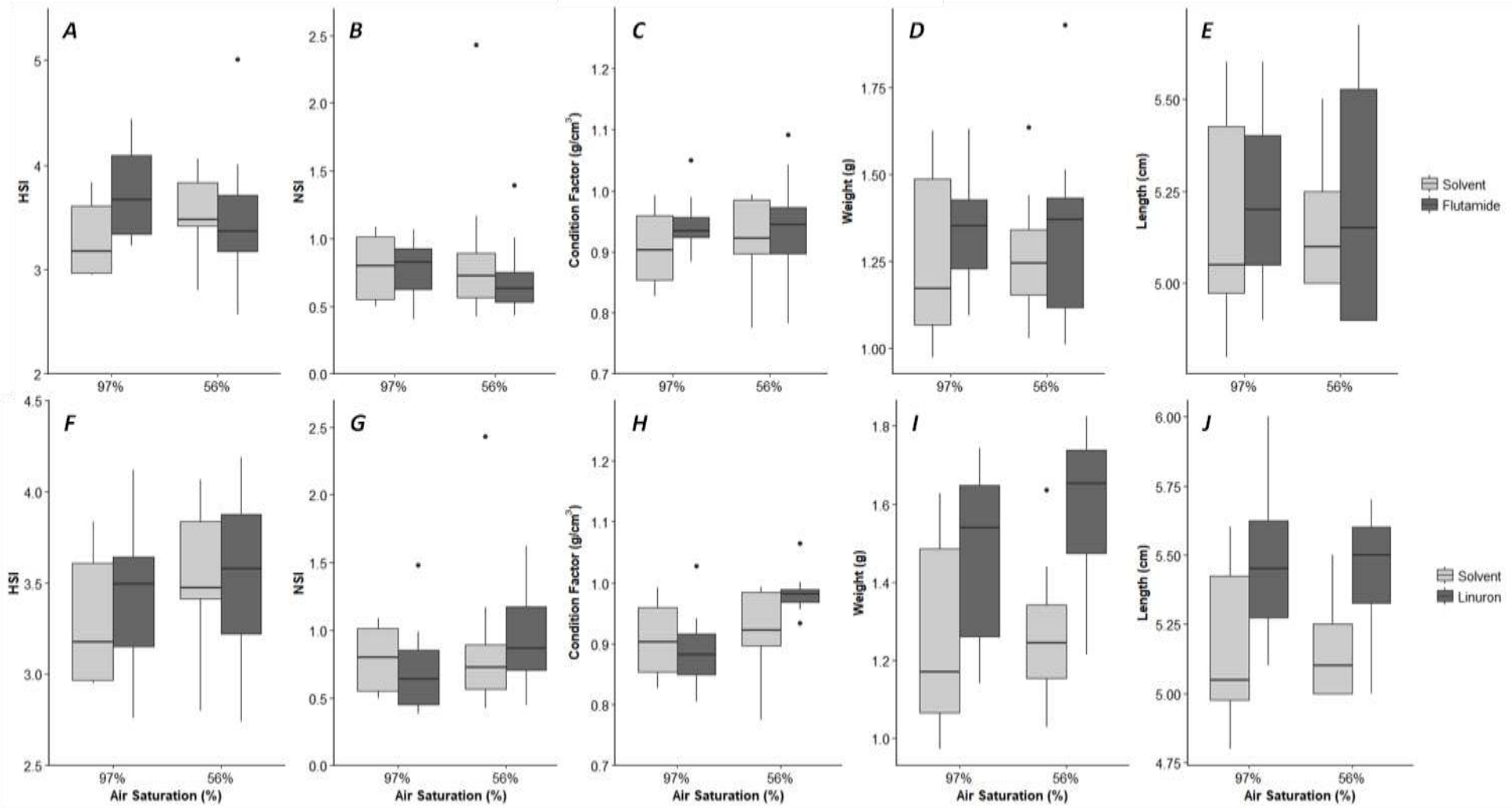
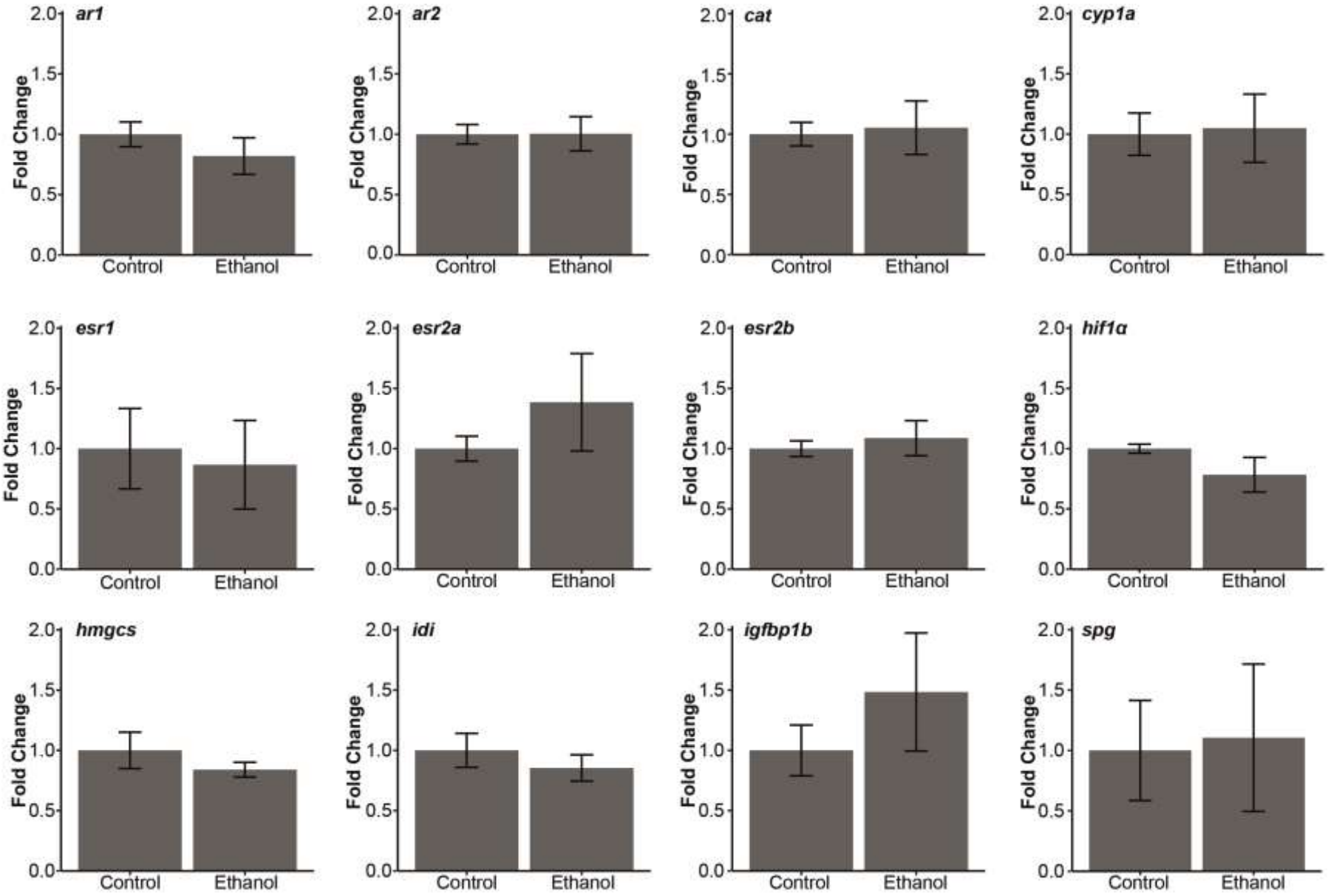


Figure S2. Morphometric parameters of fish following exposure to the anti-androgenic chemicals flutamide and linuron under different levels of air saturation. Male sticklebacks were exposed to A-E: 0 or 250 µg Flutamide/L or F-J: 0 or 250 µg Linuron/L for 7 days under

97.1 ±0.2% AS and 56.0 ±0.2% AS (n=8 individuals per treatment). Morphometric parameters measured included: heaptatosomatic index (HSI; A and F), nephrosomatic index (NSI; B and G), condition factor (g/cm^3; C and H), wet weight (g; D and I) and fork length (cm; E and J). Statistics were carried out using accepted minimum adequate models (analysis of variance model, R; $P<0.05$) with model details reported in Table S3. There was a significant effect of linuron exposure on weight and length ($P=0.004$ and $P=0.004$, respectively), and a significant effect of air saturation on the condition factor independently ($P=0.023$).


ar1
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0.0
0.5
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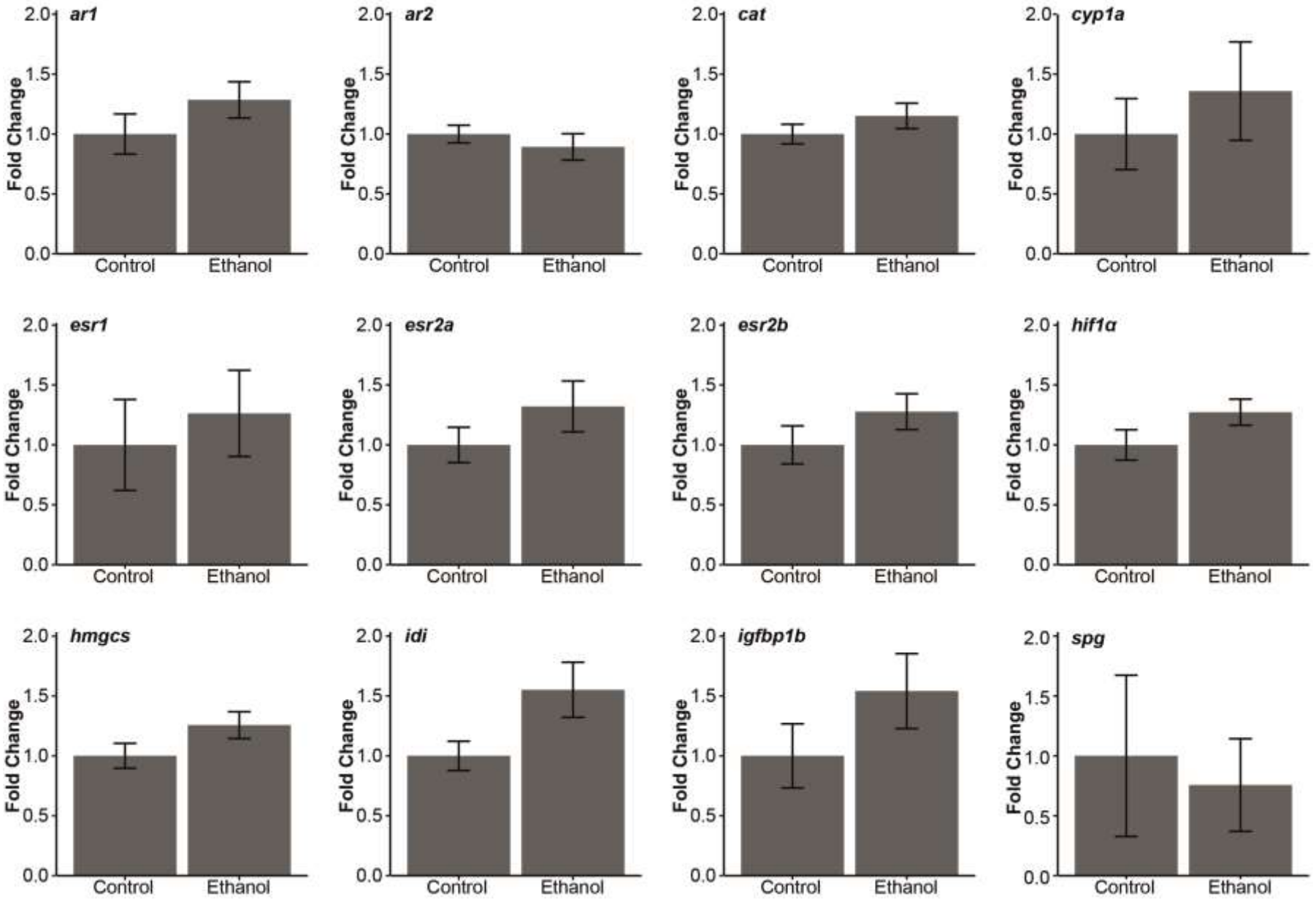
2.0
Control
Ethanol

Figure S3. Comparison between the transcript profiles for control and solvent control (0.001% ethanol) treatments under 97% AS for selected target genes. Male fish kept in either water alone or water containing 0.001% ethanol for 7 days under 97% AS are compared. 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*), hypoxia inducible factor 1α (*hif-1α*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), isopentenyl-diphosphate delta isomerase 1 (*idi*), insulin-like growth factor-binding protein 1b (*igfbp1b*) and spiggin (*spg*). Plotted data are presented as average fold change (normalised against the expression of the control gene for liver samples: ribosomal protein L8 (*rpl8*); for kidney samples: ubiquitin (*ubi*)). Points for which the transcription was below the detection limit of the assay were excluded from the analysis, resulting in a replication of n=6-8 fish per treatment group. There was no significant difference between the water control and the solvent control for any of the genes tested (*ar1*: P=0.345, *ar2*: P=0.981, *cat*: P=0.833, *cyp1a*: P=0.885, *esr1*: P=0.793, *esr2a*: P=0.394, *esr2b*: P=0.596, *hif1α*: P=0.190, *hmgcs*: P=0.355, *idi*: P=0.432, *igfbp1b*: P=0.580 and *spg*: P=0.747).


ar1
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Fold Change
0.0
0.5
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Control
Ethanol

Figure S4. Comparison between transcript profiles for control and solvent control (0.001% ethanol) treatments under 56% AS for selected target genes. Male fish kept in either water alone or water containing 0.001% ethanol for 7 days under 56.0% AS are compared. 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*), hypoxia inducible factor 1α (*hif-1α*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*hmgcs*), isopentenyl-diphosphate delta isomerase 1 (*idi*), insulin-like growth factor-binding protein 1b (*igfbp1b*) and spiggin (*spg*). Plotted data are presented as average fold change (normalised against the expression of the control gene for liver samples: ribosomal protein L8 (*rpl8*); for kidney samples: ubiquitin (*ubi*)). Points for which the transcription was below the detection limit of the assay were excluded from the analysis, resulting in a replication of n=6-8 fish per treatment group. There was no significant difference between the water control and the solvent control for any of the genes tested (*ar1*: P=0.230, *ar2*: P=0.335, *cat*: P=0.332, *cyp1a*: P=0.759, *esr1*: P=0.406, *esr2a*: P=0.248, *esr2b*: P=0.224, *hif1α*: P=0.128, *hmgcs*: P=0.116, *idi*: P=0.059, *igfbp1b*: P=0.123 and *spg*: P=0.554).

Table S1: Details of primer sequences and assay conditions for the QPCR assays

Gene Name	Gene Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Ta (°C)	PCR Efficiency
Ribosomal protein L8	*rpl8*	GGTGCGTCCCTCCTGATG	GCGTGGTGTGGCTATGAAC	93	60.5	2.059
Beta tubulin	*tubb4*	TCTTCAGACCAGACAACTT	CTCCTTCCTCACCACATC	119	60.0	2.025
Ubiquitin	*ubc*	GGAGGGCAGTAAAGTGATT	CAAGGCAGGAGATTCAGTT	161	57.0	1.967
Spiggin	*spg*	GCTTTGAAAACAGCCAGAGCATCT	TGGACAGGAACAGGTTTCAGTGAGT	212	58.5	2.001
Androgen receptor 1	*ar1*	CATACACTCTCACTAACA	GTTCATACATACTGGAAAC	90	56.0	2.008
Androgen receptor 2	*ar2*	CGGAAGGCAAACAGAAATAC	CGACAGGATGGACAGTTC	85	59.5	1.959
Estrogen receptor 1	*esr1*	TTGGAATAGAGGCAGGAG	GGAGTGGAGACGAGTATC	85	62.0	2.130
Estrogen receptor 2a	*esr2a*	GCCTCTCAGAAATCTTTG	CAGACATACTCCTCTCTC	82	56.0	2.108
Estrogen receptor 2b	*esr2b*	CAAGAACCGACGCAAAAG	TACACCGCACTTCATCAT	76	59.0	1.926
Cyctochrome P450, family 1, subfamily A	*cyp1a*	CCTTCGCCATTCTTCATTC	GACCTGCCACTGATTGAT	121	59.0	2.029
Hypoxia inducible factor 1 alpha	*hif1α*	GGCAATGGAAGACTTGGA	TGGACTGGAGAACCTTGA	135	60.5	2.083
Catalase	*cat*	CCAGAAGCGTAATCCTCAA	GAACAAGAAAGACACCTGATG	100	59.0	2.003
3-hydroxy-3-methylglutaryl-coenzyme A synthase	*hmgcs*	GGTTTTGACAGACTTGGACTTG	GGTTGATGGAGCGGAATGG	95	62.0	1.959
Isopentenyl-diphosphate delta isomerase 1	*idi*	AGAGCGTTCAGCGTGTTC	TCCGTGTGTAGAGGGTGAC	125	60.0	2.041
Insulin-like growth factor-binding protein 1b	*igfbp1b*	CCCAACTGCGACAAACAC	TTCTTGCCGTTCCAGGAG	104	61.0	2.098

Table S2: Measured concentrations for the anti-androgenic chemicals in the exposure water. Concentrations were measured for 8 replicate treatment tanks on days 1, 2 and 6 and are presented as mean values ± SEM.

Anti-Androgen	**Flutamide**				**Linuron**			
AS Treatment	**97%**		**56%**		**97%**		**56%**	
Nominal Concentration	**0µg/L**	**250µg/L**	**0µg/L**	**250µg/L**	**0µg/L**	**250µg/L**	**0µg/L**	**250µg/L**
Day 1	<0.98	257 ± 9.4	<0.98	262 ± 10.5	<0.98	260 ± 5.7	<0.98	270 ± 6.0
Day 2	<0.98	242 ± 7.1	<0.98	239 ± 6.4	<0.98	280 ± 7.6	<0.98	286 ± 5.6
Day 6	<0.98	268 ± 8.4	<0.98	264 ± 6.5	<0.98	297 ± 8.9	<0.98	257 ± 15.2
Mean	**<0.98**	**256 ± 5.1**	**<0.98**	**255 ±5.1**	**<0.98**	**278 ±5.2**	**<0.98**	**271 ± 6.1**

Table S3: Analysis of variance models for the relationships between chemical exposure, air saturation and the chemical/air saturation interaction measured in fish exposed to A) flutamide and B) linuron under 97% or 56% AS. The minimum adequate models were selected by model simplification using F tests based on analysis of variance. Minimum adequate models (F value) are shown for the parameters analysed: heaptatosomatic index (HSI), nephrosomatic index (NSI), condition factor (CF), wet weight (g) and fork length (cm). (Significance codes: * P<0.05, ** P<0.01, *** P<0.001)

	df	**Minimum Adequate Model**		
		Treatment	**Air Saturation**	**Treatment / Air Sat. Interaction**
A) Flutamide				
HSI	NS	NS	NS	NS
NSI	NS	NS	NS	NS
CF	NS	NS	NS	NS
Weight	NS	NS	NS	NS
Length	NS	NS	NS	NS
B) Linuron				
HSI	NS	NS	NS	NS
NSI	NS	NS	NS	NS
CF	30	NS	5.70*	NS
Weight	28	9.95**	NS	NS
Length	29	10.03**	NS	NS

Table S4: Details for the quantification of the target compounds performed by monitoring two characteristic multiple reaction monitoring transitions.

Compound	Parent ion (*m*/*z*)	Product ion (*m*/*z*)	Collision energy (V)
Linuron	249.0	160.1	17
		182.1	16
Flutamide	275.1	202.1	25
		205.1	24

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