Nest-defense behaviors in fathead minnows after lifecycle exposure to the antidepressant venlafaxine

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A B S T R A C T

Venlafaxine is an antidepressant and anti-anxiety drug that has been detected in municipal wastewater at low μg/L concentrations. In this study, the nest-defense behavior of adult male fathead minnows (Pimephales promelas) was observed in fish exposed for a full lifecycle to venlafaxine nominal concentrations of 0.88, 8.8, and 88 μg/L (i.e. 1, 9.3, 75 μg/L mean measured concentrations). Nest-defense behaviors quantified were the time taken to contact a dummy intruder fish (on a flexible stick, held near each nest) and the number of contacts made during a 1 min period. In male fathead minnows exposed to venlafaxine over a full lifecycle at environmentally relevant nominal concentrations (i.e. 0.88 and 8.8 μg/L) no significant effects were observed in behavior. However, in males exposed over a full lifecycle to the highest concentration of venlafaxine (i.e. 88 μg/L), nest-defense behaviors were increased in males with empty nests, as shown by the significantly elevated percent of empty-nest males that made contact with the dummy intruder fish (85%) relative to the lower percentage of contacts (65%) among the Control males (p = 0.046). Lifecycle exposure to high venlafaxine (88 μg/L) caused males to over-protect their empty nests. Environmental venlafaxine concentrations are approximately 70 x lower than this, so it is unlikely that behavioral changes from venlafaxine exposure would occur in the environment. Normal nest defense behaviors in control males varied, depending on whether they were protecting empty nests or nests with eggs. Compared to Control males with empty nests, more Control males with eggs in their nests made contact with the dummy intruder fish (p = 0.014), contact was faster (i.e. <10 s, p = 0.011), and they hit the dummy intruder fish more times in 1 min (p = 0.031). This study is the first to assess reproductive behaviors in fish exposed to an antidepressant over a full lifecycle.

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1. Introduction

Venlafaxine is an antidepressant and antianxiety drug that has been detected in the effluents of wastewater treatment plants (WWTP) at concentrations in the low μg/L range (Arnnok et al., 2017; Roberts et al., 2016). For example, venlafaxine was detected in two WWTP effluents that discharge to the Niagara River at concentrations of 0.26 μg/L and 0.94 μg/L, respectively (Arnnok et al., 2017), and was present at concentrations up to 0.362 μg/L (mean 0.162 μg/L) in WWTP effluents discharging into the Grand River, ON (Couperus et al., 2016). The concentrations of some of the biologically active transformation products of venlafaxine, such as the N-desmethyl metabolite can be even higher in WWTP effluents, with total venlafaxine concentrations up to 2–4 μg/L (Arnnok et al., 2017; Metcalfe et al., 2010). Lower concentrations of venlafaxine have been detected in surface waters downstream of WWTPs, at 0.01–0.46 μg/L in a large river (Lajeunesse et al., 2008), and 0.9–1.1 μg/L in smaller rivers (Metcalfe et al., 2010; Schultz and Furlong, 2008; Schultz et al., 2010). From a survey of small streams in the southeastern United States, Bradley et al. (2016) reported concentrations of the transformation product, N-desmethyl venlafaxine of up to 1 μg/L, with mean concentrations of 0.03 μg/L.

The effects of venlafaxine have been assessed in acute and chronic tests with fish using a variety of biological endpoints. Exposure of fathead minnows (Pimephales promelas) to venlafaxine for 21 d reduced survival, but the response was not concentration-dependent, with about 36% mortality in a treatment at 0.3 μg/L and
18% mortality at 1.1 μg/L (Schultz et al., 2011). Our previous study on fathead minnows exposed over a full cycle to venlafaxine showed no effects in fish exposed at environmentally relevant concentrations, but fish from the highest venlafaxine treatment (i.e. 88 μg/L nominal) produced 46% more eggs per female than control fish (Parrott and Metcalfe, 2017). In contrast, adult zebrafish (Danio rerio) exposed to 5–6 μg/L venlafaxine for 7 weeks had reduced egg production (Galus et al., 2013).

Venlafaxine has been shown to affect fish behavior in short-term exposures. The escape responses of larval fathead minnows were slowed after exposure to 5 μg/L venlafaxine for 12 d (Painter et al., 2009). Hybrid striped bass (Morone saxatilis × Morone chrysops) exposed to 0.36–4.65 μg/L venlafaxine for 3–6 d took longer to capture their prey (Bisesi et al., 2014). Adult mosquitofish (Gambusia holbrooki) exposed to 100 μg/L venlafaxine for 7 d had disrupted circadian rhythm and decreased swimming during daylight (Melvin, 2017). Venlafaxine exposure at 1 μg/L for 7 d reduced feeding in rainbow trout (Oncorhynchus mykiss) and increased plasma cortisol concentrations in subordinate fish (Melnyk-Lamont et al., 2014).

Venlafaxine has also been shown to affect invertebrate behaviors. Exposure of common cuttlefish (Sepia officinalis) to 0.1 μg/L venlafaxine for 20 d decreased their camouflage ability (Bidel et al., 2016). In marine snails (Chlorostoma funebralis) exposed to 157 μg/L venlafaxine for 4 h, 90% of the animals detached from the substrate, while exposure to 31 μg/L caused no significant detachment (Fong and Molnar, 2013). Fong et al. (2015) reported that brief exposure to venlafaxine (31–157 μg/L) increased crawling speed in two species of marine snail, the oyster drill (Urosalpinx cinerea) and the American starssnail (Lithopoma americanum). Marine mud snails (Ilyanassa obsoleta) exposed to 31 μg/L venlafaxine for 2 h took longer to right themselves after being inverted (Fong et al., 2017).

Male fathead minnows have a complex reproductive behavior in which they aggressively defend a nest that contains eggs that they have fertilized (Cole and Smith, 1987, 1992; Unger, 1983; Unger and Sargent, 1988). Researchers have recently begun to assess nest-defense and nest-tending in fathead minnows to determine if there are behavioral impacts from exposure to environmental contaminants (Hoover et al., 2013; Lorenzi et al. 2012, 2016; Schoenfuss et al., 2016; Schultz et al., 2012; Weinberger and Klapner, 2014).

“Dummy” fish have been used to assess defensive and nest-tending behaviors in several species of fish (Barlow et al., 1986; Barlow and Siri, 1994; Dziewczynski, 2011; Dziewczynski et al., 2006; Dziewczynski and Hebert, 2012; Lehtonen, 2014; Rowland, 1975; Slovin and Rowland, 1978; Sowersby et al., 2017). In our previous study with fathead minnows exposed to venlafaxine over a full lifecycle (Parrott and Metcalfe, 2017), we observed that the exposed male minnows would frequently “hit” the gloved hands of research personnel checking for and collecting the eggs that were laid under tile nests. In order to evaluate the potential effects of venlafaxine on male nest-defense behavior, we introduced a dummy intruder fish near each male’s nest and quantified the number of contacts and the time to contact when the dummy intruder was positioned on a flexible stick near each male’s nest tile. We validated these behavioral assessments by determining if there were differences in protective behaviors between the male fish from the Control treatment with empty nests compared to the Control males with eggs in their nests. The objective of the present study was to evaluate whether exposure of fathead minnows to venlafaxine over a full lifecycle would alter the behaviors of male fish that were defending nests with eggs, as well as the behaviors of male fish guarding empty nests. The lowest venlafaxine exposure concentration was chosen to represent an environmentally-relevant concentration (0.88 μg/L) which is close to concentrations of venlafaxine in MWWEs, and approximates the highest venlafaxine concentrations detected in surface waters. The two higher venlafaxine fish exposure concentrations were 10x and 100x this environmentally-relevant concentration.

2. Materials and methods

2.1. Venlafaxine exposures

As described previously (Parrott and Metcalfe, 2017), fathead minnows were exposed over a full lifecycle to venlafaxine using a flow-through system with a modified Mount & Brungs-type diluter at nominal concentrations of 0 (Control), 0.88, 8.8, and 88 μg/L. The mean ± standard deviation measured concentrations in the exposure tanks were 1.00 (n = 2), 9.26 ± 1.9 (n = 4), and 75.2 ± 19.5 (n = 4) μg/L, respectively, as reported previously (Parrott and Metcalfe, 2017). The stock solution was made up from venlafaxine-HCl (CAS#99300-78-4, 99.9% purity, lot number A-1237-145) purchased from SynFine Research (Richmond Hill, ON, Canada). The flow-through system consisted of a series of 12 L glass aquaria, each with 3 vol changes per day. Venlafaxine was not detected in the Control tanks at concentrations above the Limit of Detection (LOD). The LOD was 0.02 μg/L and the Limit of Quantification (LOQ) was 0.07 μg/L.

Exposures of fathead minnows were from the fertilized egg stage through to 162–163 days post-hatch (dph). There were 8 replicate control tanks and 4 replicate tanks for each venlafaxine exposure concentration. Each tank contained 5 females and 3 males during the breeding phase of the experiment (i.e. after 106 days post hatch). Data on number of fish in each tank and survival over time are shown in Supplementary Data S3. There were three breeding tiles per tank, for one tile nest per breeding male. Tanks were aerated and covered, and the temperature was maintained by a water bath. The photoperiod was 16 h light:8 h dark, with dawn and dusk dimming. Water quality was measured weekly and was stable during the exposures. Temperatures ranged from 24.2 to 24.4 °C, mean dissolved oxygen ranged from 8.3 to 8.4 mg/L, mean pH ranged from 7.79 to 7.84, and mean conductivity ranged from 367 to 370 μS/cm, as reported previously (Parrott and Metcalfe, 2017). Data on the dilution water quality is shown in Supplementary Data (Table S1).

2.2. Nest-defense behaviors

During the breeding phase of the experiment (90–162 dph), there were 3 mature males and 5 mature females per tank, and each tank had 3 PVC breeding tiles under which the eggs were laid. As reported in the description of our previous study (Parrott and Metcalfe, 2017), tiles were checked daily for presence of eggs so that daily egg production could be calculated. During the checking for eggs, the male fathead minnow males would often “hit” the gloved hand of research personnel, and many of the male fish would persist in hitting repeatedly. To quantify these nest-defense behaviors, we designed a procedure using a dummy intruder fish (Fig. 1) introduced into the tank with the males and we set up trials to assess each adult male’s nest-protecting behaviors over a period of 18 days. These behavioral trials were performed after the enumeration of egg production had ceased.

The nest defense behaviors of male fathead minnows were assessed with five independent observers, each measuring the time to contact with a dummy intruder fish and by counting the number of contacts made during a 1 min time interval. This behavioral assessment was conducted with adult male fathead minnows at the 140 dph to 158 dph stage of development that were exposed over a full lifecycle in treatments with venlafaxine at 0.88, 8.8, and 88 μg/L.
nominal concentrations, and a Control treatment. To assess nest-defense behaviors, the observers worked in tandem with one recorder person. For each replicate tank from the venlafaxine exposures, there were 3 male fish, and each male was assessed 6 times. Thus, in total there were 18 assessments made for each tank from the venlafaxine treatments or control. These 18 assessments were split into observations of males defending “nests with eggs” and males defending “empty nests”.

The dummy intruder fish was constructed of metal in the form of a fish (3.2 cm long, 1.1 cm wide), colored black with yellow fins and gold metal edges, which was fastened to a flexible stick (a white plastic cable tie) as shown in Fig. 1. An observer held the dummy intruder fish under the water and circled the tank slowly (3 times) during a 1 min interval. After circling for 1 min, the dummy intruder fish was then held on the right side of a tile nest guarded by one of the three male fathead minnows in each tank. The dummy intruder fish was held 2 cm from the bottom of the tank and 2 cm from the opening of the nest. The observer watched the male fathead minnow to determine the time of first contact with the dummy intruder fish (and called this to the recorder), and then the observer verbally indicated subsequent dummy intruder fish contacts as they occurred during the 1 min observation period. The recorder started a stopwatch as the dummy intruder fish was in position next to the tile, and wrote down the time of first contact, as well as the number and type of contacts with the dummy intruder fish during a 1 min period. There were three types of contacts with the dummy intruder fish that could be distinguished by duration of the contact; a ‘hit’ (brief contact of <0.5 s), a ‘push’ (contact of 1 s), and a ‘hold’ (sustained contact > 1 s). For the purposes of statistical analysis, these three types of contacts were summed to give the number of contacts per 1 min. If the male fathead minnow contacted the stick, instead of the dummy intruder fish, this was not counted as a contact. Contacts between male fathead minnows were very rare, as the defensive behaviors of the males were focused on the dummy intruder fish.

After the first male from the first tile nest (always the front tile) was assessed in a tank, the second male guarding a tile nest (the middle tile) was similarly assessed in the same tank, followed by the third male guarding a tile nest (the back tile). After these three 1-min observations in each tank were completed, the tiles were examined to determine whether there were eggs present. Then, the next tank was assessed similarly. Over the course of 18 days over the period from April 28, 2008 to May 16, 2008, nest-defense behaviors were assessed 6 times, by 5 different observers, with 3 different recorders. Two of the observers were familiar with the venlafaxine lifecycle experiment, and three observers were naïve technicians brought in to assess the behaviors, independent of knowledge of the treatments. Although the naïve technicians were adept at handling fish and were trained to assess the types of fish behaviors, they were otherwise unaware of the venlafaxine treatments. The assessment was done with the various tank treatments blinded. Raw data are shown in Supplemental Data, Table S2.

2.3. Validation of the nest-defense test

We first validated whether the behavioral assessment could characterize fathead minnow nest defense behaviours by assessing if the data could distinguish between control males that had eggs in their nests and control males with empty nests. We assessed the 8 replicate control aquaria 6 times each over a period of 18 days. The behavior of these 24 control male fish (3 in each replicate tank) was summarized, and the assay was able to detect significant differences in % males making contact, % males with fast contact (<10 s), and number of contacts per minute. The control males protecting nests with eggs contacted the dummy intruder fish more often, made faster contacts with the dummy intruder fish, and contacted a greater number of times in 1 min. After validating the assay, we proceeded to test the venlafaxine-exposed male fish.

2.4. Statistical analysis

All data were analyzed using Systat 11.0 (Systat Software Inc., San Jose, CA). Raw data from the behavioral trials for each of the 4 treatments (i.e. Control and 0.88, 8.8, and 88 µg/L venlafaxine) were separated into data for males that had empty nests, and data for males with eggs in the nest. For each behavioral trial, data on the time to first contact (sec) and the total number of contacts in 1 min were collated. The raw data for each tank were assessed for normality using Shapiro-Wilk normality test, and this test showed that only 30% of the data on time to first contact met the assumptions of normality. Standard transformations did not succeed in normalizing these data. The full data set is summarized in Supplemental Data (Table S2), along with illustrations of the non-normal distributions of the time to first contact data per tank for control males (Figure S1).

Because of the non-normality of the data on time to first contact, the data were reorganized into information on the numbers of fish that made contact with the dummy intruder fish and the numbers of fish that made fast (i.e. <10 s) contact with the dummy intruder fish. These data on the percentage of fish in a tank with contact, and the percentage of fish in a tank with fast contact met the assumptions of normality in 75% of behavioral trials, according to the Shapiro-Wilk normality tests, and also met the assumptions of homogeneous variances in 75% of the behavioral trials, as assessed by Levene’s tests. Therefore, these parameters were assessed for statistically significant differences between treatments using Analysis of Variance (ANOVA), followed by post-hoc Tukey’s tests. Pairwise two-sample t-tests were used to test the significance of differences in mean behavioral responses between Control fish with eggs and without eggs, and between contact data for the three venlafaxine treatments and the Control, with data always separated into responses in male fish with eggs in their nests and male fish with empty nests. All p values in the text were from pairwise tests of means (from two-sample t-tests) using Bonferroni’s adjusted p values with separate variances, except where noted.

For the other raw behavioral data on the mean number of

![Fig. 1](image-url)
contacts with the dummy intruder fish in 1 min, 78% of data per tank met the assumptions of normality according to the Shapiro-Wilk normality test, so data on the mean number of contacts in 1 min per tank were used to describe the results and the data were analyzed for statistically significant differences between treatments by ANOVA and Tukey’s post-hoc tests. For each of the 4 treatments (controls or 3 venlafaxine exposure concentrations), 88% of the data (treatment means, calculated from 8 control tank means or 4 venlafaxine tank means) were normal as assessed by the Shapiro-Wilk normality test, and 100% had homogeneous variances, as assessed by Levene’s test. The full data set is shown in Supplemental Data (Table S2), and the normal distributions for variances, as assessed by Levene’s test. The full data set is shown in Supplemental Data Table S2, and the normal distributions for number of contacts in 1 min for each control tank are illustrated in Supplemental Data Figure S2. Pairwise two-sample t-tests were used to compare mean numbers of contacts between Control fish with eggs and without eggs, and mean numbers of contacts between the three venlafaxine treatments and the Control. All p values in the text were from pairwise tests of means (from two-sample t-tests) using Bonferroni’s adjusted p values with separate variances, except where noted.

General Linear Model (GLM) statistical procedures were used for comparisons of the nest-defense behaviours of the males (i.e. number of contacts in 1 min) with egg production (i.e. mean number of eggs per female per tank). The latter data on egg production were taken from our previous data with fish from the same exposure trials (Parrott and Metcalfe, 2017). Regressions and $r^2$ values are shown in Figures, and F and p values from the GLM are indicated in the text.

3. Results

3.1. Validation of the nest defense behavior test with control males

We first examined whether nest-defense behavior protocols could assess differences in the behaviors of male fish from the Control treatments. As shown in Fig. 2 for male fish from the Control treatment, the proportion of male fish with eggs in their nests that made contact with the dummy intruder fish (i.e. 94%) was significantly greater than the proportion of fish that made contact among the males with no eggs in their nests (i.e. 65%), as tested with a two-sample t-test with a Bonferroni’s adjusted p value, with separate variances ($p = 0.014$). Similarly, there were a significantly greater ($p = 0.031$) percentage of Control males with eggs in their nests that made fast contact with the dummy intruder fish (i.e. 80%) compared to Control males with no eggs in their nests (52%). Control males with eggs in their nests also made contact with the dummy intruder fish a greater number of times (12.9 contacts in 1 min), compared to Control males with no eggs in their nests (6.7 contacts in 1 min, $p = 0.011$), as shown in Fig. 2. Using the control data, we also assessed whether there were significant differences in the results generated by the five observers. ANOVA showed that the observer was not a significant factor in assessing the time to contact ($p = 0.477$) or number of contacts in 1 min ($p = 0.352$, Data shown in Supplemental Data S4). The length of time the dummy intruder fish was held in the tank was not related to the number of contacts on the fish stick ($p = 0.459$), or to the time of the first contact ($p = 0.114$, Data shown in Supplemental Data S4).

3.2. Nest-defense behaviours of venlafaxine-exposed males

Among the treatments with lifetime exposure to venlafaxine, there were changes to behavior among male fish that had empty nests from the treatments with the highest concentration of venlafaxine (i.e. 88 $\mu$g/L). There was a significantly greater proportion of the males with empty nests that made contact with the dummy intruder fish, compared to empty-nest Control males. Comparing these males with empty nests (Fig. 3), 65% of the Control males made contact with the dummy intruder fish, while 89% of the males exposed to the highest venlafaxine treatment made contact with the dummy intruder fish ($p = 0.046$). While the data from male fish with empty nests from the treatments at lower venlafaxine exposure concentrations showed similar trends, these data were not significantly different from Control males with empty nests, as shown by levels of significance of $p = 0.148$ and $p = 0.242$, for males exposed to 0.88 and 8.8 $\mu$g/L venlafaxine, respectively (Table 1).

In general, for fish from both the Controls and venlafaxine treatments, males with eggs in their nests made very fast contact
with the dummy intruder fish, with 80–100% of males having fast contact within <10 s. Males with eggs in their nests almost always (94–100%) made contact with the dummy intruder fish during the 1 min assessment period (Table 1). On average, only 0–6% of males made no contact at all when they had eggs in their nests among the Controls and venlafaxine treatments. The number of contacts with the dummy intruder fish in 1 min ranged from 11.3 to 14.8 and did not differ among venlafaxine treatments and Controls among males that had eggs in their nests.

### 3.3. Relationship of nest-defense behaviors with egg production

Our previously reported results from the lifecycle exposure to venlafaxine showed that fish from the highest venlafaxine treatment (88 μg/L) produced significantly greater numbers of eggs per female (Parrott and Metcalfe, 2017). To test whether the nest-defense behaviors were related to the reproductive performance of each tank, the previously reported data on the average number of eggs per female for each replicate tank were regressed against the number of contacts in 1 min (Fig. 4). Assessing the relationship for the Control fish alone, the data showed a trend of higher numbers of contacts per min among males in treatments where there had previously been greater egg production (i.e. mean number of eggs per female), although there was high variability, as shown for the regression lines in blue in Fig. 4. The regression line for Control males with eggs (solid blue line, r² = 0.600, p = 0.024 from GLM) was above that of control males with empty nests (dotted blue line in Fig. 4, r² = 0.090, p = 0.470), and the two regression lines (for control males with eggs and for control males with empty nests) were significantly different (F = 11.453, p = 0.005 from GLM). This means that control males with eggs protected their nests more vigorously than control males with empty nests.

Among treatments with the highest concentration of venlafaxine (i.e. 88 μg/L), the regression lines illustrated in Fig. 4 for males with eggs (purple solid line) and for males with empty nests (dotted line) overlapped and there was no significant difference in the number of contacts in 1 min (F = 0.002, p = 0.964 from GLM). However, when a regression line was calculated for the data from all males exposed to 88 μg/L venlafaxine, with eggs and with empty-nests, the regression was significant and had good fit (r² of 0.672 and p = 0.013). For males exposed to the highest concentration of venlafaxine, whether they had empty nests or nests with eggs, there were no differences in the numbers of contacts in a 1 min interval. This means that males exposed to high venlafaxine protected their empty nests as vigorously as if the nest contained eggs.

### Table 1

Summary parameters in the behavioral assessment of adult male fathead minnows exposed to venlafaxine for a lifecycle. Data were separated in males with empty nests and males with eggs in their nests. Table shows percentages of males that made “Contact” (% males that made contact with the dummy intruder fish in 1 min), “Fast Contact” (% males with first contact in <10 s), and the “# Contacts” (number of contacts with the dummy intruder fish in 1 min”). Values are means per tank (±standard deviation), and n is the number of tanks per control or venlafaxine treatment. The value in bold indicates a mean that was significantly different between control males with empty nests compared to 88 μg/L venlafaxine-exposed males with empty nests. All raw data and data for each individual replicate tank are shown in Supplemental Data Tables S1 and S2.

<table>
<thead>
<tr>
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<tr>
<td></td>
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<td>% trials with contact</td>
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<td>% trials with &lt;10 s contact</td>
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<td># Contacts</td>
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<tr>
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<td>9 ±(4)</td>
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<tr>
<td>Eggs in Nest</td>
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<td>n</td>
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<tr>
<td>Contact</td>
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<td>% trials with contact</td>
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<td>Fast Contact</td>
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<tr>
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*Significantly different (F < 0.05 from GLM).
4. Discussion

The protocol for assessing the interaction of male fathead minnows with a dummy intruder fish proved effective in measuring the nest-protecting behaviors of male fish. The test successfully distinguished between the behaviors of Control males that were defending nests with eggs, compared to those with empty nests. Similar “dummy” fish have been used for many years to assess defensive behaviors of male Siamese fighting fish (Betta splendens) (Dzieweczynski et al., 2006, 2012), three-spined stickleback (Gasterosteus aculeatus) (Dzieweczynski, 2011), marine tilapia (Tilapia mariae) (Slovin and Rowland, 1978), and several species of cichlids (Barlow et al., 1986; Barlow and Siri, 1994; Lehtonen, 2014; Rowland, 1975; Sowersby et al., 2017). Methods for making realistic dummy fish were reviewed by Rowland (1979).

In the present experiment, there were differences observed in the behaviors of Control males with eggs in their nests and Control males with empty nests, as males with eggs in their nests were more “protective” than control fish with empty nests. Other species of fish have been shown to be more protective when eggs and fry are present. Female red devil cichlids (Amphilophus labiatus, which is a fish species where both sexes protect eggs and larval fish) showed significantly increased protective behaviors from the lowest level for empty nests, to nests with eggs, to the highest level for free-swimming fry (Sowersby et al., 2017). Similarly, male Siamese fighting fish were more aggressive after their fry had hatched than after the eggs were laid (Jaroensutasinee and Jaroensutasinee, 2003).

Exposure of males for a lifecycle to venlafaxine increased the percentage of aggressive males that made contact with the dummy intruder fish compared to control fish. This trend was seen in male fish from all treatments with venlafaxine concentrations, but was significant only for the treatments at the highest venlafaxine concentration. Interestingly, there was no difference in the degree of nest protecting behavior among males exposed to 88 µg/L venlafaxine, regardless of whether the males were guarding nests with eggs or empty nests.

Other researchers have used a similar nest defense protocols to assess the behavior of mature fathead minnow males. There were trends towards decreased aggression in fathead minnow males exposed to 17α- and 17-β estradiol, but the changes were not significant (Shappell et al., 2010) although exposures to these estrogens did increase plasma vitellogenin in male fish. In another study, increased aggression was observed in fathead minnow males exposed to low concentrations of estrone and estradiol, but the results were inconsistent over two separate experiments with different concentration ranges (Dammann et al., 2011). Exposure of adult fathead minnows to 1 - 2 µg/L triclosan and triclocarbon for 21 d decreased male aggressive behaviours and the authors that suggested this may decrease a male’s ability to defend a nest site (Schultz et al., 2012).

Exposures of fish to antidepressants have shown changes in aggressive behaviours and boldness, with most studies showing decreased aggression. Siamese fighting fish exposed to fluoxetine (0.54 µg/L nominal concentration) showed decreased aggression (Dzieweczynski and Hebert, 2012). Three-spined stickleback (Gasterosteus aculeatus) exposed to fluoxetine at concentrations of 3.2, 10, and 32 µg/L for 21 d built nests of significantly poorer quality compared to control males, but there were no significant changes in the aggressive behavior of the male fish (Sebire et al., 2015). In contrast, male fathead minnows exposed for 4 weeks to fluoxetine (100 µg/L) showed significantly increased aggression and also showed increases in repetitive behaviours such as nest cleaning compared to control males (Weinberger and Klaper, 2014). Male gulf toadfish (Opsanus beta) implanted with fluoxetine showed increases in dominant-fish aggression (McDonald et al., 2011).

The increased nest-defense behavior of males exposed to 88 µg/L venlafaxine in the present study is positively correlated with the previously reported egg production among female fish from these trials. In the present study, there were significant relationships between the average number of eggs produced per female and the “protectiveness” of the male fish. The Control fish showed a stronger relationship between these parameters when the behavior was assessed in males with eggs present in their nests. Venlafaxine (88 µg/L) exposed fish had elevated egg production, and this correlated with the level of nest-protecting behavior of the males, regardless of whether the males were protecting nests with eggs or without eggs. In the wild, fathead minnow males are territorial and defend a nest during the breeding season (Unger, 1983). Fathead minnow males also protect their existing eggs to increase their chances of attracting new females. Males protecting nests with eggs already in them are more likely to be favored by female fathead minnows over empty-nest males (Unger and Sargent, 1988). A study of fathead minnows in a natural pond setting showed that males showing aggressive care of their nests produced significantly more eggs and held their nests for longer periods compared to males demonstrating no care or passive nest-defense care (Divino and Tonn, 2008). Other fish species also show that increased nest-defensive behavior is related to reproductive success. For instance, male zebrafish that were the boldest and most aggressive were successful in fertilizing a higher percentage of eggs compared to non-aggressive males (Ariyomo and Watt, 2012).

Exposure to the highest concentration of venlafaxine increased male nest-protecting behavior and our previous studies showed that exposure also increased reproductive success. However, we cannot be sure whether these effects were both direct effects of exposure, or whether one was a secondary effect caused by the other. An increase in the number of eggs deposited in the nests may have caused the increased nest-defense behaviors of the males. Conversely, the increased nest-protectiveness in venlafaxine exposed males may have resulted in more eggs being laid by the females. Another possibility is that increased nest-defense by the venlafaxine-exposed males prevented some of the eggs from being eaten, as less of eggs due to predation from other fathead minnows is common (Vandenbos et al., 2006).

The ecological significance of the increased nest defense behavior is unknown. It is possible that the venlafaxine-exposed males’ increased protection of empty nests may result in negative ecological outcomes due to faster depletion of the male’s energy stores. It may also result in the male fish being injured by a rival male or being eaten by a predator. Competitive behavior between rival males and predator-avoidance behaviours (such as those assessed in Saaristo et al. (2017)) were not assessed in the present study, and results from such studies could help interpret the increased nest-defense behaviours of venlafaxine exposed males. Conversely, increases in protection of empty nests may have positive ecological outcomes. Increased nest defense behaviours in venlafaxine-exposed males may increase their chances of attracting female fathead minnows and thus of siring more eggs. Male minnows with eggs already in their nests are favored by female minnows (Unger and Sargent, 1988), so if a venlafaxine-exposed male acts as if he had eggs in his nest (by being more aggressive), this may also increase his attractiveness to female fish.

The increased nest-protective behaviours of male fathead minnows observed in this study were seen only at the highest venlafaxine exposure concentration (88 µg/L nominal, 75 µg/L measured). Environmental venlafaxine concentrations are approximately 70 x lower than this, so it is unlikely that behavioral changes from venlafaxine exposure would occur in the environment. However, in small rivers near WWTP discharges, fish are exposed to a variety of antidepressants and their metabolites, and
so these real-life antidepressant mixtures may be more important to assess in long term fish exposures and behavioral studies.

5. Conclusions

The present study showed that nest-defense behavior in fathead minnows can effectively be measured by assessing the time and the number of contacts with a dummy intruder. The behavior in Control males showed increased nest-protection when eggs were present, but the difference in nest-protecting behaviors between males with nests with eggs and without eggs was not observed in treatments with the highest concentration of venlafaxine. Venlafaxine exposure at 88 µg/L significantly increased the proportion of males that protected their empty nests. Nest-protective behaviors were positively correlated with egg production in females in the Control treatments and the treatments with the highest venlafaxine concentration. To assess the relevance of the increased nest-protection of venlafaxine-exposed males, future studies should measure predator avoidance or nest-competition among fathead minnow males. However, when considering the real environment and the variety of antidepressants discharged, perhaps a more important approach would be to assess antidepressant mixtures.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.11.049.

References

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