Environmental Pollution 270 (2021) 116286

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Transcriptome-wide changes associated with the reproductive behaviour of male guppies exposed to 17α-ethinyl estradiol☆

Minna Saaristo [a, b, c, *], John A. Craft [d], Sonika Tyagi [b], Christopher P. Johnstone [b], Mayumi Allinson [e], Khalid S. Ibrahim [d, f], Bob B.M. Wong [b]

[a] EPA Victoria, Water Sciences, Melbourne, Australia
[b] School of Biological Sciences, Monash University, Australia
[c] Department of Biosciences, Åbo Akademi University, Finland
[d] Life Sciences, Glasgow Caledonian University, UK
[e] Department of Chemical Engineering, University of Melbourne, Australia
[f] Department of Biology, University of Zakho, Kurdistan Region, Iraq

ARTICLE INFO

Article history:
Received 25 September 2020
Received in revised form
24 November 2020
Accepted 9 December 2020
Available online 13 December 2020

Keywords:
Adverse effects
Behavioural ecotoxicology
Emerging contaminant
Mate choice
Pharmaceutical
Sexual selection

ABSTRACT

Although many pharmaceutical compounds (and their metabolites) can induce harmful impacts at the molecular, physiological and behavioural levels, their underlying mechanistic associations have remained largely unexplored. Here, we utilized RNA-Seq to build a whole brain transcriptome profile to examine the impact of a common endocrine disrupting pharmaceutical (17α-ethinyl estradiol, EE2) on reproductive behaviour in wild guppies (*Poecilia reticulata*). Specifically, we annotated 16,791 coding transcripts in whole brain tissue in relation to the courtship behaviour (i.e. sigmoid display) of EE2 exposed (at environmentally relevant concentration of 8 ng/L for 28-days) and unexposed guppies. Further, we obtained 10,960 assembled transcripts matching in the non-coding orthologous genomes. Behavioural responses were assessed using a standard mate choice experiment, which allowed us to disentangle chemical cues from visual cues. We found that a high proportion of the RNAseq reads aligned back to our *de novo* assembled transcriptome with 80.59% mapping rate. Behavioural experiments showed that when males were presented only with female visual cues, there was a significant interaction between male treatment and female treatment in the time spent in the preference zone. This is one of the first studies to show that transcriptome-wide changes are associated with the reproductive behaviour of fish: EE2 exposed male guppies that performed high levels of courtship had a gene profile that deviated the most from the other treatment groups, while both non-courting EE2 and control males had similar gene signatures. Using Gene Ontology pathway analysis, our study shows that EE2-exposed males had gene transcripts enriched for pathways associated with altered immunity, starvation, altered metabolism and spermatogenesis. Our study demonstrates that multiple gene networks orchestrate courting behaviour, emphasizing the importance of investigating impacts of pharmaceuticals on gene networks instead of single genes.

1. Introduction

Pharmaceutical pollution is an issue of global environmental concern (Arnold et al., 2014), with discharge of effluent from wastewater treatment plants being a primary source of contamination (Verlicchi et al., 2012). Since pharmaceuticals are manufactured to produce therapeutic effects at low concentrations and can retain pharmacological activity due to their incomplete metabolism in the body (Ort et al., 2010), they have the potential to impact non-target organisms (Brodin et al., 2013; Vasquez et al., 2014; Martin et al., 2017; Bertram et al., 2018; Lagesson et al., 2019). One pharmaceutical of global concern is 17α-ethinyl estradiol (EE2), a synthetic estrogen used commonly in the oral contraceptive pill. Given its widespread use, EE2 has now been reported in surface and effluent waters worldwide at concentrations ranging from 0.1 ng/L (Hannah et al., 2009) to 11 ng/L

☆ This paper has been recommended for acceptance by Dr. Da Chen.

* Corresponding author. School of Biological Sciences, Monash University, Australia.
E-mail address: minna.saaristo@monash.edu (M. Saaristo).

https://doi.org/10.1016/j.envpol.2020.116286
0269-7491/

(Tiedeken et al., 2017). Since the biological pathways targeted by EE2 are evolutionarily conserved (Ankley et al., 2007), EE2 has been shown to have adverse effects on a wide range of organisms, from ovarian regression in frogs (Vu et al., 2015) and reduced sperm count in mice (Porro et al., 2015) to eggshell thinning in birds (Holm et al., 2006), and population collapse in fish (Kidd et al., 2007, 2014).

Sex hormones play an important role in regulating and modulating sex-specific behaviours (Xu et al., 2012). Although, in mammals and birds, sexual differentiation of the brain is considered permanent (Arnold and Breedlove, 1985; McCarthy and Arnold, 2011), in fish, neuroendocrine systems and brain programming is plastic (Rosenfeld et al., 2017). Gonadal hormones act via nuclear and membrane receptors (Zhu et al., 2003), effects of which are modulated by neurotransmitters, neuropeptides and neurohormones (Rosenfeld et al., 2017). Indeed, the expression of specific behaviours is often based upon both steroid and non-steroid hormones acting via the neuroendocrine system (Goncalves and Oliveira, 2011). In this regard, teleost fish sexual expression (Godwin et al., 1996) and sex determination patterns (Munday et al., 2006) can both be mediated by social and environmental cues driving rapid phenotypic responses (Todd et al., 2016). This is a serious concern, because chemical contaminants, such as EE2, with a capacity to disrupt neuroendocrinology, can directly and indirectly impact sexual differentiation of the brain (Rosenfeld et al., 2017). For instance, previous studies have demonstrated that exposure to EE2 can alter brain structure (Vosges et al., 2010), neurosteroidogenesis (Lyssimachou et al., 2006), neuron network morphology, and synaptic potentiation (Martyniuk et al., 2010). Although there is increasing evidence of the impact of pharmaceuticals on neuroendocrine function in non-target organisms (Volkova et al., 2015; Rosenfeld et al., 2017), much less attention and research effort has been given to investigating how this might affect behaviour.

Behaviour can often be the product and the driver of underlying genetic mechanisms interacting with the environment. For instance, social information triggers alterations in brain and behaviour, which, in turn, affects neural and neuroendocrine regulation, including the transcriptome (Robinson et al., 2008). Much of this evidence comes from research suggesting an apparent link between the expression of specific genes to behaviours, such as sociality (Filiano et al., 2016), aggression (Loveland and Fernald, 2017) and mate choice (Cummings et al., 2008). Genes, however, do not define behaviour directly (Robinson et al., 2008), and the complex patterns of activation between a gene and a specific behaviour are known to vary across and within major taxonomic groups, and even between closely related species (Young et al., 1997). To date, some studies have linked gene responses in the brain to endogenous hormones (Robinson et al., 2008; Rosenfeld et al., 2017), but no study has tested for an association between pharmaceutical-induced behavioural changes and underlying gene signatures. This is true despite research showing that EE2 can alter both gene expression (e.g. gonads: Gardner et al., 2012; liver: De Wit et al., 2010; brain: Saaristo et al., 2017; Porseryd et al., 2018) and behaviour (Söffker and Tyler, 2012; Porseryd et al., 2019; Fenske et al., 2020) of non-target organisms.

Guppies (*Poecilia reticulata*) are an ideal species for exploring the behavioural and transcriptomic responses of wildlife to chemical contaminants. Guppies are a small, sexually dimorphic livebearing freshwater fish and their reproductive behaviours are well characterized. Specifically, male guppies employ two alternate mating strategies, either courting females by performing so called 'sigmoid displays' (Houde, 1997), or forcing copulations by thrusting their modified anal fin towards the female's genital pore (Luyten and Liley, 1991). In this regard, males are known to enjoy higher insemination success through solicited, rather than unsolicited, copulations (Luyten and Liley, 1991). Although previous studies of mate choice in guppies have tended to focus on female choice, male guppies can also be choosy (Dosen and Montgomerie, 2004), and use both visual and chemical cues in mate assessment (Guevara-Fiore et al., 2009).

Behaviour aside, guppies present a promising model to examine the genetic basis of phenotypic variation (Hughes et al., 2005) and the transcriptome of guppies has been the focus of recent investigations. For instance, the following resources now exist: a *de novo* assembly (454 sequencing) of the male and female guppy transcriptomes (brain and body: Fraser et al., 2011), non-barcoded and barcoded library (RNA-Seq) from whole embryos and tissues of male and female guppies (brain, tail, gonad: Sharma et al., 2014), and transcriptome (RNA-Seq) of male and female guppy brains using normalized libraries (Saaristo et al., 2017). In particular, we previously discovered 285 transcripts specific for EE2-treated fish and evidence of sex-specificity (Saaristo et al., 2017). The existence of such resources provides an excellent opportunity with which to unravel the effects of EE2 on the genetic mechanisms and gene signatures underlying phenotypic (i.e. behavioural) variation in the guppy.

Accordingly, in the first study of its kind, we set out to investigate the impacts of EE2-exposure on sexual behaviour, and potential gene signatures in the brain transcripts of, male guppies. To provide an environmentally relevant scenario, we exposed both females and males to EE2, but only focus on the male part of the study here. First, we tested whether exposure to EE2 impacts male mate choice. Based on previous studies in other fish (Bell, 2001; Larsen et al., 2009; Saaristo et al., 2009a,b; Saaristo et al., 2010), we predicted EE2-exposure to either have no effect or decrease male courtship behaviour. Secondly, using a transcriptome wide analysis, we tested whether behaviour is associated with differences in male gene expression profile, and whether any EE2-induced behavioural changes were associated with altered gene expression patterns. According to our previous study (Saaristo et al., 2017), we predict transcript abundances to be specific to EE2-treatment- and sex.

2. Methods

Collection and housing. Wild adult guppies were collected using dip nets from Alligator Creek (19°26′17.94″S, 146°57′1.09″E), which is located adjacent to the Bowling Green Bay National Park in Queensland, Australia. Water quality testing at this location carried out across multiple locations and years confirmed that the guppy population used in our study had not been previously exposed to pharmaceuticals (ALS group, unpubl. data). Mature fish were separated by sex and brought back to Monash University, Melbourne, where they were acclimated to laboratory conditions (+26 °C ± 1, 12: 12 h light: dark regime) for two months in 54 L tanks (20 fish per tank) prior to exposure. Fish subsisted on an *ad libitum* diet of commercial fish pellets. This study was approved by the Biological Sciences Animal Ethics Committee at Monash University (BSCI/2013/09) and complies with all relevant State and Federal laws of Australia.

Exposure design. Guppies were exposed to EE2 for 28-days via a flow-through system using the design of Saaristo et al. (2013). Briefly, the exposure set-up comprised 16 exposure tanks, which was made up of 8 control tanks (a solvent control: 0.00004% ethanol) and 8 exposure tanks (EE2: measured mean concentration of 8 ng/L, SE = 1.13, n = 22 water samples). During the exposure period, water samples were taken weekly from the exposure tanks and measurement of EE2 was conducted using a commercial kit (Ecologiena, EE2 ELISA Kit, Tokiwa Chemical Industries, Japan) in

accordance with the manufacturer's instructions, with minor modifications (Saaristo et al., 2013). A total of 448 fish (224 males, 224 females) were randomly taken from the holding tanks and placed into the exposure tanks, with the sexes kept separately (60 × 30 × 24 cm; 28 fish per tank). All exposure tanks were maintained at a temperature of 25–27 °C, on a 12: 12 h light: dark regime, with fish fed once a day during the exposure period.

Behavioural assays. We examined male mate choice using a standard mate choice experiment (Guevara-Fiore et al., 2009). Specifically, a male (control or EE2) was presented with a dichotomous choice between two size-matched females (one control and one EE2-female; 19.85 ± 0.36 mm and 19.90 ± 0.37 mm). The glass tank (60 × 30 × 28 cm) had 2 cm layer of gravel on the bottom and was filled with filtered freshwater. The tank was divided with transparent acrylic dividers (without perforations to prevent chemical communication) into three sections, with the focal male in the middle compartment and one female in each of the two end compartments. The side compartment occupied by the exposed and control females were alternated between trials to eliminate side bias (Guevara-Fiore et al., 2009).

Prior to each trial, females were introduced first and allowed to acclimate for 5 min, after which, the male was placed in the centre of his compartment inside a plastic container and, after a further 5 min of acclimation, the male was gently released from the container into the compartment and video recorded, over a 15 min period. Trials were video recorded to eliminate the presence of a human observer from disturbing the fish. For each male, the following behaviours were quantified: total time the male spent within a 5 cm preference zone (equivalent to approximately 1.5 body length) in front of each female's compartment and actively interacting with the female, the number of times the male entered the zone, as well as, the number of sigmoid displays performed. Time spent in the preference zone associating with females is widely used in studies of mate choice in fishes (Houde, 1997), and is the standard measure of mating preferences in poeciliids, including guppies (Jeswiet and Godin, 2011). After the trial, the females and dividers were removed and the male was left in the tank overnight. Total sample size for this part of the experiment was: control = 24 males, EE2 = 24 males.

To examine whether a male (control or EE2) is able to discriminate between control and EE2-exposed females, we collected female olfactory cues from 5 L glass tanks holding a mix of five females (small, medium and large in size). The preparation of the chemical cue water started by setting up a large glass tank (60 × 30 × 28 cm) with aquarium corner filters, aquarium heaters and filtered freshwater. The corner filters were used to remove any traces of cues from the filtered tap water. This water was used the next day (24 h later) to fill up the 5 L glass olfactory cue tank after which five females were introduced. During the 24 h the females spent in the tank, they were not fed, but they had visual contact with 5 males in an adjacent glass tank. After 24 h, the females were taken out of the tank and the holding water was used as the olfactory cue tank in the trials. The cue water was used on the day females were removed. Every day we prepared four olfactory cue tanks: 2 x control females and 2 x EE2-exposed females.

The chemical cue assay followed the visual cue assay (i.e. 24 h after). The trials were carried out in the same tank as the visual cue assays (but without the Perspex dividers), because we wanted to minimize handling between trials. Two olfactory cue tanks (one control and one EE2) were placed on a shelf directly above the trial tank. The side the cue was on, was alternated between the trials. Each cue tank had a silicon tube connected to its base, which was fed through a peristaltic pump (Watson-Marlow), which controlled the drip rate (1.5 mL/min) to the actual trial tank. The rate at which the olfactory cues flowed was checked before each trial. After the olfactory cues had been set up, the male was given a 5 min acclimation period. Following this we introduced the olfactory cues into the tank and recorded male behaviour for 15 min. Trials were video recorded to eliminate the presence of a human observer from disturbing the fish. To quantify the behaviours of the focal fish, we watched the recordings blind to treatment using the key-logging, event-recording software JWatcher V1.0. For each male, the following behaviours were quantified: total time the male spent in each preference zone, total time the male spent in the neutral zone and frequency of these behaviours. A total of 48 trials were conducted (n = 24 control males, n = 24 EE2-males).

RNA isolation and quality assessment. After the behavioural assays were completed, both males and females were euthanized, the weight and length measured, whole brain tissue removed under a stereomicroscope and stored in RNALater. Total RNA was isolated from the whole brain using a combination of the Trizol method and the NucleoSpin RNA II kit (Marchery-Nagel, Germany) following the protocol of Saaristo et al. (2017). RNA isolation and quality were determined (Nanodrop ND-1000 and Agilent Bioanalyzer) and the concentrations varied between 15 and 90 ng/uL, 260/280 ratios were >1.8, and RIN were >8.

RNA-Seq library construction and sequencing. Selected RNA samples were sent to LGC Genomics (Berlin, Germany) for cDNA library preparation and next generation sequencing. Specifically, we used RNA samples from individual fish (6 control males, 6 EE2-exposed males), to create biological replicates. Because we had behavioural data for each male, we divided males into two groups based on their degree of sexual interest in the females: (1) those that had performed sigmoid displays during the trial (note: courting males also spent >50% of their time associating with females) versus (2) those that did not perform any sigmoid displays and spent significantly less time associating with females. For sequencing, we chose 3 control males that performed sigmoid display (i.e. male sigmoid control; hereafter 'MSC'), 3 control males that did not perform sigmoid display (i.e. male non-courting control; hereafter MNC), 3 EE2-exposed males that performed sigmoid display (i.e. male sigmoid exposed; hereafter 'MSE') and 3 EE2-exposed males that did not perform sigmoid display (i.e. male non-courting exposed; hereafter 'MNE'). Our sequencing strategy was based upon the finding of EE2-exposed males showing high levels of courtship (Liu et al., 2013). Also, to cover estrogen-specific rare transcripts, we used both female and male samples for building the transcriptome. Poly-A containing mRNA was isolated via oligo-dT selection on mRNA Catcher Plates (Invitrogen) and used for the preparation of strand-specific cDNA libraries with the Ovation® Universal RNA-Seq System 1–16 (NuGEN) but without DNase treatment (Borodina et al., 2011) and individual libraries were prepared by amplification and barcoding before pooling. Sequencing was carried out on an Illumina Hiseq 2000 platform with 100bp paired-end (read length = 2 × 100bp), which was run on 2 lanes and 6 samples per channel (LGC Genomics, Berlin, Germany).

Illumina data analysis and sequence assembly. Initial sequence processing was conducted at LGC. All libraries were demultiplexed using the Illumina bcl2fastq (1.8.4) software to generate raw reads (FASTQ format). Mismatches of 1 or 2 Ns were permitted in the barcode read when the barcode distances between all libraries on the lane allowed for it. Clipped reads were produced by removing sequencing adapter remnants from all raw reads, as well as, reads with final length <20 bases. FastQC reports were produced for all FASTQ files and confirmed phred quality values > 20. Read counts for the demultiplexed data are presented in Supplementary Table S1.

We used the published guppy reference genome (Guppy_female_1.0_MT, INSDC Assembly GCA_000633615.2) (Künstner et al.,

2016) to perform alignment of RNAseq reads using HISAT2 aligner (v2.1.0) (Kim et al., 2015). On average we found 40–60% reads aligning back to the reference genome (Supplementary Table S1). Further we checked the coverage of the known guppy genes (Guppy_female_1.0_MT; dated April 24, 2014) in our RNAseq data but found only 20–30% mapped reads overlapping the published annotation (Supplementary Table S1). Given low mapping rates of our RNAseq reads with the published genome and gene annotation, this suggested that a large snapshot of our RNAseq experiment had not been covered in the existing annotation. We therefore decided to run a *de novo* assembly of the transcriptome using published reference genome information as a guide. We analysed both the coding and non-coding parts of the transcriptome.

A *de novo* transcriptome was assembled using TRINITY (v2.8.5) (Grabherr et al., 2011) in parallel mode on a high-performance compute cluster at Monash eResearch Centre with 16 CPUs and memory set at 300 GB. Additional analysis was also computed on ARCHIE-WeSt High Performance Computer (www.archie-west.ac.uk) based at the University of Strathclyde. All reads (both male and female) were concatenated into a single set, the directionality of the strand-specific library specified and run TRINITY in genome-guided settings (Bryant et al., 2017). Females were included to incorporate female-specific transcripts that might be produced in EE2-treated males. The reads were then normalized in-silico prior to assembly generation (default settings).

The assembled transcripts underwent processing through best practice blastx and blastn pipelines suggested in the TRINITY workflow to find orthologous matches in three different databases i.e. uniprot (The UniProt Consortium, 2019), nucleotide database at NCBI and ncRNA database generated using RNAcentral (PMID: 30395267). Further, we used TransDecoder (Haas et al., 2013) to identify candidate coding regions within the assembled transcript sequences. Transdecoder looks for the presence of an Open Reading Frame (ORF) and predicts a transcript to be coding based on several computational coding potential criteria. The coding transcripts from each sample were then used to perform gene co-expression analysis to explore system-level functionality of genes. We used Salmon (Patro et al., 2017) to quantify expression of assembled transcripts using an alignment free approach.

2.1. Gene signatures

We examined groups of co-expressed genes (aka “modules”) to generate gene expression signatures related to each condition (Zhang and Horvath, 2005). Specifically, we generated gene expression signatures related to each treatment group by considering co-expressed gene modules using a R based tool called CEMiTool (Russo et al., 2018). Four co-expressed modules with trait significance resulted from the analysis, namely M1, M3, M7 and M8. To identify if the co-expressed genes in a module belong to a certain pathway or a function, we obtained overrepresented biological processes from Gene Ontology (GO) knowledgebase (http://geneontology.org/). We used the *human* database as a reference to perform the GO analysis which gave us the highest number of matching gene terms. Finally, using gene set enrichment analysis (GSEA) we assessed whether the genes in a module showed significant, concordant difference between treatment groups. This is measured as a net enrichment score (NES) shown as positively correlated (red) or negatively correlated (blue).

3. Results

Behavioural assays. Exposure to EE2 had no effect on the frequency of male sigmoid courtship displays (courting control: $n = 8$; non-courting control: $n = 16$; courting EE2: $n = 7$; non-courting EE2: $n = 8$; $\chi^2 = 0.1$, $df = 1$, $p = 0.756$). When males were presented only with female visual cues, there was a significant interaction between male treatment and female treatment ($df = 87$, $t = 2.29$, $p = 0.024$) in the time spent in the preference zone. Specifically, EE2 males spent more time in the preference zone of EE2 females compared to control females, while control males showed no preference in either direction (Fig. 1a). There was no difference in the number of times males (control or EE2) entered the preference zones ($z = 1.88$, $p = 0.061$). When males were presented only with female chemical cues, EE2 exposure had no effect on the total time males (control or EE2) spent in the preference zone (interaction term ‘male treatment x chemical cue’: $df = 79$, $t = -1.105$, $p = 0.272$). However, EE2 males showed a preference for control-female chemical cues over EE2 female

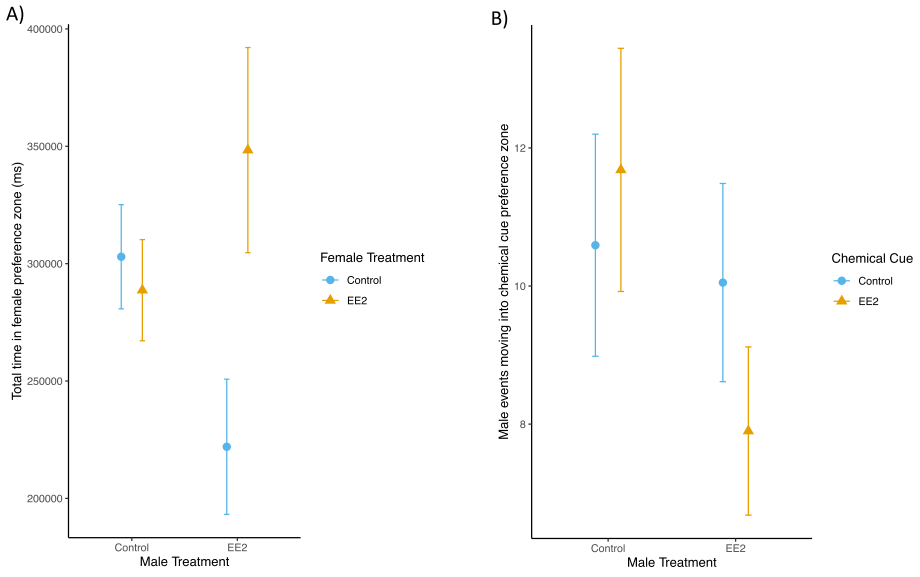

Fig. 1. (A) Mean total time (+SE) male associated with females when presented with female visual cues only and (B) mean number of times (+SE) male visited the chemical cue preference zone when presented with female chemical cues only. Blue = control female; Yellow = EE2-exposed female. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

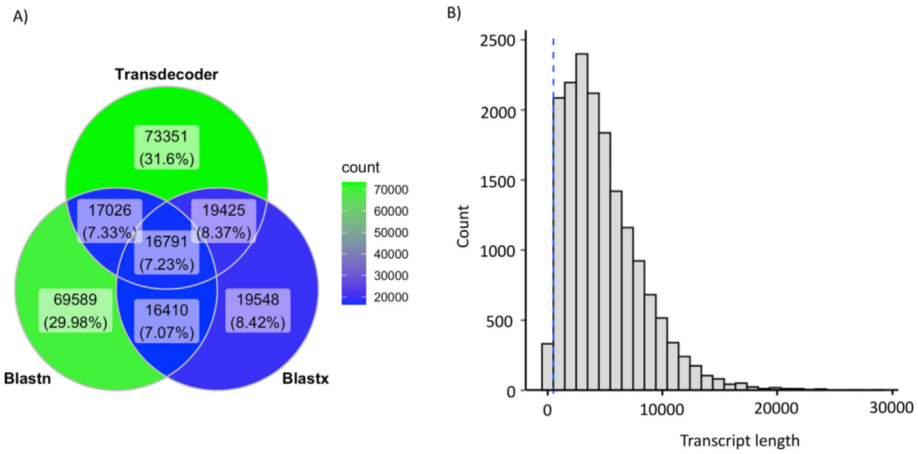

Fig. 2. A) Venn diagram shows the number of common coding transcripts obtained from three different approaches (Blastn, Blastx, Transdecoder). B) Histogram of common coding transcripts lengths. Blue vertical intercept marks 500 nucleotides position. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

chemical cues (interaction between 'male treatment x chemical cue': $z = -2.45$, $p = 0.014$; Fig. 1b).

Illumina data analysis and sequence assembly. A total of 345M demultiplexed, joined and quality filtered reads were obtained from the twelve male guppies (Supplementary Table S1). The assembled transcriptome contained 554,661 putative transcripts (including reads from twelve females). To assess the quality of the *de novo* assembly, we computed an Ex90N50 value, which was computed to be 3.2 kb and consisted of 184,738 transcripts representing 90% of the total normalized gene expression captured in our RNAseq experiment (Supplementary Table S1). A high proportion of the RNAseq reads could be aligned back to our *de novo* assembled transcriptome with 80.59% mapping rate of which 71% showed concordant alignment with expected read pairing and orientation. We obtained matches for 119,816 transcripts using blastn, 72,174 matches with blastx and 160,799 transcripts were predicted to contain an ORF (Supplementary Table S2). Additionally, we performed blastn with the non-coding RNA database (ftp://ftp.ebi.ac.uk/pub/databases/RNAcentral) to confirm composition of noncoding transcripts in our data. We further filtered these blast results (10,960 transcripts) with a minimum 50% query length matching criteria giving us 2219 non-coding transcripts, of which, 637 are known long-noncoding-RNA (Supplementary Table S3). High numbers of non-coding RNA transcripts suggest that these may have a role in gene expression regulation under the EE2 condition. A more targeted in-depth sequencing of the non-coding transcriptome may be performed in future to explore their expression and to confirm their role. In general, there were a large number of transcripts obtained from our analysis that can further be annotated and studied for their function. A total of 16,791 coding transcripts were confirmed by intersecting results from transdecoder, blastn and blastx (Fig. 2A), and were used for downstream functional analysis. Although the minimum length cutoff of the transcripts was selected to be 200 nucleotides, the majority of the intersecting transcripts were longer than 500 nucleotides long (Fig. 2B).

Gene signatures: Functional analyses based on gene expression signatures revealed that M1 was associated with treatment and behaviour (Fig. 3). Specifically, courting control males had a strong positive signature, non-courting control males and courting EE2 males had a negative signature, while the signature was absent in non-courting EE2 males. Further, M7 appeared to be associated with treatment; non-courting control males had a positive signature and courting EE2 males had a negative signature, while this signature was missing in non-courting EE2 and courting control males (Fig. 3). M3 and M8 were associated with non-courting behaviour (Fig. 3). In both cases, non-courting control males had a negative signature, non-courting EE2 males had a positive signature, while the signatures were missing from both courting control and courting EE2 males (Fig. 4).

4. Discussion

This study demonstrated that exposure to an environmentally relevant concentration of a common pharmaceutical endocrine disruptor, EE2, alters the brain gene profile of male guppies. Importantly, we showed, for the first time, that transcriptome-wide changes are associated with the reproductive behaviour of fish: EE2 exposed male guppies that performed high levels of courtship had a gene profile that deviated the most from the other treatment groups, while both non-courting EE2 and control males had similar gene signatures.

We found that exposure to EE2 did not alter male courtship behaviour but, instead, altered their mate choice. Specifically, when males were presented only with visual cues of females, EE2 males spent more time associating and performing courtship towards EE2 females than control females. The observation that courtship behaviour seems resilient to the effects of EE2 could be partially explained by the fact that, in teleost fish, estrogens are locally produced within the brain itself (Pellegrini et al., 2016). However, it is worth noting that this resistance to the feminizing effects of EE2 is consistent with some fish studies (Larsen et al., 2009; Coe et al., 2010; Henriksen et al., 2016; Saaristo et al., 2019), but not with others (Bell, 2001; Bjerselius et al., 2001; Oshima et al., 2003; Kristensen et al., 2005). The reasons for these differences are not well understood. One possibility could be due to the complex actions of gonadal hormones and how they are modulated by neurotransmitters, neuropeptides, and neurohormones (Rosenfeld et al., 2017). Further, differences between studies could be due to species differences, the variety of experimental designs used, as well as the exposure concentrations employed (Saaristo et al., 2018). In any case, altered mate choice, as was evidenced in our study, might have a direct bearing on male fitness. Indeed, when males were presented only with chemical cues of females, we found that EE2 males preferred the control female cues and made more visits to the preference zone of that cue. This mismatched

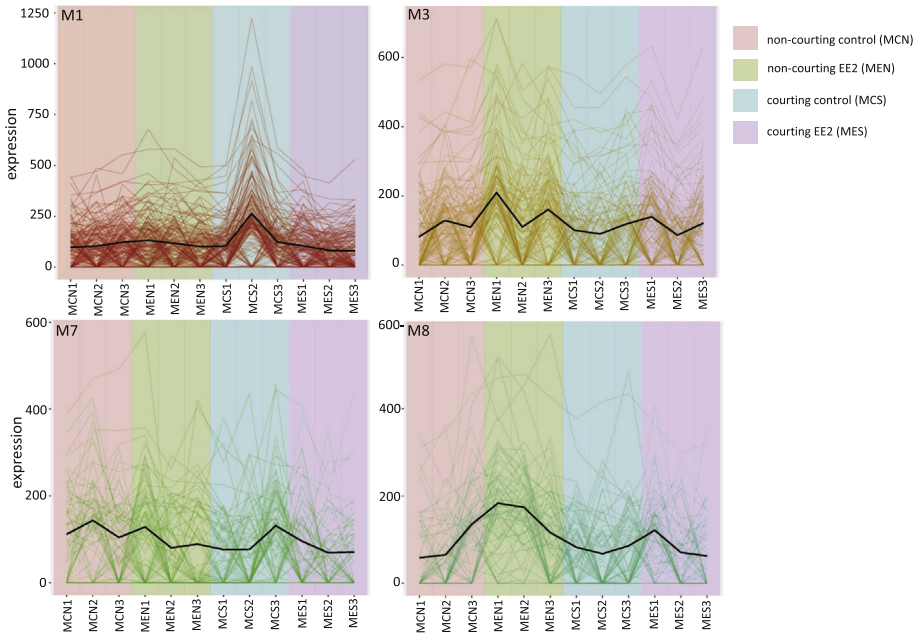

Fig. 3. Gene expression signatures are displayed as expression profiles of individual genes in each sample group (shown in different coloured blocks) and median gene expression trend is plotted as a solid black line. Male groups: non-courting control males, non-courting EE2 males, courting control males, courting EE2 males. Each group represents expression of three individual males (e.g. MCNI1 = non-courting control male 1; MCNI2 = non-courting control male 2; MCNI3 = non-courting control male 3).

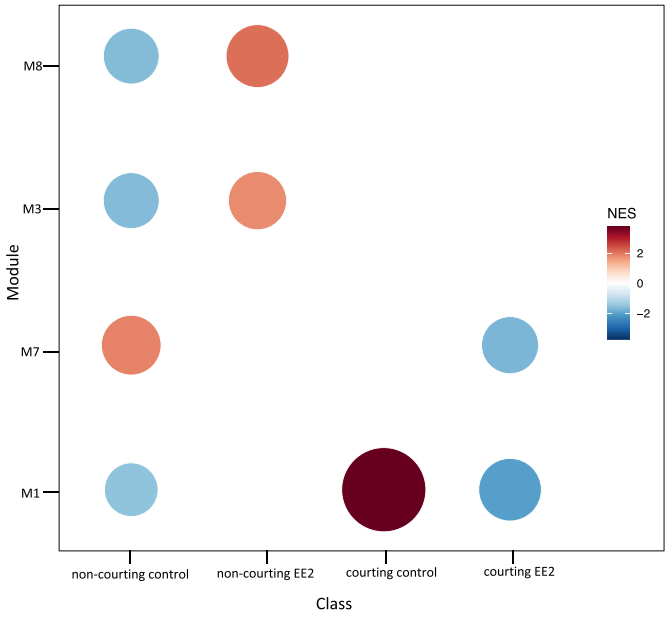

Fig. 4. Gene set enrichment analysis (GSEA) associates activity of each module to its sample class represented as Net Enrichment Scores (NES). The NES corresponds to a shifting of gene set constituents of a module towards either end of the ranked list representing strongly positive or negative correlations. Red represents higher (positive) activity and blue represents lower (negative) activity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

preference between visual and chemical cues is supported by our recent study where we found that guppy males (control and EE2) preferred control females paired with an EE2 female chemical cue, and EE2 females when paired with a control female chemical cue (Saaristo et al., 2019).

Courtship displays are generally considered to be an honest signal of male quality because they can be energetically demanding to perform (Candolin, 2000), and are often reflective of current physical condition (O'Steen et al., 2010). In the literature, it has been suggested that so-called 'dishonest' signaling can arise as a terminal effort, whereby males in poor condition increase their investment into current reproduction when the prospects of future reproduction are grim (Candolin, 1999; Sadd et al., 2006; Nielsen and Holman, 2012). In this regard, it is well established that exposure to EE2 reduces body weight and size (Van den Belt et al., 2003), and depresses immunity (Ahmed, 2000). Consequently, our result could be evidence of dishonest sexual signaling, as has been reported in other studies of wildlife exposed to environmental contaminants, including effects of eutrophication on sticklebacks (*Gasterosteus aculeatus*) (Wong et al., 2007) and EDC mixtures on starlings (*Sturnus vulgaris*) (Markman et al., 2008). From an ecological perspective, dishonest signaling has direct links to fitness because it affects female choice and can therefore influence both offspring quantity and quality (Candolin and Wong, 2012). Also, the fact that some EE2 males in the current study still courted provide us with further evidence that not all individuals are affected equally when exposed to the same contaminants. Indeed, it is becoming increasingly evident that differences in susceptibility between individuals due to life-stage, size, behaviour, and metabolism can all lead to differences in exposure loads (Henriksen et al., 2016; Windsor et al., 2017; Martin et al., 2019; Tan et al., 2020).

With the functional analysis, for module M1, we found that courting control males had a gene profile that was distinct from the other male groups. In particular, a strong positive signature was revealed for genes related to movement and muscle membrane stability. For example, in control courting males, the gene encoding protein *noelin-2* (also known as olfactomedin), which is involved in transforming growth factor beta-induced smooth muscle differentiation, was strongly positive. Previous studies have shown *noelin-2* to be an essential regulator of exploration, locomotion, olfactory sensitivity and visual stimuli, in both mice (Sultana et al., 2014) and zebrafish (Lee et al., 2008). Based on our study, the gene

signature of EE2 males was negative in regard to *noelin-2* and could therefore partially explain the mismatched preference between visual and chemical female cues in the current and earlier studies (Saaristo et al., 2019). However, further research is required to confirm this. Another gene that was strongly positive in courting control males was a gene encoding *zeta-sarcolycan,* which plays a role in maintenance of muscle membrane stability. Sarcoglycans are part of a dystrophin and dystrophin-associated protein (DAP) complex, which is a system protecting the plasma membrane of striated muscle fibers against contraction-induced mechanical stress (Ozawa et al., 2000; Shiga et al., 2006). Intriguingly, in contrast to the positive signature in control courting males, the gene signature of courting EE2 males was negative in relation to the above listed movement-related genes, as well as to *ubiquitin carboxyl-terminal hydrolase 42*, a gene that plays an important role during spermatogenesis in fish (Hu et al., 2017). The reasons for these differences are unknown and warrant further investigation.

We found that non-courting behaviour was associated with a specific gene signature. Specifically, modules M3 and M8 had gene signatures that were only present in non-courting control and EE2 males. For example, *GAGE12J* encoding protein G antigen 12J, which belongs to a multigene family expressed in a large variety of tumors (Lee et al., 2015), was strongly positive in non-courting EE2 males. Another gene that was strongly positive in non-courting EE2-males was *CLEC18A* encoding C-type lectin domain family 18A protein, which has been suggested to contribute to host immunity against pathogens (Huang et al., 2009; 2015). These findings suggest altered immunity in non-courting EE2-males, which is consistent with recent studies in fish showing that exogenous exposure to EE2 can impair immune competence and immunomodulatory pathways (marine medaka, *Oryzias melastigma*) (Ye et al., 2018), as well as genes encoding inflammatory response (trout, *Oncorhynchus mykiss*) (Massart et al., 2014). Interestingly, another gene that was highlighted in module M3, the gene *QRICH2*, encodes for glutamine-rich protein 2 and plays an essential role in reproduction due to its function in the formation of sperm flagella and flagellar structure maintenance. Indeed, a recent knockout mouse study confirmed the importance of *QRICH2* in spermatogenesis and the implications of its malfunction to male fertility (i.e. homozygous *QRICH2* knock-out males were infertile; Kherraf et al., 2019). It remains to be tested, however, if malfunction to formation of sperm flagella or altered immunity induced by chemical contaminants affects the reproductive success of male guppies.

We found that module M7 captured gene signatures associated with EE2 treatment. Specifically, it showed a positive gene signature in non-courting control males and a negative signature in courting EE2 males, while being absent in both control and non-courting EE2 males. In particular, present in this signature were genes linked to immune response, T-cell mediated cytotoxicity, locomotory behaviour, and insulin-like growth factor stimulus and starvation. For example, *PFR1* encoding protein perforin-1, plays a key role in secretory granule-dependent cell death, in defense against virus-infected cells, and as an important mediator of immune cytotoxicity (Chowdhury and Lieberman, 2008; Cullen and Martin, 2008; Ovadya et al., 2018). A recent study showed that *PFR1* knockout mice accumulated more senescent cells in their tissues, which was accompanied by a progressive state of chronic inflammation, increased tissue fibrosis, tissue damage, and compromised organ functionality (Ovadya et al., 2018). Such findings could suggest compromised immunity in non-courting control males, indicative of an honest sexual signaling function for male courtship displays in guppies (Lopez, 1998; Grether et al., 2001; O'Steen et al., 2010).

By contrast, altered metabolism did not appear to have an effect on courtship in EE2 males. Specifically, a gene strongly positive in non-courting control males and negative in courting EE2 males, was *FBN1*. Encoding the protein fibrillin-1, *FBN1* is an important component of both elastic and nonelastic connective tissues throughout the body (Lönnqvist et al., 1998; Milewicz et al., 1995). Interestingly, its C-terminal cleavage product, asprosin, is a protein hormone that regulates glucose homeostasis. This recently discovered fasting-induced hormone modulates hepatic glucose release (Romere et al., 2016; Duerrschmid et al., 2017), resulting in appetite stimulation, and an urge to accumulate adiposity and body weight (Duerrschmid et al., 2017). Malfunction of this protein hormone has profound effects on the pathway that leads to glucose release into circulation (Lönnqvist et al., 1998; Romere et al., 2016). This indicates, perhaps, that pathways related to glucose release and appetite were negatively affected in courting EE2 males. Hence, the fact that such males engaged in courtship regardless of altered metabolism, suggests that courting EE2 males may be signaling dishonestly as to their metabolic condition. These associations warrant further investigation, with our study identifying important gene signatures for disentangling the mechanistic pathways underpinning behavioural responses to EE2-exposure.

5. Conclusions

We highlight a previously unknown association between environmentally relevant EE2-exposure, gene signature and reproductive behaviour. This is the first study to show that transcriptome-wide changes are associated with the reproductive behaviour of fish. Specifically, we found that EE2 exposed male guppies that performed high levels of courtship had a gene profile that deviated the most from the other treatment groups. Using Gene Ontology pathway analysis, our study shows that EE2-exposed males had gene transcripts enriched for pathways associated with altered immunity, starvation, altered metabolism and spermatogenesis. Our study demonstrates that multiple gene networks orchestrate courting behaviour, emphasizing the importance of investigating impacts of pharmaceuticals on gene networks instead of single genes. Overall, our study contributes pivotal insights into the molecular and neurobiological consequences of a widespread endocrine disruptor.

Author contribution

Minna Saaristo: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing – original draft, Project administration, Funding acquisition; John A Craft: Conceptualization, Investigation, Formal analysis, Data curation, Writing – review & editing, Funding acquisition; Sonika Tyagi: Formal analysis, Formulated bioinformatic analysis workflow, Writing – review & editing; Christopher P. Johnstone: Formal analysis, Data curation, Writing – review & editing; Mayumi Allinson: Methodology, Investigation, Writing – review & editing; Khalid S. Ibrahim: Investigation, Writing – review & editing; Bob B.M. Wong: Conceptualization, Methodology, Resources, Supervision, Project administration, Writing – review & editing, Funding acquisition.

Data accessibility

All sequence data and assemblies submitted to public repositories at DDBJ/EMBL/GenBank = MCNI:SRR5277659; MENI:SRR5262324; MCS:SRR5262322; MES: SRR5262321.

Declaration of competing interest

The authors declare that they have no known competing

financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Rowan Jacques-Hamilton, Simon Michnowicz and Philip Chan for technical and logistical support, Dr Richard Martin and his team for technical assistance at Archie-West, Jake Martin for support with the graphical abstract, and the Academy of Finland (265629 to MS) and the Australian Research Council (DP130100385, DP160100372, FT190100014 to BBMW) for funding.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.116286.

References

Ahmed, S.A., 2000. The immune system as a potential target for environmental estrogens: a new emerging field. Toxicology 150, 191–206.
Ankley, G.T., Brooks, B.W., Huggett, D.B., Sumpter, J.P., 2007. Repeating history: pharmaceuticals in the environment. Environ. Sci. Technol. 41, 8211–8217.
Arnold, A.P., Breedlove, S.M., 1985. Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. Horm. Behav. 19, 469–498.
Arnold, K.E., Brown, A.R., Ankley, G.T., Sumpter, J.P., 2014. Medicating the environment: assessing risks of pharmaceuticals to wildlife and ecosystems. Phil. Trans. R. Soc. B. 369, 2013.0569.
Bell, A.M., 2001. Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, *Gasterosteus aculeatus*. Anim. Behav. 62, 775–780.
Bertram, M.G., Saaristo, M., Martin, J.M., Ecker, T.E., Michelangeli, M., Johnstone, C.P., O'Bryan, M.K., Wong, B.B.M., 2018. Field-realistic exposure to the androgenic endocrine disruptor 17β-trenbolone alters ecologically important behaviours in female fish across multiple contexts. Environ. Pollut. 243, 900–911.
Bjerselius, R., Lundstedt-Enkel, K., Olsen, H., Mayer, I., Dimberg, K., 2001. Male goldfish reproductive behaviour and physiology are severely affected by exogenous exposure to 17 beta-estradiol. Aquat. Toxicol. 53, 139–152.
Borodina, T., Adjaye, J., Sultan, M., 2011. A strand-specific library preparation protocol for RNA sequencing. Methods Enzymol. 500, 79–98.
Brodin, T., Fick, J., Jonsson, M., Klaminder, J., 2013. Dilute concentration of a psychiatric drug alter behavior of fish from natural populations. Science 339, 814.
Bryant, D.M., Johnson, K., DiTommaso, T., Tickle, T., Couger, M.B., Payzin-Dogru, D., Whited, J.L., 2017. A tissue-mapped de novo transcriptome enables identification of limb regeneration factors. Cell Rep. 18, 762–776.
Candolin, U., Wong, B.B.M., 2012. Behavioural Responses to a Changing World. Oxford University Press, Oxford, U.K.
Candolin, U., 2000. Increased signalling effort when survival prospects decrease: male-male competition ensures honesty. Anim. Behav. 60, 417–422.
Candolin, U., 1999. The relationship between signal quality and physical condition: is sexual signalling honest in the three-spined stickleback? Anim. Behav. 58, 1261–1267.
Chowdhury, D., Lieberman, J., 2008. Death by a thousand cuts: granzyme pathways of programmed cell death. Annu. Rev. Immunol. 26, 389–420.
Coe, T.S., Söffker, M.K., Filby, A.L., Hodgson, D., Tyler, C.R., 2010. Impacts of early life exposure to estrogen on subsequent breeding behavior and reproductive success in zebrafish. Environ. Sci. Technol. 44, 6481–6487.
Cullen, S.P., Martin, S.J., 2008. Mechanisms of granule-dependent killing. Cell Death Differ. 15, 251–262.
Cummings, M.E., Larkins-Ford, J., Reilly, C.R.L., Wong, R.Y., Ramsey, M., Hofmann, H.A., 2008. Sexual and social stimuli elicit rapid and contrasting genomic response. Proc. R. Soc. B. 275, 393–402.
De Wit, M., Keil, D., van der Ven, K., Vandamme, S., Witters, E., De Coen, W., 2010. An integrated transcriptomic and proteomic approach characterizing estrogenic and metabolic effects of 17α-ethinylestradiol in zebrafish (*Danio rerio*). Gen. Comp. Endocrinol. 167, 190–201.
Dosen, L.D., Montgomerie, R., 2004. Female size influences mate preferences of male guppies. Etholol 110, 245–255.
Duerrschmid, C., He, Y., Wang, C., Li, C., Bournat, J.C., Romere, C., Saha, P.K., Lee, M.E., Phillips, K.J., Jain, M., et al., 2017. Asprosin is a centrally-acting orexigenic hormone. Nat. Med. 23, 144–1453.
Fenske, L., Concato, A.C., Vanin, A.P., Tamagno, W.A., Sofiatti, J.R.D., Treichel, H., da Rosa, J.G.S., Barcellos, L.J.G., Kaizer, R.R., 2020. 17-α-Ethinylestradiol modulates endocrine and behavioral responses to stress in zebrafish. Environ. Sci. Pollut. Res. 27, 29341–29351.
Filiano, A.J., Xu, Y., Tustison, N.J., Marsh, R.L., Baker, W., Smirnov, I., Kipnis, J., 2016. Unexpected role of interferon-gamma in regulating neuronal connectivity and social behaviour. Nature 535, 425–429.
Fraser, B.A., Weadick, C.J., Janowitz, I., Rodd, F.H., Hughes, K.A., 2011. Sequencing and characterization of the guppy (*Poecilia reticulata*) transcriptome. BMC Genom. 12, 202.
Gardner, L.D., Jayasundara, N., Castilho, P.C., Block, B., 2012. Microarray gene expression profiles from mature gonad tissues of Atlantic bluefin tuna, *Thunnus thynnus* in the Gulf of Mexico. BMC Genom. 13, 530.
Godwin, J., Crews, D., Warner, R.R., 1996. Behavioural sex change in the absence of gonads in a coral reef fish. Proc. R. Soc. B. 263, 1683–1688.
Goncalves, D.M., Oliveira, R., 2011. Chapter 7 - hormones and reproduction of vertebrates. In: Norris, D.O., Lopez, K.H. (Eds.), Fishes, vol. 1. Academic Press, pp. 119–147. Hormones and reproduction of vertebrates.
Guevara-Fiore, P., Skinner, A., Watt, P.J., 2009. Do male guppies distinguish virgin females from recently mated ones? Anim. Behav. 77, 425–431.
Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Regev, A., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 29, 644–652.
Grether, G.F., Hudon, J., Endler, J.A., 2001. Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual coloration in guppies (*Poecilia reticulata*). Proc. R. Soc. B. 268, 1245–1253.
Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., et al., 2013. De novo transcript sequence reconstruction from RNA-seq using the trinity platform for reference generation and analysis. Nat. Protoc. 8, 1494–1512.
Hannah, R., D'Aco, V.J., Anderson, P.D., Buzby, M.E., Caldwell, D.J., Cunningham, V.L., Sumpter, J.P., 2009. Exposure assessment of 17α-ethinylestradiol in surface waters of the United States and Europe. Environ. Toxicol. Chem. 28, 2725–2732.
Henriksen, P.G., Beedholm, K., Baatrup, E., 2016. Differences in reproductive behavior between spawning and non-spawning zebrafish pairs and the effects of 17α-ethinylestradiol. Toxics 4, 22.
Holm, L., Blomqvist, A., Brandt, I., Brunstrom, B., Ridderstrale, Y., Berg, C., 2006. Embryonic exposure to o,p '-DDT causes eggshell thinning and altered shell gland carbonic anhydrase expression in the domestic hen. Environ. Toxicol. Chem. 25, 2787–2793.
Houde, A.E., 1997. Sex, Color, and Mate Choice in Guppies. Princeton University Press, Princeton.
Hu, F.Z., Xu, K., Zhou, Y.F., Wu, C., Wang, S., Xiao, J., Wen, M., Zhao, R.R., Luo, K.K., Tao, M., Duan, W., Liu, S.J., 2017. Different expression patterns of sperm motility-related genes in testis of diploid and tetraploid cyprinid fish. Biol. Reprod. 96, 907–920.
Huang, Y.L., Pai, F.S., Tsou, Y.T., Mon, H.C., Hsu, T.L., Wu, C.Y., Chou, T.Y., Yang, W.B., Chen, C.H., Wong, C.H., Hsieh, S.L., 2015. Human CLEC18 gene cluster contains C-type lectins with differential glycan-binding specificity. J. Biol. Chem. 290, 21252–21263.
Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nat. Protoc. 4, 44–57.
Hughes, K.A., Rodd, F.H., Reznick, D.N., 2005. Genetic and environmental effects on secondary sex traits in guppies (*Poecilia reticulata*). J. Evol. Biol. 18, 35–45.
Jeswiet, S.B., Godin, J.G., 2011. Validation of a method for quantifying male mating preferences in the guppy (*Poecilia reticulata*). Ethology 117, 422–429.
Kherraf, Z.E., Cazin, C., Coutton, C., Amiri-Yekta, A., Martinez, G., Boguenet, M., Mustapha, S.F.B., Kharouf, M., Gourabi, H., Hosseini, S.H., et al., 2019. Whole exome sequencing of men with multiple morphological abnormalities of the sperm flagella reveals novel homozygous QRICH2 mutations. Clin. Genet. 96, 394–401.
Kidd, K.A., Paterson, M.J., Rennie, M.D., Podemski, C.L., Findlay, D.L., Blanchfield, P.J., Liber, K., 2014. Direct and indirect responses of a freshwater food web to a potent synthetic oestrogen. Phil. Trans. R. Soc. B. 369, 20130578.
Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. Unit. States Am. 104, 8897–8901.
Kim, D., Langmead, B., Salzberg, S.L., 2015. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12, 357–360.
Kristensen, T., Baatrup, E., Bayley, M., 2005. 17β-ethinylestradiol reduces the competitive reproductive fitness of the male guppy (*Poecilia reticulata*). Biol. Rep. (Wash. D C) 72, 150–156.
Künstner, A., Hoffmann, M., Fraser, B.A., Kottler, V.A., Sharma, E., Weigel, D., et al., 2016. The genome of the trinidadian guppy, *Poecilia reticulata*, and variation in the Guanapo population. PloS One 11, e0169087.
Lagesson, A., Saaristo, M., Brodin, T., Fick, J., Klaminder, J., Martin, J.M., Wong, B.B.M., 2019. Fish on steroids: temperature dependent effects of 17β-trenbolone on anti-predator, risk-taking and exploratory behaviors. Environ. Pollut. 245, 243–252.
Larsen, M.G., Bilberg, K., Baatrup, E., 2009. Reversibility of oestrogenic sex-changes in zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 28, 1783–1785.
Lee, E.K., Song, K.A., Chae, J.H., Kim, K.M., Kim, S.H., Kang, M.S., 2015. GAGE12 mediates human gastric carcinoma growth and metastasis. Int. J. Canc. 136, 2284–2292.
Lee, J.A., Anholt, R.R.H., Cole, G.J., 2008. Olfactomedin-2 mediates development of the anterior central nervous system and head structures in zebrafish. Mech. Dev. 125, 167–181.

Liu, Y., Zhou, J., White, K.P., 2013. RNA-seq differential expression studies: more sequence or more replication? Bioinformation 30, 301–304.
Lopez, S., 1998. Acquired resistance affects male sexual display and female choice in guppies. Proc. R. Soc. B. 265, 717–723.
Loveland, J.L., Fernald, R.D., 2017. Differential activation of vasotocin neurons in contexts that elicit aggression and courtship. Behav. Brain Res. 317, 188–203.
Lönnqvist, L., Reinhardt, D., Sakai, L., Peltonen, L., 1998. Evidence for furin-type activity-mediated C-terminal processing of profibrillin-1 and interference in the processing by certain mutations. Hum. Mol. Genet. 7, 2039–2044.
Luyten, P.H., Liley, N.R., 1991. Sexual selection and competitive mating success of male guppies from four Trinidad populations. Behav. Ecol. Sociobiol. 28, 329–336.
Lyssimachou, A., Jenssen, B.M., Arukwe, A., 2006. Brain cytochrome P450 aromatase gene isoforms and activity levels in atlantic salmon after waterborne exposure. Toxicol. Sci. 91, 82–92.
Markman, S., Leitner, S., Catchpole, C., Barnsley, S., Müller, C.T., Pascoe, D., Buchanan, K.L., 2008. Pollutants increase song complexity and the volume of the brain area HVC in a songbird. PloS One 3, e1674.
Martin, J.M., Bertram, M.G., Saaristo, M., Fursdon, J.B., Hannington, S.L., Brooks, B.W., Burket, R., Mole, R.A., Deal, N.D.S., Wong, B.B.M., 2019. Antidepressants in surface waters: fluoxetine influences mosquito fish anxiety-related behavior at environmentally relevant levels. Environ. Sci. Technol. 53, 6035–6043.
Martin, J.M., Saaristo, M., Bertram, M.G., Lewis, P.J., Coggan, T.L., Clarke, B.O., Wong, B.B.M., 2017. The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish. Environ. Pollut. 222, 592–599.
Martyniuk, C.J., Kroll, K.J., Doperalski, N.J., Barber, D.S., Denslow, N.D., 2010. Environmentally relevant exposure to 17α-ethinylestradiol affects the telencephalic proteome of male fathead minnows. Aquat. Toxicol. 98, 344–353.
Massart, S., Milla, S., Redivo, B., Flamion, E., Mandiki, S.N.M., Kestemont, P., 2014. Influence of short-term exposure to low levels of 17 alpha-ethynylestradiol on expression of genes involved in immunity and on immune parameters in rainbow trout, *Oncorhynchus mykiss*. Aquat. Toxicol. 157, 57–69.
McCarthy, M.M., Arnold, A.P., 2011. Reframing sexual differentiation of the brain. Nat. Neurosci. 14, 677–683.
Milewicz, D.M., Grossfield, J., Cao, S.N., Kielty, C., Covitz, W., Jewett, T., 1995. A mutation in FBN1 disrupts profibrillin processing and results in isolated skeletal features of the Marfan syndrome. J. Clin. Invest. 95, 2373–2378.
Munday, P.L., Buston, P.M., Warner, R.R., 2006. Diversity and flexibility of sex-change strategies in animals. Trends Ecol. Evol. 21, 89–95.
Nielsen, M.L., Holman, L., 2012. Terminal investment in multiple sexual signals: immune-challenged males produce more attractive pheromones. Funct. Ecol. 26, 20–28.
Ort, C., Lawrence, M.G., Rieckermann, J., Joss, A., 2010. Sampling for pharmaceuticals and personal care products and illicit drugs in wastewater systems: are your conclusions valid? Environ. Sci. Technol. 44, 6024–6035.
Oshima, Y., Kang, I.J., Kobayashi, M., Nakayama, K., Imada, N., Honjo, T., 2003. Suppression of sexual behavior in male Japanese medaka exposed to 17b-estradiol. Chemosphere 50, 429–436.
O'Steen, S., Eby, S.L., Bunce, J.A., 2010. Dynamically honest displays: courtship locomotor performance indicates survival in guppies. Funct. Ecol. 24, 1045–1053.
Ovadya, Y., Landsberger, T., Leins, H., Vadai, E., Gal, H., Biran, A., Yosef, R., Sagiv, A., Agrawal, A., Shapira, A., et al., 2018. Impaired immune surveillance accelerates accumulation of senescent cells and aging. Nat. Commun. 9, 5435.
Ozawa, E., Imamura, M., Noguchi, S., Yoshida, M., 2000. Dystrophinopathy and sarcoglycanopathy. Neurosci. News 3, 13–19.
Patro, R., Duggal, G., Love, M.I., Irizarry, R.A., Kingsford, C., 2017. Salmon provides fast and bias-aware quantification of transcript expression. Nat. Methods 14, 417–419.
Pellegrini, E., Diotel, N., Vaillant-Capitaine, C., Maria, R.P., Gueguen, M.M., Nasri, A., Nicolau, J.C., Kah, O., 2016. Steroid modulation of neurogenesis: focus on radial glial cells in zebrafish. J. Steroid Biochem. Mol. Biol. 160, 27–36.
Porro, V., Pagotto, R., Harreguy, M.B., Ramirez, S., Crispo, M., Santamaria, C., Bollati-Fogolin, M., 2015. Characterization of Oct4-GFP transgenic mice as a model to study the effect of environmental estrogens on the maturation of male germ cells by using flow cytometry. J. Steroid Biochem. Mol. Biol. 154, 53–61.
Porseryd, T., Larsson, J., Kellner, M., Bollner, T., Dinnétz, P., Hällström, I.P., 2019. Altered non-reproductive behavior and feminization caused by developmental exposure to 17α-ethinylestradiol persist to adulthood in three-spined stickleback (*Gasterosteus aculeatus*). Aquat. Toxicol. 207, 142–152.
Porseryd, T., Volkova, K., Caspillo, N.R., Källman, T., Dinnetz, P., Hällström, I.P., 2018. Persistent effects of development exposure to 17α-ethinylestradiol on the zebrafish brain transcriptome and behavior. Front. Behav. Neurosci. 11, 69.
Robinson, G.E., Fernald, R.D., Clayton, D.F., 2008. Genes and social behavior. Science 322, 896–900.
Romere, C., Duerrschmid, C., Bournat, J., Constable, P., Jain, M., Xia, F., Saha, P.K., Solar, M.D., Zhu, B., York, B., et al., 2016. Asprosin, a fasting-induced glucogenic protein hormone. Cell 165, 566–579.
Rosenfeld, C.S., Denslow, N.D., Orlando, E.F., Gutierrez-Villagomez, J.M., Trudeau, V.L., 2017. Neuroendocrine disruption of organizational and activational hormone programming in poikilothermic vertebrates. J. Toxicol. Environ. Health B. 20, 276–304.
Russo, P.S.T., Ferreira, G.R., Cardozo, L.E., Burger, M.C., Arias-Carrasco, R., Maruyama, S.R., Hirata, T.D.C., Lima, D.S., Passos, F.M., Fukutani, K.F., et al., 2018. CEMiTool: a Bioconductor package for performing comprehensive modular co-expression analyses. BMC Bioinf. 19, 56.
Saaristo, M., Johnstone, C.P., Xu, K., Allinson, M., Wong, B.B.M., 2019. The endocrine disruptor, 17α-ethinyl estradiol, alters male mate choice in a freshwater fish. Aquat. Toxicol. 208, 118–125.
Saaristo, M., Brodin, T., Balshine, S., Bertram, M.G., Brooks, B.W., Ehlman, S.M., McCallum, E.S., Sih, A., Sundin, J., Wong, B.B.M., Arnold, K.E., 2018. Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife. Proc. R. Soc. B. 285, 20181297.
Saaristo, M., Wong, B.B.M., Craig, A., Johnstone, C.P., Allinson, M., Craft, J.A., 2017. Characterisation of the transcriptome of brain of guppy with RNASeq: comparison between sexes and treatment with the pharmaceutical 17α-ethinyl estradiol. Aquat. Toxicol. 186, 28–39.
Saaristo, M., Tomkins, P., Allinson, M., Allinson, G., Wong, B.B.M., 2013. An androgenic agricultural contaminant impairs female reproductive behaviour in a freshwater fish. PloS One 8, e62782.
Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2010. An endocrine disrupting chemical changes courtship and parental care in the sand goby. Aquat. Toxicol. 97, 285–292.
Saaristo, M., Craft, J.A., Lehtonen, K.K., Björk, H., Lindström, K., 2009b. Disruption of sexual selection in sand gobies (*Pomatoschistus minutus*) by 17α-ethinyl estradiol, an endocrine disruptor. Horm. Behav. 55, 530–537.
Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2009a. Sand goby males exposed to an endocrine disrupting chemical fail in nest and mate competition. Horm. Behav. 56, 315–321.
Sadd, B., Holman, L., Armitage, H., Lock, F., Marland, R., Siva-Jothy, M.T., 2006. Modulation of sexual signalling by immune challenged male mealworm beetles (*Tenebrio molitor, L.*): evidence for terminal investment and dishonesty. J. Evol. Biol. 19, 321–325.
Sharma, E., Kunstner, A., Fraser, B.A., Zipprich, G., Kottler, V.A., Henz, S.R., Dreyer, C., 2014. Transcriptome assemblies for studying sex-biased gene expression in the guppy, *Poecilia reticulata*. BMC Genom. 15, 400.
Shiga, K., Yoshioka, H., Matsumiya, T., Kimura, I., Takeda, S., Imamura, M., 2006. zeta-Sarcoglycan is a functional homologue of gamma-sarcoglycan in the formation of the sarcoglycan complex. Exp. Cell Res. 312, 2083–2092.
Sultana, A., Nakaya, N., Dong, L., Abu-Asab, M., Qian, H., Tomarev, S.I., et al., 2014. Deletion of Olfactomedin 2 induces changes in the AMPA receptor complex and impairs visual, olfactory, and motor functions in mice. Exp. Neurol. 261, 802–811.
Söffker, M., Tyler, C.R., 2012. Endocrine disrupting chemicals and sexual behaviors in fish—a critical review on effects and possible consequences. Crit. Rev. Toxicol. 42, 653–668.
Tan, H., Polverino, G., Martin, J.M., Bertram, M.G., Wiles, S.C., Palacios, M.M., Bywater, C.L., White, C.R., Wong, B.B.M., 2020. Chronic exposure to a pervasive pharmaceutical pollutant erodes among-individual phenotypic variation in a fish. Environ. Pollut. 263, 114450.
The UniProt Consortium, 2019. UniProt: a worldwide hub of protein knowledge. Nucleic Acids Res. 47, D506–D515.
Tiedeken, E.J., Tahar, A., McHugh, B., Rowan, N.J., 2017. Monitoring, sources, receptors, and control measures for three European Union watch list substances of emerging concern in receiving waters: a 20-year systematic review. Sci. Total Environ. 574, 1140–1163.
Todd, E.V., Liu, H., Muncaster, S., Gemmell, N.J., 2016. Bending genders: the biology of natural sex change in fish. Sex. Develop. 10, 223–241.
Van den Belt, K., Verheyen, R., Witters, H., 2003. Effects of 17α-ethynylestradiol in a partial life-cycle test with zebrafish: effects on growth, gonads and female reproductive success. Sci. Total Environ. 309, 127–137.
Vasquez, M.I., Lambrianides, A., Schneider, M., Kummerer, K., Fatta-Kassinos, D., 2014. Environmental side effects of pharmaceutical cocktails: what we know and what we should know. J. Hazard Mater. 279, 169–189.
Verlicchi, P., Aukidy, M.A., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment–a review. Sci. Total Environ. 429, 123.
Volkova, K., Caspillo, N.R., Porseryd, T., Hallgren, S., Dinnetz, P., Porsch-Hällström, I., 2015. Developmental exposure of zebrafish (*Danio rerio*) to 17α-ethinylestradiol affects non-reproductive behavior and fertility as adults, and increases anxiety in unexposed progeny. Horm. Behav. 73, 30–38.
Vosges, M., Le Page, Y., Chung, B.C., Combarnous, Y., Porcher, J.M., Kah, O., Brion, F., 2010. 17α-ethinylestradiol disrupts the ontogeny of the forebrain GnRH system and the expression of brain aromatase during early development of zebrafish. Aquat. Toxicol. 99, 479–791.
Vu, M., Navarro-Martin, L., Gutierrez-Villagomez, J.M., Trudeau, V.L., 2015. Development of an in vitro ovary culture system to evaluate endocrine disruption in wood frog tadpoles. J. Toxicol. Environ. Health A. 78, 1137–1141.
Windsor, F.M., Ormerod, S.J., Tyler, C.R., 2017. Endocrine disruption in aquatic systems: up-scaling research to address ecological consequences. Biol. Rev. 93, 626–641.
Wong, B.B.M., Candolin, U., Lindström, K., 2007. Environmental deterioration compromises socially enforced signals of male quality in three-spined sticklebacks. Am. Nat. 170, 184–189.
Xu, X.H., Coats, J.K., Yang, C.F., Wang, A., Ahmed, O.M., Shah, N.M., 2012. Modular genetic control of sexually dimorphic behaviors. Cell 148, 596–607.
Ye, R.R., Peterson, D.R., Kitamura, S.I., Segner, H., Seemann, F., Au, D.W.T., 2018. Sex-specific immunomodulatory action of the environmental estrogen 17 alpha-ethynylestradiol alongside with reproductive impairment in fish. Aquat. Toxicol. 203, 95–106.
Young, L.J., Winslow, J.T., Nilsen, R., Insel, T.R., 1997. Species differences in

V1areceptor gene expression in monogamous and nonmonogamous voles: behavioral consequences. Behav. Neurosci. 111, 599.
Zhu, Y., Rice, C.D., Pang, Y.F., Pace, M., Thomas, P., 2003. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc. Natl. Acad. Sci. Unit. States Am. 100, 2231–2236.
Zhang, B., Horvath, S., 2005. A general framework for weighted gene co-expression network analysis. Stat. Appl. Genet. Mol. Biol. 4, 1544.