Social hierarchy modulates biomarker expression in fish exposed to contaminants of emerging concern

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Social hierarchy modulates biomarker expression in fish exposed to contaminants of emerging concern

Jelena Ivanova

Male fathead minnows (*Pimephales promelas*) establish social hierarchy. The social status of individual fish is determined by the expression of secondary sex characteristics (SSCs) that develop during the reproductive window, and is vital for reproductive success during the spawning season. The social rank of each male is linked to the concentrations of circulating androgens: testosterone and 11-ketotestosterone that influences the expression of SSCs, and is under the control of hypothalamo-pituitary-gonadal (HPG) axis. Since the dominant and subordinate males are initially under different physiological conditions, we proposed that they belong to two significantly different subpopulations. Here we demonstrate that male fish population is indeed heterogeneous. This is in contrary to the previously held assumption that single sex exposure populations are homogeneous.

Our results demonstrate that social hierarchy influences the fish responses to the contaminants of emerging concern (CECs). We anticipate our study to be a starting point for re-evaluation of toxicological data analysis in single sex exposure experiments.
Acknowledgements

We would like to thank all authors of manuscripts that were used in this study, all undergraduate and graduate students who assisted with these experiments, Dr. Cook for support, SETAC for recognition of the importance of this study, and US Fish & Wildlife Services and National Science Foundation for grants that made these studies possible.
Chapter I

LITERATURE REVIEW

In toxicology, as in many life sciences, experiments frequently rely on the use of a laboratory organism whether they are bacteria, plants, or animals. Their production in laboratory cultures and their use as units of replication implies homogeneity. Indeed, the reporting of a sample size (for example, n=10) carries the implied assumption that these animals are similar enough to serve as replicates (non-withstanding the statistical issue of pseudo-replication). This assumption, however, runs counter to the foundation of evolutionary biology with its tenant of individual variability (unless parthenogenetic organisms such as Daphnia magna are used for toxicological experiments). Consequently, we ought to ask whether individual variability needs to be accounted for when data are analyzed in toxicological studies.

One frequent cause of intraspecies variability is the result of dominant/subordinate relationship between two individuals of the same species where one has an advantage over the other in resource access (Cubitt et al., 2008; Danylchuk & Tonn, 2001). When the same pattern is established within an entire population, it takes the form of social hierarchy where the rank of each individual is based on outcomes of aggressive encounters (Cubitt et al., 2008). Social hierarchies are fluent and may change for individuals in a population, for example when a dominant male looses its advantage. In this case, the subordinate male takes over the territories, and initiates the physiological transformation that leads to enabling of reproduction (Fox et al., 1997). Social hierarchy can develop under wild and laboratory conditions (Kah et al., 1993) and may have implications for some toxicological studies as the modulations of endocrine physiology of an animal is dependent upon its social status (Cubitt et al., 2008; Gilmour et al.,
2005), and is sensitive to external stressors, such as exposures to contaminants of emerging concern (CECs) that are ubiquitous in our environment (Goodman, 2005).

Multiple pathways interact in the intricate modulation of the endocrine system with the brain integrating external and internal stimuli to establish an appropriate endocrine status of each individual (Kah et al., 1993). The hypothalamo-pituitary-gonadal (HPG) axis regulates the production of sex hormones that guide in sexual maturation and success of reproduction (Ankley & Johnson, 2004). The hypothalamo-pituitary-adrenal (HPA) axis, in turn, engages in response to external and internal stressors often through the release of cortisol hormone. Differing levels of stress are imposed upon an animal based on its social status, especially on the subordinate individuals (Gilmour et al., 2005; Fox et al., 1997). Persistently high concentrations of cortisol are associated with reduced abilities of an individual to access the food, which results in decreased body mass and body condition factor (BCF), and chronically activated HPA axis that leads to production of even higher concentrations of cortisol (Gilmour et al., 2005). Recent studies have even suggested that neurological circuits in the central nervous system may be altered as a result of interactions between dominant and subordinate conspecifics (Desban & Wyart, 2016).

The most widely studied CECs include the naturally occurring estrogens $\beta$-Estradiol (E2) and its less potent metabolite Estrone (E1) (Dammann et al., 2011; Hyndman et al., 2010; Kolok et al., 2012; Rearick et al., 2013; Schultz et al., 2012; Shapell et al., 2010). Also well studied are several mood altering pharmaceuticals acting as selective serotonin reuptake inhibitors (SSRIs) (Larson & Summers, 2001; Schultz et al., 2011; Somoza & Peter, 1991; Winberg et al., 1997; Winberg & Lepage, 1998). Estrogen hormones have multi-faceted and wide-ranging effects in vertebrate animals, and are the end products of HPG axis activation.
Neurons located within preoptic area (POA) secrete the gonadotropin-releasing hormone (GnRH) that stimulates the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that directly influence the gonadal growth and steroidogenesis (Trudeau, 1997). Estrogens modulate the activity of HPG axis via negative/positive feedbacks (Ankley & Johnson, 2004; Trudeau, 1997). In addition, sex steroids either directly or indirectly modulate the effects on a number of neurons involved in regulation of GnRH and catecholamines like serotonin (Kah et al., 1993). In some vertebrates, and especially in fish, GABA has a stimulatory effect on GnRH release that initiates the cascade reaction and secretion of sex steroids. It has been shown that high levels of estrogens block this stimulatory effect of GABA that reduces the levels of end products (Kah et al., 1993).

SSRIs are inherently biologically active and often target areas of the brain also involved in influencing the dominant-subordinate relationship among con-specifics. The subordinate behavior is physiologically determined by a decrease of HPG axis activity and a chronic increase of brain serotonin (5-Hydroxytryptamine, 5-HT) levels (Cubitt et al., 2007; Overli et al., 2007). The elevation of 5-HT concentration corresponds to an increase in cortisol concentration due to chronic activation of HPA axis under stressful conditions (Winberg et al., 1997). Social stress from aggressive interactions is expressed differently in brain regions of dominant and subordinate males (Larson & Summers, 2001). The response to serotonin stimulus in dominant males is rapid. However, it takes longer for serotonergic neurons to conduct the electric stimulus in subordinate males but the effects are prolonged (Larson & Summers, 2001). It was established that serotonin can either impose its effects on hypothalamus influencing the secretion of GnRH (Schulz & Goos, 1999) or directly affecting pituitary gland bypassing hypothalamus; therefore, it stimulates the secretion of FSH and LH hormones actively involved in animal sexual maturation.
and reproduction (Somoza & Peter, 1991). In addition, three subtypes of 5-HT receptors have been identified: substrate binding to 5-HT\textsubscript{1A} receptor results in increase in aggression, whereas binding to 5-HT\textsubscript{2} results in suppression of aggression and enhancement of GnRH release (Johnson et al., 2009; Kah et al., 1993; Somoza & Peter, 1990). The differential effects of serotonin on brains of animals of different social statuses presumably reflect the differential responses of these animals to SSRIs that prolong serotonin presence in the synaptic clefts.
Chapter II

Social hierarchy modulates biomarker expression in fish exposed to contaminants of emerging concern

INTRODUCTION

The molecular pathways involved in initiation of toxic responses are highly conserved across vertebrate species and the effects of CECs on these pathways have been studied extensively in model laboratory species such as the fathead minnow (Ankley & Johnson, 2004; Harries et al., 2000). Previous research (Rantala & Kortet, 2003) has established the high resistance and/or tolerance to pathogens in dominant males and in males with elaborate secondary sexual characteristics (Jacob et al., 2008) whose development is induced by androgens, and peak during the summer breeding season (Smith, 1978). Reproduction is dependent upon the males’ ability to acquire and defend a high quality nesting territory in the presence of other male competitors (Martinovic-Weigelt et al., 2012). Thus larger, more dominant individuals may interfere with the general reproductive functions of subordinate fishes, particularly in their ability to hold a territory. In addition, smaller fishes require more time to replenish over-winter energy deficits than do larger fishes because of their lower energy stores, which results in a delayed reproduction (Danylchuk & Tonn, 2000). The social status imposes great effects on fish physiology and several studies have quantified differences in concentrations of circulating cortisol (DiBattista et al., 2006), androgens (Maruska et al., 2010) and various neuromodulators (Greenwood et al., 2008) in fishes of different social status. Estrogens are important in oviparous vertebrates because they control the production of vitellogenin (VTG), a yolk pre-cursor protein, in the liver of adult females (Munakata & Kobayashi, 2010). However, estrogens as well as estrogen agonists can induce VTG synthesis in males (Filby et al., 2012;
Panter et al., 1998), cause changes in SSC expression of adult male fish (Ankley & Johnson, 2004) that may result in incidences of intersex (Jobling et al., 1998). As described by Martinovic et al., (2003) VTG itself does not reduce the reproductive success but rather the combination of the several factors does. First, VTG diverts energy from reproduction to its own production. Second, in Martinovic et al., (2003) demonstrated that male fathead minnows exposed to E2 contained lower levels of circulating androgens, which could explain the reduction in aggressive behavior and impaired ability to acquire a nest site under competition pressure. Another explanations suggests that subordinate animals, fish in particular, might be more sensitive to CECs because levels of GnRH-secreting cells in subordinate males are eight-fold lower compared to dominant males. (Gilmour et al., 2005; Fox et al., 1997). This implies the reduction in FSH and LH release that directly affects the levels of circulating androgens and estrogens (Trudeau, 1997). Moreover, the increased metabolism and release of 5-HT in teleost fish typically occurs in response to social stress, levels of which differ between the individuals of different social statuses (Larson & Summers, 2001; Overli et al., 2007).

Researchers have noted before that complexity of social structure among the vertebrates may have implications on their responses to CECs (Hyndman et al., 2009; Larson & Summers, 2001; Schultz et al., 2011). In the mid-1990’s biologists begun to combine the results of several studies to explore the heterogeneity of animal population that often was not resolved by small sample sizes of individual studies (Fernandez-Duque & Vallegia, 1994). Meta analysis, as one of statistical techniques, has been employed by the scientists to discover the patterns in populations. However, there must be the degree of similarity between the studies required in order for the pooled results to be meaningful. The “quality” of data sets based on the number and measurement criteria of variables that match other studies of interest is often choses as a
preferred filtering method (Luppino et al., 2010). The studies that involve manipulations of the environment, such as the presence of CECs focusing on assessment of morphological changes need to evaluate the degree of phenotypic fluctuations at both population and individual levels. The goal of this study is aimed at filling this gap. Multivariate statistics were chosen as tools to achieve this goal. The hypothesis of this study proposes that the male fathead minnow population is heterogeneous, and this variability decreases upon exposure to estrogenic compounds E1 and E2, but would increase upon exposure to SSRIs. This information will allow filling in the gap and re-evaluating the current approach to data analysis in toxicology.
Methodology

In toxicological studies, fish within a treatment are usually assumed to be homogeneous in their response to CEC exposure. However, considering the establishment of social hierarchy among male fish (Cubitt et al., 2008), it is plausible to suggest that male fish population within a treatment is rather heterogeneous. The goal of this study is aimed at exploring the heterogeneity of male fish population within a treatment group by applying multivariate statistics across ten studies conducted in the same exposure laboratory under largely similar conditions.

Study Conditions

A Meta analysis integrating ten previously conducted studies (Table 1) was used to explore the heterogeneity of male fathead minnow populations subjected to estrogen or SSRI exposures. The similarities between the studies were based on common exposure conditions and similarities in the endpoints that were assessed across all studies: whole body weight, length, liver weight, testis weight, secondary sex characteristics and plasma vitellogenin concentration. In all studies three indices of interest were calculated using the same formulas: hepatosomatic index \((\text{HSI} = \frac{\text{liver weight}}{\text{whole body weight}})\), gonadosomatic index \((\text{GSI} = \frac{\text{gonadal weight}}{\text{whole body weight}})\), and body condition factor \((\text{BCF} = (\frac{\text{whole body weight}}{\text{length}^3}) \times 100,000)\).
Table 1. The list of the studies used in the project, from which data were pooled. These studies were chosen for the project because all experiments were conducted at the Aquatic Toxicology lab at St. Cloud State University; fish for all studies were received from the fish supplier (Environmental Testing and Consulting laboratory, Superior, WI). During the exposure, fish were subject to the same environmental conditions including similar photoperiod (16:8 light:dark), temperature (20-23°C), dissolved oxygen (5.0-7.5 mg/L), and pH (7.2-8.3). These studies include the treatments of interest: Control, E1, E2 and SSRIs.

<table>
<thead>
<tr>
<th>Study</th>
<th>Compounds</th>
<th>Concentration (ng/L)</th>
<th># of treatments</th>
<th># fish/treatment</th>
<th>Water t°C ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Control Estrone (E1) 17 β-Estradiol (E2)</td>
<td>12.5 - 50.0 5.0 - 30.0</td>
<td>7</td>
<td>20</td>
<td>21.5 ± 0.25</td>
</tr>
<tr>
<td>67</td>
<td>Control 17 α-Estradiol 17 β-Estradiol (E2)</td>
<td>27.0 - 150.0 9.0 - 44.0</td>
<td>7</td>
<td>10</td>
<td>21.57 ± 0.37</td>
</tr>
<tr>
<td>61</td>
<td>Genistein Daidzein Formononetin Mix</td>
<td>1000 1000 1000.0 - 3000.0</td>
<td>7</td>
<td>12</td>
<td>21.08 ± 0.36</td>
</tr>
<tr>
<td>63</td>
<td>Triclosan Triclocarban Mix 17 β-Estradiol (E2)</td>
<td>170.0 - 450.0 560.0 - 1600.0 739.0 - 2050.0 30</td>
<td>8</td>
<td>24</td>
<td>21.13 ± 0.40</td>
</tr>
<tr>
<td>64</td>
<td>Bupropion Venlafaxine Sertralin Fluoxetine Mix</td>
<td>7.4 - 57.0 305.0 - 1104.0 1.6 - 5.2 2.5 - 28.0 170.5 - 1314.0</td>
<td>11</td>
<td>10</td>
<td>22.92 ± 0.19</td>
</tr>
<tr>
<td>17</td>
<td>Control Estrone (E1)</td>
<td>16.4-86.3</td>
<td>8</td>
<td>10</td>
<td>23.2 ± 0.5</td>
</tr>
<tr>
<td>62</td>
<td>Control 4-Nonylphenol</td>
<td>150.0 - 15000.0</td>
<td>9</td>
<td>12</td>
<td>24.0 ± 0.36</td>
</tr>
<tr>
<td>30</td>
<td>Control 17 β-Estradiol (E2)</td>
<td>6.5 - 17.0</td>
<td>6</td>
<td>15</td>
<td>21.8 ±0.37</td>
</tr>
<tr>
<td>41</td>
<td>Control 17 β-Estradiol (E2)</td>
<td>13.2 - 36.4</td>
<td>11</td>
<td>15</td>
<td>21.3 ± 0.25</td>
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**Exposure**

The test organisms used in the studies were 6-month old mature male fathead minnows from the same laboratory fish supplier (Environmental Testing and Consulting laboratory, Superior, WI). All fish were subject to 21-day in-house flow-through exposure at constant environmental conditions including similar photo-period (16:8 light:dark), temperature (20-23°C), dissolved oxygen (5.0-7.5 mg/L), and pH (7.2-8.3) (Dammann et al., 2011; Feifarek et al., 2015; Hyndman et al., 2010; Kolok et al., 2012; Rearick et al., 2013; Shapell et al., 2010; Schoenfuss et al., 2008; Schultz et al., 2011; & Schultz et al., 2012). Exposure experiments were conducted at the St. Cloud State University Aquatic Toxicology Laboratory (St. Cloud, MN) following established and published flow-through exposure protocols (Schoenfuss et al., 2008). Briefly, male fathead minnows were randomly assigned to control or exposure aquaria at fish density of 10 per 16L tank. Fish were maintained following US EPA guidelines (Denny, 1987) throughout the experiments at constant environmental conditions and fed frozen brine shrimp (*Artemia fransiscana*, San Francisco Bay Brand, Inc., Newark, CA) twice daily *ad libitum*. The St. Cloud State University Animal Use and Care Committee (IACUC) approved all experimental protocols.

**Chemical Compounds**

Four treatment groups were examined in the present study: an ethanol carrier control, E1, E2 and SSRIs. The SSRI treatment group contained the data from the exposures to Sertraline, Fluoxetine and Venlafaxine. The concentrations of the exposure chemicals were within the environmentally relevant concentration ranges.
**Biological Endpoints**

At the end of the experiments series of biological endpoints were assessed, including whole body weight, length, liver weight, gonadal weight, HSI, GSI, and BCF. SSCs were evaluated based on the blind scoring system described by Smith (1978). The prominence of nuptial tubercles, dorsal pad and the banding pattern were scored on a 0-3 scale. Plasma VTG concentrations were measured via a competitive antibody-capture ELISA. The obtained values were log-transformed for the analyses.

**Data Organization**

Data points from the retained studies were separated into tables based on the treatment (see Supplemental Table S1). Data were tested for the presence of outliers based on Mahalanobis distances (JMP Pro 11 for Mac software). Any detected outliers (Mahalanobis distance > 3.068) were removed from the subsequent analyses.

**Statistical Analyses**

Although there was a high degree of similarity among the variables used in the studies, we also examined the similarities of rearing conditions. As differences in water temperature can have extensive physiological implications for ectotherm vertebrates (Brian et al., 2008; Brian et al., 2011), a one-way Anova was conducted to ensure there were no significant differences between the studies in water temperature. The raw data of the remaining studies were pooled into separate tables for each treatment (Table S1). Ethanol carrier control treatment data were used for model building. The distribution platform was first run for each variable separately to explore patterns (JMP Pro 11 for Macintosh). The normality test was performed on SSC and logVTG variables to confirm the observed distribution patterns. Raw data from all studies were available for analyses, and therefore, weighted averages, a common approach of Meta-analysis, were not
used (Luppino et al., 2010). For the next step principle component analysis (PCA) was conducted on all continuous variables to reduce the dimensions and prevent losing any important information (JMP Pro 11 for Mac software) (Jolliffe, 1986; Yeung & Ruzzo, 2001). The number of retained principle components (PCs) was determined based on the following criteria: eigenvalues > 1, overall variation explained > 80% (Jolliffe, 1986). Five PCs were chosen for K-Means cluster analysis to observe the natural separation of data points. (JMP Pro 11 for Mac software) (Kaufman & Rousseeuw, 1990; Yeung & Ruzzo, 2001). The number of clusters to retain was determined based on either highest positive or lowest negative cubic clustering criterion (CCC). To evaluate the obtained clusters, MANOVA was run as a follow-up test (JMP Pro 11 for Mac software) (Adams et al., 1994). First, MANOVA was run on clusters and PCs then MANOVA was run on clusters and the variables of interest: SSC and logVTG. The same steps were then repeated for other treatments: E1, E2 and SSRIs.
Results

The data from ten previously studies were pooled to obtain a large sample sizes to investigate the heterogeneity of male fish populations within a treatment. We argue that social hierarchy imposes physiological pressure on males within the same community that consequently leads to a formation of more than one distinct population within a treatment.

One-way ANOVA was conducted on temperature to exclude study environmental conditions as a confounding factor. Schoenfuss et al. (2008) was found to be significantly different from the other studies and was removed from further analysis. The remaining studies were within a 2°C range of water temperature (Figure 1).

Figure 1. Selected studies were compared based on the temperatures, at which fish were reared during the exposure experiments. One-way Anova with all pairs Tukey-Kramer post-test (α = 0.05) were conducted to investigate the differences between the studies.

As a next step of the data analysis, the distribution platform of all variables was examined to establish the distribution pattern. While most variables were normally distributed, logVTG
resembled a bimodal distribution. These results were comparable between all treatments. That was also confirmed with a normality test (Figures 2-4).

Figure 2. Results of the distribution pattern of “length” variable followed by normality quantile plot from four exposure treatment groups: (A) Control, (B) E1, (C) E2 and (D) SSRIs.
Figure 3. Results of the distribution pattern of “SSC” variable followed by normality quantile plot from four exposure treatment groups: (A) Control, (B) E1, (C) E2 and (D) SSRIs.
Figure 4. Results of the distribution pattern of “logVTG” variable followed by normality quantile plot from four exposure treatment groups: (A) Control, (B) E1, (C) E2 and (D) SSRIs.

**Control Treatment**

The output of PCA yielded in retention of five PCs (first five eigenvalues > 1 and explain 81% of variability). K-Means cluster analysis run on five PCs yielded two clusters (CCC = -3.2424) as an optimal solution, which supports our hypothesis of the presence of more than one distinct population of male fish within a treatment group. Clusters were saved and a MANOVA was conducted as a follow-up test. MANOVA of two clusters versus five PCs showed that clusters are significantly different from each other while already accounting for 81% of variability (p-value < 0.0001). For all analyses significance was set at $\alpha = 0.05$. Another MANOVA output resulted in two clusters being significantly different relative to SSC and
logVTG variables (p-value < 0.0001). The cluster separation relative to SSC was from 5.71 to 5.76, whereas logVTG distributed from 0.86 to 2.14, respectively (Figure 5A).

Figure 5. Naturally occurring data separation from single sex male exposures was observed with an aid of K-Means cluster analysis performed following PCA. Five PCs were retained based on the number of eigenvalues >1 and minimum percentage of variability explained by these PCs and used for clustering. The most representative variables with contribution percentage driving the PCs are shown on 3D biplots. The treatment groups are: (A) Control, (B) E1, (C) E2 and (D) SSRI.
E1 Treatment

As with the E2 treatments, five PCs were retained for the E1 treatment (five eigenvalues $> 1$ and 85% of variability explained). The retention of five PCs once again resulted in data separation into two groups. While two clusters were significantly different from each other (p-value < 0.0001, MANOVA), clusters were not significantly different relative to SSC and logVTG variables (p-value = 0.1748). The mean SSC values decreased to 4.33 and 4.86, whereas logVTG values remained within 1.76 and 1.81 range, respectively (Figure 5B). We saw an increase in VTG synthesis but to a lesser degree comparing to E2. These findings also support the hypothesis that variability within fish populations would decrease upon exposure to E1.

E2 Treatment

Similarly to the control treatment, five PCs were retained for E2 treatment ($5^{th}$ eigenvalue $= 0.8924$ and 87% of variation is explained). The insertion of five PCs into K-Means cluster analysis yielded fish separation into two distinct populations, similar to Control treatment group. MANOVA on two clusters versus five PCs showed that clusters were significantly different (p-value < 0.0264), whereas they were no longer significantly different relative to SSC and logVTG variables (p-value = 0.1236). The mean SSC decreased to 4.8 and 5.37 values while logVTG values increased to 3.15 and 2.88, respectively, which showed that fish maturation slowed down constraining the separation while inducing VTG synthesis (Figure 5C). This supports hypothesis that variability within fish population would decrease upon exposure to E2.
**SSRI Treatment**

Five PCs of SSRI treatment explained 87% of variation while fifth eigenvalue was at 0.92. Two clusters were identified after running cluster analysis on retained PCs. Both MANOVA outputs yielded significant results for clusters versus PCs (p-value < 0.0001) and clusters versus SSC and logVTG (p-value = 0.0009) (Figure 5D). Secondary sex characteristic separation increased to 5.21 and 6.5 while logVTG values remained within Control range of 0.70 and 2.17, respectively, which supports the hypothesis that variability within fish populations would increase upon exposure to SSRIs.
Chapter III

DISCUSSION AND CONCLUSION

The purpose of this study was to explore the variability within single sex fish populations that establish the social hierarchy under the environmental conditions. Multivariate statistical methods were utilized to investigate whether fish would fall into distinct subpopulations based on their social status, and whether status influences the response to CECs by modulating VTG synthesis. The data were extracted from nine studies, all of which utilized fish from the same breeding colony and all of which were conducted at the same exposure laboratory.

Brain is the primary target organ of endocrine modulation in socially diverse animals. In fact, social behavior network in vertebrates, including fish, birds and mammals, is established within the basal forebrain and midbrain (Goodson, 2005; Kah et al., 1993). The nodes within these network house receptors of sex steroid hormones, activation of which involves the coordination of sexual differentiation and the modulation of multiple forms of social behavior of an animal (Goodson, 2005). Moreover, the homologues of these nodes: POA, anterior hypothalamus (AH), ventromedial hypothalamus (VMH) and midbrain are preserved across vertebrate species, and were found to control many forms of social behavior, such as aggression, forms of communication, social recognition and affiliation, sexual behavior and responses to social stressors (Goodson, 2005). Both axes are critical in establishment of individual’s social status; however, both are susceptible to adverse effects of CECs (Ankley & Johnson, 2004).

During sexual maturation male fathead minnows develop SSCs: nuptial tubercles, dorsal pad and the banding pattern which are used to acquire a nest site during the spawning season and protecting their offspring until hatching (Smith, 1974; Watanabe et al., 2007). It is well established that these characteristics are under androgenic control (Margiotta-Casaluci &
Sumpter, 2011) which, in turn, is regulated by HPG axis via negative/positive feedback (Trudeau, 1997) The production of androgens is initiated in hypothalamus which releases GnRH that acts on the pituitary gland to release FSH and LH and then act on of the reproductive organs to produce sex steroids (Ankley & Johnson, 2004). The establishment of socially complex community has been documented for many fishes, including fathead minnows (Danylchuk & Tonn, 2001), and is driven by the changes in HPG axis. The dominance of the male is determined by the concentration of circulating male sex steroids that ultimately lead to the enhanced SSC expression (Billard et al., 1982; Ankley & Johnson, 2004), which is consistent with observed results where some fish became dominant while others remained subordinate.

Although male fathead minnows do not typically produce VTG, they possess the quiescent vg genes that trigger protein synthesis upon activation (Miracle et al., 2006). The concentrations of VTG, often very low, can sometimes be detected in unexposed males perhaps due to low levels of E2 present in male fish (Jensen et al., 2001; Thorpe et al., 2007). The physiological role of VTG presence in untreated males is unclear. The relatively high VTG concentration in a dominant group of males from the Control group could in part be explained by the sensitivity of ELISA and the antibodies utilized for the analysis (Jensen et al., 2001).

Based on the outputs of the cluster analysis and MANOVA, the social hierarchy constrained the variability within the male fish population exposed to estrogenic compounds: E1 and E2 when compared to Control treatment group. VTG synthesis was induced in fish exposed to both compounds with higher VTG concentrations in E2-exposed males. In other words, the dominance in male fish was shown to be suppressed pushing males towards subordinate spectrum. For estrogens to exert their effects at the molecular level, they first need to interact with their respective ERs where estrogen acts either as a transcription factor within a cell nucleus
(Thornton, 2001) or utilizes an alternative signaling pathway involving a rapid, non-genomic route initiated by membrane-bound estrogen receptors located on the cell surface (Nagler et al., 2007). The nuclear estrogen receptors belong to the superfamily of intracellular steroid hormone receptors (Kah et al., 1997) that includes androgen, progesterin, and mineralocorticoid receptors. There are two subtypes of ERs: ERα (NR3A1) and ERβ (NR3A2). The ERβ subtype is found in two different isoforms in some vertebrate species because of gene duplication event (Garcia-Revero et al., 2009; Thornton, 2001). However, two isoforms of ERs show differences in tissue distribution, receptor expression between the sexes, and can also dimerize to generate a functional unit (Socorro, 2000). The differential expression suggests tissue-specific roles for estrogen receptor subtypes (Filby & Tyler, 2005), and that different effects may be mediated by homo- and heterodimers of the two receptors (Kuiper et al., 1997). The differential expression suggests the sensitivity to estrogen (Flouriot et al., 1997), the tissue-specific roles for estrogen receptor subtypes, and that different effects may be mediated by homo- and heterodimers of the two receptors (Garcia-Reyero et al., 2009; Kuiper et al., 1997). Experiment conducted by Nelson & Habibi (2013) showed that β form of the estrogen receptors has a higher than ERα affinity for estrogens, and could serve as a sensor to estrogenic compounds (Filby & Tyler, 2005), which was confirmed by Filby et al. (2012) that ERα expression was induced upon exposure to exogenous estrogenic compounds.

In addition, the constraint of social hierarchy among males in part can also be explained by previous observations that male fish exposures to exogenous estrogens result in the suppression of 11-Ketotestosterone (11-KT) production, a primary androgen in teleost fish associated with dominance and aggressive behavior (Filby et al., 2012; Harries et al., 2000). The reduced synthesis of 11-KT was attributed to the inhibition of expression genes directly involved
in androgen synthesis pathway, such as cyp17, cyp19a1b, Hsd17b3 (Filby et al., 2012). It is plausible that inhibition of androgen synthesis in males results in either the loss or down grading of the social status consequently imposing the effects on their social structure (Filby et al., 2012; Harries et al., 2000). Alternatively, this shift of dominance towards sub ordinance involves the perturbations in HPG axis. It is know that estrogens modulate the activity of HPG axis via negative/positive feedback (Ankley & Johnson, 2004; Trudeau, 1997) that is continuously involved in informing the brain of the physiological status of distantly located organs (Kah et al., 1997). Male fish exposure to exogenous estrogenic compounds interferes with the stimulatory effects of GABA that either acts on hypothalamus inducing the release of GnRH (Kah et al., 1992) or acts directly on pituitary gland inducing the release of FSH-like and LH-like hormones (Kah et al., 1993). The previous studies showed that high levels of estrogens, but not testosterone (Schulz et al., 1993), block the stimulatory effect of GABA that ultimately leads to a reduction of end product synthesis, androgens for males (Martinovic et al., 2007). In addition, estrogens inhibit the expression of ar gene that codes for androgen receptors (AR) in the testis (Ikeuchi et al., 2001) consequently leading to the reduced production of male sex steroids (Filby et al., 2012). The reduction in male sex hormone production in either case affects the reproductive status of the males (Jobling et al., 1998; Martinovic et al., 2007) that can be expressed in a form of suppression of aggressive behavior and impaired ability to acquire a nest site under competition pressure (Martinovic et al., 2003) as well as in the expression of less prominent SSCs (Ankley & Johnson, 2004), which is consistent with the observed results.

Previous studies also demonstrated that transcription activation of ER gene synthesis starts at low estrogen concentration producing more ERs, which initiates the transcription of vitellogenin gene (Vg) (Jobling et al., 1997; Miracle et al., 2006). The primary location of ER up-
regulation upon exposure to exogenous estrogentic compounds is liver where the synthesis of VTG begins (Jobling et al., 1997; Martinovic et al., 2007).

In contrast to the observed effects of estrogenic exposure, the social hierarchy within male fish population exposed to SSRIs was liberated while maintaining plasma VTG concentrations similar to the Control group. This is consistent with the previously conducted study by Schultz et al. (2011) that documented no VTG induction upon SSRI exposure, with exception of exposure to FLX. Fish exposed to FLX on average scored lower on SSC and showed the induction of VTG production. The observed increase in separation between the two male populations based on the SSCs in the present study could be explained by on average lower SSC scores of fish exposed to FLX.

Nevertheless, serotonin is known to impose its effects via both HPG and HPA axes (Schultz et al., 2011; Winberg et al., 1997). Previous studies have shown the presence of a relationship between serotonin and HPA axis (Winberg et al., 1997; Winberg & Lepage, 1998). HPA axis is involved in mediating stressful conditions while altering 5-HT system that is conserved among the vertebrate species (Winberg et al., 1997). The direct contact between serotonin and CRH-containing neurons in paraventricular nucleus has been shown in mammals (Winberg et al., 1997; Winberg & Lepage, 1998). The studies on teleost fish showed an increase in 5-HT activity in socially subordinate individuals and during other stressful events while inducing the release of cortisol (Winberg et al., 1997; Winberg & Lepage, 1998). The HPA axis activity is activated upon serotonin by predominantly binding to 5-HT$_{1A}$ receptor in hypothalamus and 5-HT$_2$ receptors in the pituitary (Winberg et al., 1997). Thus exposure to SSRIs chronically elevates 5-HT concentrations in the synaptic cleft prolonging the effects of
serotonin, which either mimics the stressful event or the social subordination accompanied by increased 5-HT activity.

Serotonin, associated with a display of aggressive behavior (Larson & Summers, 2000) in male fish, also influences the critical stages of their reproductive development (Schultz et al., 2011). It has a close association with HPG axis by inducing LH and FSH release upon stimulation (Larson & Summers, 2000). Thus exposures to SSRI can indirectly affect spermatogenesis in fish by stimulating the release of LH from the pituitary gland (Trudeau, 1997) through 5-HT$_2$ receptor (Somoza & Peter, 1991). Although 5-HT has a stimulatory effect on LH hormone in vertebrates that influences the production of testosterone and consequently spermatogenesis (Schultz et al., 2011), it shows mixed results between vertebrates in its effects on intraspecific aggression. Serotonin exhibits an inverse relationship with aggression in some lizard species (Deckel, 1996) whereas its presence result in an increase in aggression in crustaceans (Pyle, unpublished study). The study conducted by Johnson et al. (2009) confirmed that the type of the receptor, to which serotonin binds, mediates the aggressive behavior. These authors found that 5-HT$_2$ receptor activation reduces the aggression in males (Johnson et al., 2009), the same receptor that induces the release of LH from the pituitary (Kah et al., 1993; Schultz et al., 2011). The binding of serotonin to 5-HT$_{1A}$ receptor induced the aggressive behavior in males (Johnson et al., 2009).

In addition, there is a temporal difference between socially variable individuals to serotonergic stimulus. Dominant males show rapid brief response whereas subordinate males show a delayed (up to a week) prolonged response to 5-HT (Larson & Summers, 2000). Moreover, the study conducted by Larson & Summers (2000) showed the reversal of social
status when only dominant males were exposed to SSRIs but no such effect was observed when both dominant and subordinate males were exposed to SSRI.

Based on all information presented above, it is plausible that the maintenance of social status as well as regulation of aggressive behavior is modulated by HPG, HPA axes, differential receptor expression and substrate binding, and temporal differences in responses to serotonergic stimuli between the individuals of different social statuses. Although serotonin tends to suppress dominance in males, there are multiple factors that affect the outcome, such as the concentration of the chemical, the length of exposure and the interplay between the signaling mechanisms that yet to be elucidated.

We were able to demonstrate that male fathead minnows separate into significantly different subpopulations within each treatment group. This finding suggests that a treatment of fish population as homogeneous may lead to the increased chance of Type II error, which implies that effects of some endocrine-disrupting compounds may have been underestimated. This in turn may lead to under regulation of these chemicals and under protection of our environment.
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