

*Pharmaceuticals and Personal Care Products in the Environment*ANTIDEPRESSANTS AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS
AFFECT PREDATOR AVOIDANCE BEHAVIOR OF LARVAL FATHEAD MINNOWS
(*PIMEPHALES PROMELAS*)

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Abstract—The effects of embryonic and larval exposure to environmentally relevant (ng/L) concentrations of common antidepressants, fluoxetine, sertraline, venlafaxine, and bupropion (singularly and in mixture) on C-start escape behavior were evaluated in fathead minnows (*Pimephales promelas*). Embryos (postfertilization until hatching) were exposed for 5 d and, after hatching, were allowed to grow in control well water until 12 d old. Similarly, posthatch fathead minnows were exposed for 12 d to these compounds. High-speed (1,000 frames/s) video recordings of escape behavior were collected and transferred to National Institutes of Health Image for frame-by-frame analysis of latency periods, escape velocities, and total escape response (combination of latency period and escape velocity). When tested 12 d posthatch, fluoxetine and venlafaxine adversely affected C-start performance of larvae exposed as embryos. Conversely, larvae exposed for 12 d posthatch did not exhibit altered escape responses when exposed to fluoxetine but were affected by venlafaxine and bupropion exposure. Mixtures of these four antidepressant pharmaceuticals slowed predator avoidance behaviors in larval fathead minnows regardless of the exposure window. The direct impact of reduced C-start performance on survival and, ultimately, reproductive fitness provides an avenue to assess the ecological relevance of exposure in an assay of relatively short duration.

Keywords—Pharmaceuticals mixtures Behavior Larvae Embryos Fathead minnow

INTRODUCTION

A wide variety of pharmaceuticals are discharged into waterways via treated wastewater effluent [1–3]. Since pharmacological function usually requires metabolic stability, these drugs are often resilient to microbial degradation, can pass through the wastewater treatment process with limited removal, and remain biochemically active after they reach the aquatic ecosystem [4,5]. Although many pharmaceuticals are designed to target specific pathways of the endocrine and immune systems, little is known regarding the effects of these drugs in nontarget aquatic life [6–8].

Among pharmaceuticals, antidepressants are a frequently prescribed class of psychotherapeutic drugs that persist in wastewater effluent and have been reported at nanogram per liter to low microgram per liter concentrations in the aquatic environment [9,10]. The modification of neurotransmitter regulation is an inherent trait of antidepressants. Neurotransmitters, such as serotonin (5-hydroxytryptamine; 5-HT), norepinephrine (NE), and dopamine (DA), are involved in major homeostatic processes throughout the central and peripheral nervous systems [11], and any alteration of neurotransmitter regulation through exogenous sources may have multisystem ramifications.

The group of antidepressants known as selective serotonin reuptake inhibitors (SSRIs) includes drugs such as fluoxetine

(FLX) and sertraline (SER). Serotonin is one of the most abundant neuromodulators in vertebrates and regulates immune system function, homeostasis of neural tissues, and survival behaviors [7,12,13]. Other popularly dispensed groups of antidepressants are the selective serotonin and NE reuptake inhibitors (SSNRIs), such as venlafaxine (VEN), that affect the function of both serotonergic and noradrenergic systems. Antidepressant drugs such as bupropion (BUP) also inhibit reuptake of NE (important in control of sleep and wakefulness, attention and feeding behavior) [11] and suppress the reuptake of the neurotransmitter DA (a key regulator of movement, learning and memory, motivated behavior, and hormonal signaling) [14]. Aquatic persistence of these compounds in treated municipal wastewater effluent has been reported with concentrations as high as 65, 80, 2,100, and 700 ng/L for FLX, SER, VEN, and BUP, respectively [10].

Both mammals and teleost fish exhibit a close relationship between the brain neurotransmitter systems and the hypothalamic–pituitary–interrenal axis (the teleost equivalent of the hypothalamic–pituitary–adrenal axis). Therefore, both taxa have a similar need to maintain autonomic, behavioral, and neuroendocrine stress responses through neurotransmitter systems [15]. Through modulation of neurotransmitter systems by exogenous compounds, reproductive fitness may be altered via effects on nonreproductive behavior and physiology. Reproductive fitness critically depends upon the optimal expression of nonreproductive traits that influence survivorship to reproduction, including predator avoidance responses [16]. Predation is one of the primary sources of mortality at all

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life stages but is most pronounced in fish during the larval stage [17]. Predator avoidance responses include coordinated bursts of swimming known as fast-starts [18]. One well-studied fast-start, the C-start, is innate and conserved across teleost lineages as a predator avoidance strategy [19]. C-starts are reflex behaviors that begin with a short latency period during which the threat stimulus is being perceived by the animal, are followed by a dramatic bending of the body into a C-shape, and end with an explosive burst of high-velocity locomotion away from the threat stimulus [18].

C-start behavior is regulated by a sensory-motor axis that integrates auditory, visual, and vibrational information and transduces these stimuli into musculoskeletal activation via special neurons called Mauthner cells located in the hindbrain [19]. The axon of a Mauthner cell extends into the spinal cord, and stimulation excites primary and secondary motor neurons and interneurons, which then excite the white fibers of the lateral musculature [20]. Evidence demonstrates that the Mauthner cell not only activates this fast-start response but also serves as a command neuron linked to a lower-level central pattern generator (CPG) [21]. Autonomic locomotor activities such as swimming and walking use repetitive movements reliant on groups of spinal interneurons that synchronize the activation and inhibition of motor neuron signaling to muscles [22,23]. In the case of the Mauthner cell, this command neuron triggers a CPG that creates a fixed action pattern of muscle contraction and relaxation, similar to an oscillating wave, which causes the fish to swim away from an approaching predator. Moreover, when a stimulus triggers the command neuron, the CPG directs the fixed action pattern devoid of peripheral feedback [22]. In this manner, an escape behavior may be the result of activation of a single Mauthner cell [21].

Mauthner cell signaling in vertebrates is hormone responsive [24] and requires coordination of multiple neuronal signaling systems [25]. Toxicants can impair neurotransmitters by altering presynaptic neurotransmitter production, by binding to the neurotransmitters, or by blocking postsynaptic receptors. Any of these alterations can disrupt communication between the afferent sensory receptors and the efferent motor systems, resulting in reduced behavioral performance [26]. Exposure to multiple pharmaceuticals with similar modes of action may generate additive or synergistic effects [6]. However, the effects are unknown in mixtures of pharmaceuticals representing multiple modes of action. In the aquatic environment, these chemicals are often present in complex mixtures, therefore supporting the need for assessment of their combined effects.

The present study aimed to test three hypotheses. First, larval fathead minnows exposed to antidepressants, singularly or in mixture, suffer a reduced ability to perform an innate C-start behavior when faced with a threat stimulus. Second, additive effects increase the potency of antidepressant mixtures, and, third, the sensitivity to antidepressants varies among developmental stages.

MATERIALS AND METHODS

The present study exposed fathead minnow (*Pimephales promelas*) embryos (from fertilization to hatching) or larvae (from hatching to 12 d posthatch) to varying concentrations of antidepressants, singularly and in mixture, to determine the effect of early life-stage antidepressant exposure on predator avoidance behavior. Regardless of the exposure window, all

animals were assessed for their ability to respond to a standardized threat stimulus 12 d posthatch. Assessment of the escape behavior included delay in response (latency period), velocity during the first 40 ms after the initiation of an evasive maneuver, and total escape response (a product of latency and velocity). Research was conducted in accordance with Saint Cloud State University Institutional Animal Care and Use permits.

Embryo exposures

Embryos (<12 h postfertilization) were obtained from Environmental Consulting and Testing. Fathead minnows are nest spawners whose eggs are deposited on submerged surfaces, in this case small nest tiles made from half sections of 7.6-cm diameter polyvinyl chloride pipe. Embryos from six spawning nests were removed from the tiles, combined in one beaker, and used to randomly populate 9-cm-diameter glass Petri dishes ($n = 25$ embryos per dish; six replicate Petri dishes; total $n = 150$). Each dish was filled to a 1-cm depth with conditioned well water (aerated for at least 24 h at room temperature prior to use). Treatment-specific chemicals were added to the conditioned well water prior to daily water exchange (100% daily static renewal). Embryos were maintained at $23 \pm 0.4^\circ\text{C}$ (mean \pm standard error) under a constant photoperiod (16:8 h light:dark). Larvae were fed 2 ml of hatched brine shrimp (Brine Shrimp Direct[®]) twice daily and allowed to feed ad libitum. After hatching on the fifth day of exposure, 40 larvae (singular compound exposures) or 60 larvae (mixture exposure) representing all six Petri dishes, were randomly transferred to two 1-L Pyrex[®] glass beakers and allowed to grow in conditioned well water until 12 d old. At that time, it was possible to observe the behavior of the animals in the filming arena (animals were too small to be filmed earlier than day 12 posthatch).

Larval exposures

Posthatch fathead minnow larvae (<48 h old) were obtained from Environmental Consulting and Testing. Groups of 20 larvae (singular compound exposures) or 30 larvae (mixture exposure) in duplicate ($n = 40$ or 60 per treatment) were exposed for 12 d in 1-L Pyrex glass beakers with conditioned well water at $22.8 \pm 0.4^\circ\text{C}$ under a constant photoperiod (16:8 h light:dark). Larvae were fed 2 ml of hatched brine shrimp twice daily and allowed to feed ad libitum. The exposure length was chosen to provide the shortest posthatch exposure window that would allow the filming of the animals immediately following exposure (animals were too small to be visible in the filming arena prior to day 12 posthatch). Treatment-specific chemicals were added to the conditioned well water prior to daily water exchange (50% daily static renewal).

Test chemicals

Organisms were exposed to the singular antidepressants FLX, SER, VEN, and BUP at three concentrations (low, medium, and high) to establish a response baseline and then in mixture (Table 1). Exposure concentrations for each chemical were chosen to approximate measured environmental concentrations [9,10]; they spanned a 10-fold difference between low and high doses to account for temporal and spatial concentration variability found in the aquatic environment. Concentrated stock solutions were prepared in the U.S. Geological

Table 1. Exposure concentrations used in embryonic and larval fathead minnow exposure experiments

	Ethanol solvent control	Low (ng/L)	Medium (ng/L)	High (ng/L)
Fluoxetine (FLX)	—	25	125	250
Sertraline (SER)	—	25	125	250
Venlafaxine (VEN)	—	500	2,500	5,000
Bupropion (BUP)	—	200	1,000	2,000
Mixture				
FLX	—	25	125	250
SER	—	25	125	250
VEN	—	500	2,500	5,000
BUP	—	200	1,000	2,000

Survey laboratories and used to draw daily spike aliquots. Briefly, masses of neat standards of BUP hydrochloride, FLX hydrochloride, SER hydrochloride, and VEN hydrochloride, of between 98 and 99.9% minimum purity, were individually dissolved in unadulterated anhydrous ethanol to produce 10-ml stock single-component solutions ranging in concentration between 0.18 and 2.91 mg/L. A variable volume micropipettor was used to dispense appropriate volumes of each stock solution into 1.7-ml microcentrifuge vials. Vials were filled with deionized well water to 1 ml, and an adequate supply of these daily aliquots was made and stored at 4°C to last for the entire experiment. For the daily static renewal exchanges, one aliquot of the pertinent chemical was dissolved in 1 L of conditioned well water and gently agitated to ensure proper mixing. Ethanol solvent concentrations in the treatments did not exceed ethanol levels of 8.5 µL/L well water, which was below solvent concentrations used in previous experiments [27,28] and lower than observed no-effect concentrations [29]. An ethanol solvent control was added to each experiment and mimicked the maximum amount of ethanol present within the chemical spikes. Well water was routinely tested and was free of emerging contaminants.

Experimental design

The C-start performance of exposed larvae was measured using a trigger-activated system with a small light-emitting diode and a vibrating electronic chip attached to the base of the filming arena to provide a stimulus [30]. When activated, the system caused a short vibrational stimulus (<1 s) marked in the field of view by the appearance of the light-emitting diode used to determine time 0 for data analyses. The filming arena consisted of a 5-cm diameter glass Petri dish positioned on top of a 1-mm grid. The larval escape behavior in the filming arena was recorded using a high-speed digital video camera (Redlake MotionScope M1®) at 1,000 frames/s. The camera was positioned approximately 50 cm above the arena with the field of view encompassing the 1-mm grid.

Larvae were fed 0.5 h prior to testing, and a resultant time limit of 6 h was set for data collection to avoid an observed drift in response due to time since last feeding [30]. Individuals were randomly placed into the filming arena, and water temperature was kept constant (22.4 ± 0.6°C) throughout the testing period. Once placed in the swimming arena, a larva was allowed to acclimate while the system was primed for another recording (usually 30–60 s). A larva was tested from each treatment in a sequential pattern (solvent control, high, medium, low, solvent control, high, etc.) until the behaviors of approximately 22 larvae per treatment (representing all replicates) were observed. When a larval fish swam into the

center portion of the grid (marked with a square), the trigger was depressed and the vibrational stimulus provoked a C-start response (Fig. 1). Each larval fish was used for only one performance recording.

High-speed video sequences of C-start behaviors were saved as AVI files and transferred to the public domain National Institutes of Health Image program for Apple Macintosh™. The positions of landmarks on the bodies of the larvae were digitized using the QuickImage modification (developed by J. Walker and available at www.usm.maine.edu/~walker/software.html). For each larva, the anteriormost tip of the snout and posteriormost tip of the tail were marked in addition to two points representing the 1-mm grid. The resultant coordinate data were exported and used to calculate the time to induction of behavior (latency period), escape velocity (velocity during the first 40 ms after the initiation of an evasive maneuver, body length per millisecond to exclude any size differences as confounding factors among individual fish [31]), and total escape response [body length/(latency in ms + 40 ms)]. Videos were not considered if the latency response was less than 5 ms (false start). The actual *n* value for latency period, escape velocity, and total escape response varied slightly among treatments due to factors such as survival, maximum allotted time for filming, and number of videos not considered due to false starts.

Statistical analysis

The assumption of normality for latency period, escape velocity, and total escape response was tested with the Lilliefors test for normality prior to any additional analysis (Prism 4.01™ statistical package, GraphPad Software). Many treatments violated the assumption of normality, so all data were analyzed using the nonparametric Kruskal-Wallis test followed by Dunn's posttest (Prism 4.01). In addition, the total escape response was assessed for dose-dependent trends using the rank-based procedure of the Jonckheere-Terpstra test (SAS 9.1®). The nonparametric Jonckheere-Terpstra test was conducted using a one-tailed approach with the assumption of greatest C-start performance consistent with the all-or-nothing (survival or death) functionality of this behavior. This approach follows convention in the biomechanical performance literature, which assumes that unique behaviors can be underestimated in laboratory settings but cannot be overestimated due to the physiological and mechanical constraints of the observed organism [32–34]. The total escape responses of treatment-specific solvent controls were compared using the Kruskal-Wallis test followed by Dunn's posttest (Prism 4.01) in order to determine whether differences among controls were within the realm of normal variation. A probability of *p* < 0.05 was set as level of significance for all comparisons.

RESULTS

Escape performance: Embryonic exposure

When tested 12 d posthatch, no statistical difference was found among total escape responses of treatment-specific solvent control larvae exposed as embryos (*p* = 0.07, data not shown). Within each experiment, each treatment was compared to its respective solvent control since no differences were found among controls. Larvae exposed singularly to FLX, VEN, and the antidepressant mixture as embryos exhibited alterations in their C-start performance when compared to control animals (Fig. 2). The latency period before a C-start

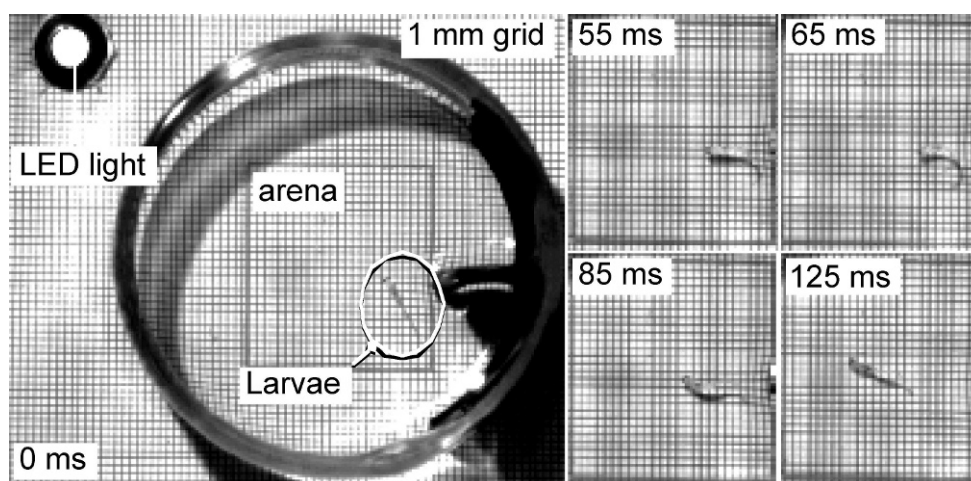


Fig. 1. Multiple still frames extracted from a representative C-start behavior filmed at 1,000 frames/s. The 12-d posthatch larva is hovering within the square outlining the center of the field of view of the swimming arena as the system is activated at 0 ms, indicated by the illumination of the light-emitting diode (LED) in the upper left corner of the field of view. During the following 125 ms, the larva first bends the body into the characteristic C-shape (65 ms) before darting off at an angle to the prestimulus position. Brightness and contrast have been enhanced digitally to improve clarity of images.

commenced was significantly longer in the VEN-low (500 ng/L) treatment than in the control treatment ($p = 0.025$). Escape velocity was significantly slower in the FLX-high (250 ng/L) and MIX-high (7,500 ng/L) treatments than in the control ($p = 0.04$ and $p = 0.003$, respectively; Fig. 2). The diminished performance carried over to the total escape responses for FLX, VEN, and antidepressant mixture exposures (Fig. 2). The total escape responses of FLX-low (25 ng/L), VEN-low, and MIX-high treatments were all significantly slower than in control animals ($p = 0.021$, $p = 0.046$, and $p = 0.004$, respectively). The Jonckheere-Terpstra test revealed that at these experimentally selected concentrations a dose-dependent reduction in total escape response for antidepressant-exposed animals was observed in the FLX ($p = 0.045$) and mixture experiments ($p = 0.0006$). In the present study, neither SER nor BUP affected behavior of larvae after embryonic exposure.

Escape performance: Larval exposure

When tested 12 d posthatch, no statistical difference was found among total escape responses of treatment-specific solvent control larvae ($p = 0.23$, data not shown). Within each experiment, each treatment was compared to its respective solvent control since no differences were found among controls. Similar to embryo exposures, adverse effects were seen in larvae exposed to antidepressants for 12 d posthatch. The mean latency period was adversely affected by exposure to VEN (VEN-high treatment of 5,000 ng/L vs. control, $p = 0.048$; Fig. 3). Escape velocity was affected by BUP exposure ($p = 0.034$ Kruskal-Wallis, not resolved using Dunn's posttest) and the antidepressant mixture (MIX-low treatment of 750 ng/L, $p = 0.037$; Fig. 3). Similar to embryo exposures, the total escape response was affected by exposure to VEN (VEN-high treatment; $p = 0.043$) and the antidepressant mixture (MIX-low treatment, $p = 0.014$; Fig. 3). The Jonckheere-Terpstra test revealed that at these experimentally selected concentrations, a dose-dependent reduction in total escape response for antidepressant-exposed animals was observed in the VEN ($p = 0.005$) and BUP experiments ($p = 0.020$). In the present study, neither FLX nor SER affected escape behavior of larvae after larval exposure.

Body length

Although not within the original scope of the study, differences in total body length in larvae, but not embryos, exposed to FLX, BUP, and the antidepressant mixture were observed. In the FLX exposure, the mean total length of control larvae was 7.3 mm compared to the FLX-low (25 ng/L) mean of 8.2 mm ($p = 0.043$, Kruskal Wallis; not resolved using Dunn's posttest). Similarly, in the BUP experiment, a statistically significant difference was observed between the control (mean 6.4 mm) and BUP-high treatment (2,000 ng/L; mean 8.2 mm; $p = 0.004$). However, a contradictory trend was seen within the antidepressant mixture. Mean total lengths of control and MIX-low larvae were similar (8.0 and 8.1, respectively), which differed significantly from the smaller MIX-high larvae (mean 7.3; $p = 0.010$).

DISCUSSION

The present study aimed to determine whether predator avoidance performance diminished in fathead minnows exposed as embryos or larvae to environmentally relevant concentrations [9,10] of FLX, SER, VEN, and BUP, singularly or in mixture. Escape behaviors require a larval fish to convert sensory receptor information into activation of the lateral musculature. To survive, a larva must be able to respond quickly and swim rapidly, and any deviation in the normal afferent to efferent signaling may have lethal consequences.

To investigate the ability of larvae to respond quickly to a stimulus, latency period was measured (time to induction of behavior). In the present study, larvae and embryos exposed to SSNRIs (VEN), but not SSRIs (FLX, SER) or DA/NE reuptake inhibitors (BUP), exhibited delayed latency periods compared to controls. In the VEN embryo exposure, a prolonged latency period was seen at the lowest concentration (mean \pm standard error; control 67.4 ± 7.5 ms and VEN-low group 116.3 ± 11.7 ms); however, these low-dose adverse effects were not observed at higher concentrations. This observation reveals a unique relationship whereby latency period increases with decreasing VEN doses. In the larval exposures, the response was dose dependent, with the VEN-high group responding on average 66.8 ms later than

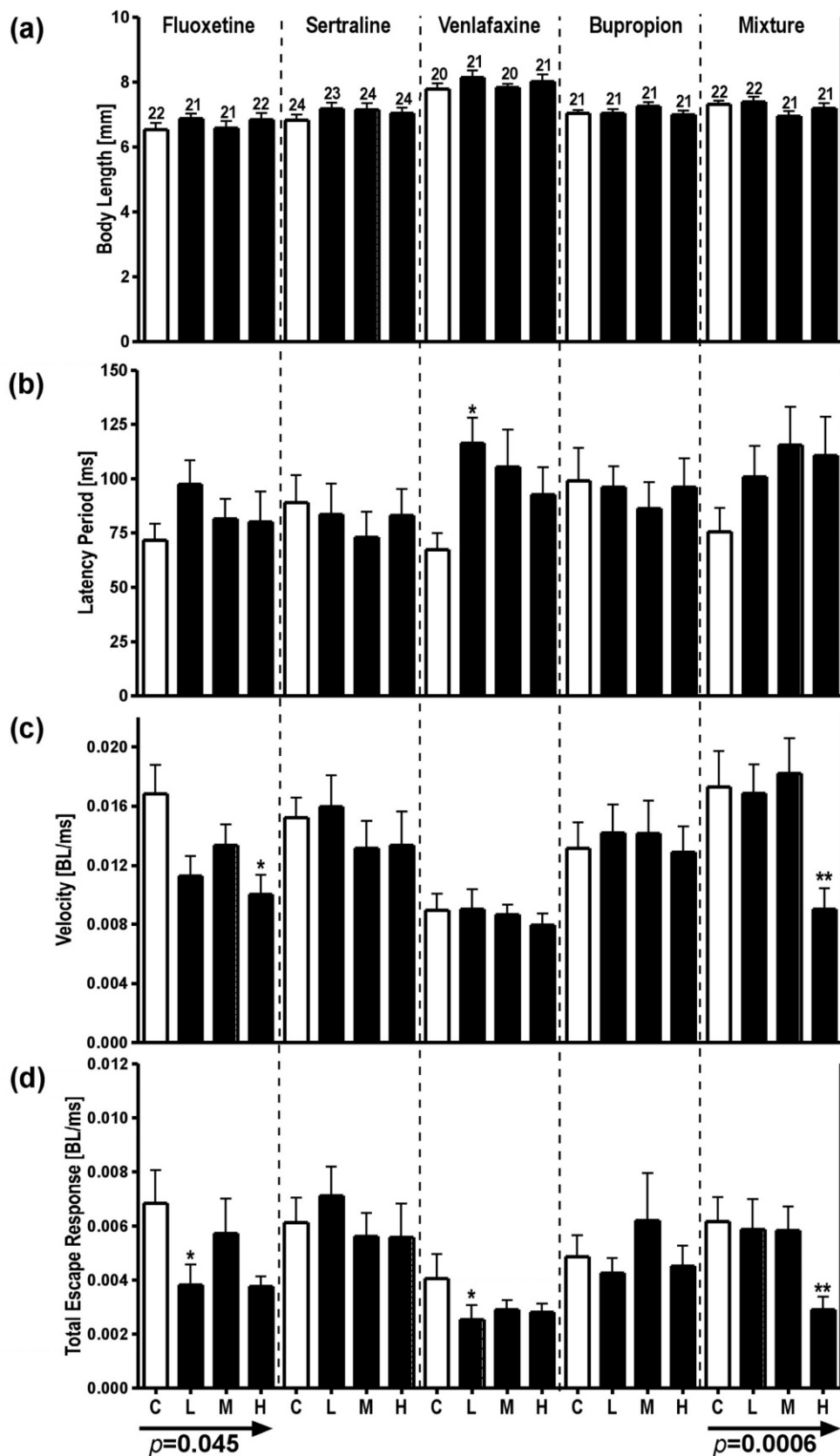


Fig. 2. Effects of embryonic exposure to antidepressants on C-start performance in 12-day-old fathead minnows. Body length (BL, mm; a); latency period (ms) from the time of the stimulus, indicated by the illumination of the light-emitting diode, to commencement of C-start behavior (b); swimming velocity for the 40 ms following commencement of C-start behavior (BL/ms; c); total escape response (BL/ms) from the stimulus to 40 ms past the commencement of the C-start behavior (d). This measure takes into account both the length of the latency period and the speed of the ensuing escape response. Sample sizes for all measures (a–d) are indicated above each column in panel a. The asterisk (*) indicates significance at $p < 0.05$; two asterisks (**) indicate significance at $p < 0.01$ (Kruskal-Wallis test with Dunn's posttest). Dashed lines indicate separate experiments (see text for additional details). The arrow below the total escape response indicates significant dose-dependent effects (Jonckheere-Terpstra test). For treatment concentrations, ethanol solvent control (C), low (L), medium (M), and high (H), consult Table 1.

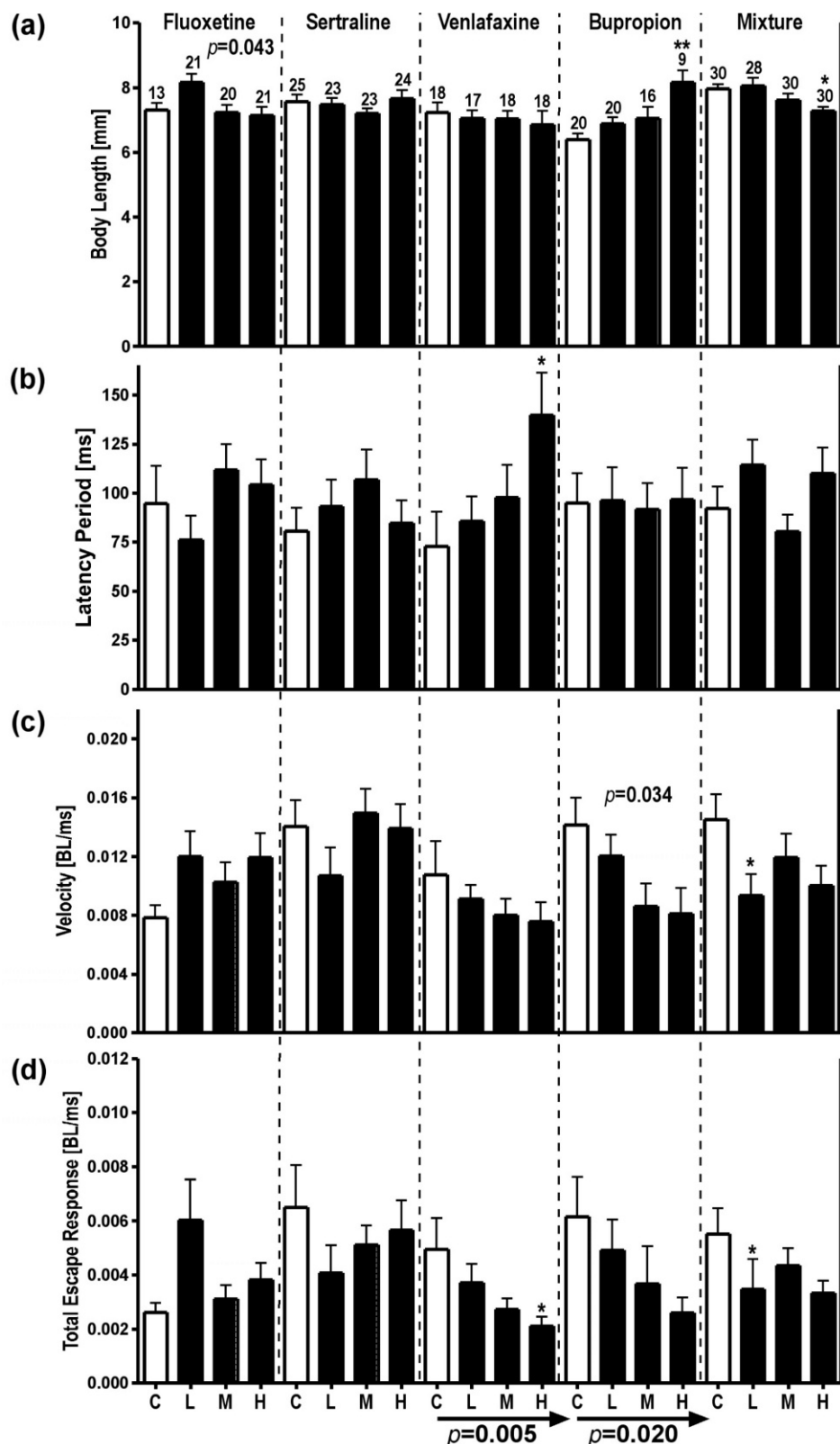


Fig. 3. Effects of larval exposure to antidepressants on C-start performance in 12-day-old fathead minnows. Body length (BL, mm; a); latency period (ms) from the time of the stimulus, indicated by the illumination of the light-emitting diode to commencement of C-start behavior (b); swimming velocity for the 40 ms following commencement of C-start behavior (BL/ms; c); total escape response (BL/ms) from the stimulus to 40 ms past the commencement of the C-start behavior (d). This measure takes into account both the length of the latency period and the speed of the ensuing escape response. Sample sizes for all measures (a–d) are indicated above each column in panel a. The asterisk (*) indicates significance at $p < 0.05$; two asterisks (**) indicate significance at $p < 0.01$ (Kruskal-Wallis test with Dunn’s posttest). The p values above columns (a, c) indicate significant differences determined through the Kruskal-Wallis analysis that remained unresolved after the Dunn’s posttest. Dashed lines indicate separate experiments (see text for additional details). The arrow below the total escape response indicates significant dose-dependent effects (Jonckheere-Terpstra test). For treatment concentrations, ethanol solvent control (C), low (L), medium (M), and high (H), consult Table 1.

controls (control 72.9 ± 17.3 ms and VEN-high group 139.7 ± 21.6 ms), which represented a 92% longer latency period. In the embryo experiments, embryos were only exposed to VEN for 5 d (compared to 12 d for larval exposures) and posthatch were allowed a 12-d depuration period to grow in control well water. Therefore, these results suggest a critical developmental disruption occurring during short-term exposure to environmental concentrations. Considering that neither the SSRIs nor BUP (DA/NE reuptake inhibitor) adversely affected latency response, it is possible that the combined reuptake inhibition of the neurotransmitters 5-HT and NE may have caused a delayed latency period.

To successfully evade a predator, a larval fish must be able to swim rapidly after a C-start has been initiated. Evidence demonstrates that the Mauthner cell not only activates the C-start response but also triggers a swimming CPG that synchronizes motor neuron signaling to muscles [21–23]. In this way, the Mauthner cell acts as a command neuron, and when command inputs result in the release of neurotransmitters, the membrane properties of generator neurons will be altered and the neurons will take on an active, rhythm-generating state [22]. Early studies performed on lamprey (*Petromyzon marinus*) [35] suggest that endogenous 5-HT modifies the CPG for locomotion. Experimental data demonstrate that brainstem serotonergic projections to the spinal cord appear to be evolutionarily conserved among other vertebrate species, with the development of these projections occurring in utero [36,37]. In addition to 5-HT, the neurotransmitter DA influences activation of the CPG for locomotion, and inhibiting DA receptors prevent the locomotor pattern generated by both DA and 5-HT [38]. The etiology of Parkinson's disease in humans further demonstrates DA's role in motor function as degeneration of dopaminergic neurons leads to the characteristic bradykinesia (slow movements) associated with this condition [11,14].

To investigate the role of neurotransmitter involvement within the CPG for larval locomotion, escape velocity was measured. In the embryonic exposure experiments, organisms exposed to FLX and the antidepressant mixture responded significantly slower to the vibrational stimulus than control larvae. Larvae exposed as embryos in the MIX-high treatment revealed an approximate 50% reduction in performance compared to controls. In the larval experiments, exposure to BUP and the antidepressant mixture resulted in a decreased escape velocity. All compounds that adversely affected escape velocity (FLX, SSRI; BUP, DA/NE reuptake inhibitor; and the antidepressant mixture) have been shown to modulate neurotransmitters involved in CPG activation [38]. It is noteworthy that FLX delayed escape velocity, but SER, which is assumed to have a similar mode of action, albeit with substantial structural differences, did not affect the escape velocity of the exposed larvae.

By combining performance parameters for latency period and escape velocity, total escape response was calculated. Total escape response provided an overall behavioral description of signaling as sensory receptor input travels to the command neuron (Mauthner cell), triggering muscle activation and then passing within the synchronized activities of the CPG. Therefore, a reduction in total escape performance indicated multisystemic disruption. Dose-dependent disruption of this kind was seen in organisms exposed to FLX, VEN, BUP, and the antidepressant mixture. Notably, a reduction in total escape performance was observed at the lowest experimental doses for FLX (embryo exposed), VEN (embryo exposed), and the antidepressant

mixture (larval exposed); however, these low-dose adverse effects were not observed at higher concentrations. This observation reveals a unique relationship whereby total escape response is most severely reduced as antidepressant dose decreases.

In addition to their role in vertebrate locomotion, the neurotransmitters 5-HT and DA are critical food-intake regulators. By studying pre- and postsynaptic dopaminergic systems in lean and obese rats, an increase in brain DA levels was observed in obese rats, which, in turn, consumed larger meals [39]. A strong correlation exists between dopaminergic and serotonergic systems, and abnormal distribution of these neurotransmitters in the hypothalamus relates to increased appetite and weight gain. In addition to these two neurotransmitters, increased NE levels in fathead minnows correlated with time spent feeding [40]. Significant increases in body length were observed in the study for larval fish exposed to FLX and BUP. However, a reduction in overall body length was evident in larvae exposed to the antidepressant mixture. Larval exposures appear to have a greater effect on body length than do embryonic exposures. This may be a function of either confounding variables (longer exposure period for larvae; 12-d depuration period for exposed embryos) or true differences in the developmental timeframe (the larval fish is now self-reliant on foraging skills instead of yolk sac lipoprotein content). Further investigation of this phenomenon is warranted.

The effects matrix resulting from these experiments suggests complex interactions between the individual antidepressant drugs and the mechanisms underlying C-start behavior. Furthermore, and beyond the scope of the current study, additional confounding factors may be present, including the biotransformation of the exposure compounds within the test animals, in the treatment waters, or both; the bioavailability of the test compounds to the embryos and larvae; the persistence of these compounds in the test environment; and finally, antagonistic interactions of the tested compounds in the mixture treatments.

The mechanics of C-start behaviors have been investigated for many years and are fundamentally understood. This neural infrastructure appears to be evolutionarily conserved among other vertebrate species. Consequently, disruption within a single process of this afferent to efferent axis jeopardizes evolutionarily orchestrated molecular control.

Reproductive fitness critically depends upon the optimal expression of behaviors that influence survivorship to reproduction. The present study demonstrates that larval survival behaviors are imperiled by their current environmental exposure to antidepressant mixtures. Of further detriment, anatomical variations in body length were also produced. The sensitivity of this performance assay is noteworthy as it provides robust behavioral responses at low chemical concentrations not usually associated with *in vivo* assays. The direct impact of reduced survival probability, and ultimately, its effect on evolutionary fitness provides an avenue to assess the effects of aquatic pollutants in an assay of ecological relevance.

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