The Impacts of Embryonic Arsenic Exposure of Fundulus heteroclitus

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THE IMPACTS OF EMBRYONIC ARSENIC EXPOSURE ON FUNDULUS HETEROCLITUS

By
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B.A. University of Maine, 2015

A THESIS
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Arsenic is a toxic metalloid that exceeds safe drinking water standards in groundwater in many locations worldwide. Arsenic exposure in fish has been linked to destruction of gill tissues, impairment of growth, decreased muscle mass, memory impairment, increased aggression, and avoidance behaviors. We examined the behavior of mummichogs (*Fundulus heteroclitus*) following arsenic exposure during development in two studies. Embryos were collected from fish from three reference sites: Scorton Creek (SC), Massachusetts, Wells Harbor (WE), Maine, and Block Island (BLOC), Rhode Island and two contaminated sites: Callahan Mine (CM), Brooksville, Maine, and New Bedford Harbor (NBH), Massachusetts. Embryos were exposed to 0, 10, 50, or 500 ppb (parts per billion) sodium arsenite. These levels represent a control, the current EPA (Environmental Protection Agency) and WHO (World Health Organization) Maximum Contaminant Level (MCL) of arsenic in drinking water, the previous regulatory standard, and the upper level of arsenic found in Maine ground water, respectively.

We used five different standard tests to assess fish behavior: An Open Field Test to measure basic motor function; a Light/Dark Preference Test as a measurement of anxiety; a Novel Object Test to measure the response to a new variable in the environment; a Sociability Test to examine how an individual interacts with a group of conspecifics; and a Light/Dark Startle Response Test to look for differences in activity post exposure. We hypothesized that exposure to arsenic would alter fish behavior by decreasing activity, increasing the light preference, decreasing the time spent investigating the novel object, and
decreasing the time spent socializing. Analysis of the Open Field Test showed an effect of location but not treatment. Fish from CM were less active than fish from the SC reference site. Results of the Light/Dark Preference Test showed that fish from CM exposed to arsenic spent less time in the light than fish from SC. The Novel Object Test showed no impact of treatment but a possible trend for location effect with fish from SC spending more time away from the novel object than fish from CM. The Sociability Test showed no differences in group behaviors. Finally, no differences in behavior were noted during the Light/Dark Startle Response Test.

Overall, these results suggest that there are location-based differences in some of the behaviors explored here. The data also suggest that there is little impact of environmentally relevant levels of arsenic on mummichog behavior. This may be due to several reasons, including the ability of this fish to withstand low levels of arsenic exposure either by natural tolerance to environmental stressors or increased detoxification processes. Further research would be needed to distinguish which process, if any, is present in these populations to support that idea.
ACKNOWLEDGMENTS

Thank you for all the help and support provided by my committee and my family. Special thanks to Dr. Diane Nacci and her lab for providing me with embryos for my research and allowing me to visit them in Rhode Island to perform collections. Funding was provided by University of Maine Agricultural and Forest Experiment Station (MAFES) to RJVB and through the Coke Fellowship (TJB). Financial support was also provided through multiple teaching assistantships from the School of Marine Science.
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LIST OF ABBREVIATIONS AND DEFINITIONS

AHR – aryl hydrocarbon receptor
BLOC – Block Island, RI
CM – Callahan Mine, ME
DLC – dioxin-like compounds
DMA$^{15}$ – dimethylated arsinic acid
DMA$^{13}$ – dimethylated arsinoic acid
Dpf – days post fertilization
EPA – Environmental Protection Agency
Hpf – hours post fertilization
MCL – maximum contaminant level
MMA$^{15}$ – monomethylated arsionic acid
MMA$^{13}$ – monomethylated arsonous acid
NBH – New Bedford Harbor, MA
SCO – Scorton Creek, MA
TCDD – 2,3,7,8 – tetrachlorodibenzo-p-dioxin
TMAO (trimethylated arsenic acid)
PAH – polycyclic hydrocarbons
PCB – polychlorinated biphenyls
ppb – parts per billion (ug/liter)
ppm – parts per million (mg/liter)
WHO – World Health Organization
Arsenical keratosis: the thickening of skin caused by prolonged arsenic exposure

Euryhaline: able to tolerate a wide range of salinity

Eurythermal: able to tolerate a wide range of temperature

Location: in the context of this study, location refers to the area the parental population originates from

Thigmotaxis: open space avoidance
1.1. Arsenic and human exposure

Arsenic, a naturally occurring metalloid, is found in a wide variety of places throughout the world: in drinking water, household products, foods, soil, and in the air. As awareness of the impacts of arsenic exposure increases, it has become apparent that this is a global concern.

Arsenic has been found at high concentrations in ground water around the world (Fig. 1.1). The amount of arsenic that enters the water is believed to be related to the acidity of the water. Arsenic is commonly found in water as either arsenate (AsV) or arsenite (AsIII; Fig. 1.2). Organic forms of arsenic are also found in water but in lower concentrations (Fig. 1.2). In some countries, such as India, arsenic levels are up to 500 times the World Health Organization (WHO) provisional guidelines for drinking water of 10µg/L. Symptoms of arsenic poisoning vary, dependent on the mode of exposure, duration, concentration, and even the life stage at which exposure happens. Signs of acute arsenic exposure in humans include a loss of balance, hearing impairment, irritability, headaches, nausea, and short-term memory loss. In New England, high levels of arsenic in drinking water correlate with occurrences of bladder cancer (Fig. 1.3). Children that have been continually exposed to arsenic-contaminated water have impaired cognitive functions and behavioral problems such as shortened attention span and decreased learning ability. Prolonged skin exposure can also lead to arsenical keratosis, the formation of callouses and sores. This occurs particularly on the palms of the hands or soles of the feet where repeated contact with the water would commonly occur.
Figure 1.1 Global arsenic levels in ground water worldwide\textsuperscript{1}. 
Figure 1.2 Arsenic species typically found in natural waters\textsuperscript{11}.
Figure 1.3 Spatial distribution of arsenic concentrations in water samples collected from domestic and public-supply wells in New England crystalline rock aquifers, 1995-2007. Map modified from Flanagan. 


There are multiple hot spots of high arsenic concentrations in the United States\textsuperscript{1}. Studies have shown that in Maine specifically, 12-18\% of private wells have arsenic concentrations greater than the U.S. Environmental Protection Agency (EPA) recommended safe levels that are not exceed 10$\mu$g/L\textsuperscript{12,13,14}(Fig. 1.4). The cost of testing and a lack of awareness have contributed to the reluctance of citizens to test their drinking water\textsuperscript{13,15}. Healthy adult humans who experience acute arsenic exposure will excrete almost 80\% of the arsenic in as little as three days. Arsenic detoxification involves generating the organic forms of arsenic with the help of arsenite methyltransferase by stepwise methylation\textsuperscript{16}. This pathway creates MMA$^{+5}$ (monomethylated arsionic acid), MMA$^{+3}$ (monomethylated arsonous acid), DMA$^{+5}$ (dimethylated arsinic acid), DMA$^{+3}$ (dimethylated arsinous acid), and TMAO (trimethylated arsenic acid) as methylated forms are created, reduced, and then additional methyl groups added. Arsenic is primarily excreted through the urine, predominantly as DMA. TMAO is the least common form of arsenic found as most arsenic is excreted before it can reach this step in the detoxification process\textsuperscript{16}.

Arsenobetaine (AsB) is the most common form of arsenic is in many marine species. Researchers believe fish generate AsB from DMA during the detoxification process\textsuperscript{17}. The presence of AsB in other marine organisms is believed to be linked to their either eating fish or detritus containing AsB\textsuperscript{18}. This nontoxic form of arsenic can accumulate in the muscles\textsuperscript{17}. Arsenobetaine is believed to be generated only from the ingestion of arsenic through food, not from environmental exposure\textsuperscript{19}. 
Figure 1.4 Map of median arsenic concentrations for towns with five or more sampled private wells in Maine, 2005-09. Map modified from Nielsen 14.
1.2. Introducing *Fundulus heteroclitus*

Model organisms such as the zebrafish (*Danio rerio*) and the mummichog (*Fundulus heteroclitus*) provide opportunities to study how organisms are impacted by arsenic. Zebrafish are a more commonly used model and have a vast array of information and research associated with them\textsuperscript{20}. Mummichogs also present as a useful model for studying early life toxicity. One of the most abundant fish species in the estuaries of New England\textsuperscript{21}, *F. heteroclitus*, can be found all along the east coast of North America from Nova Scotia to northern Florida\textsuperscript{22}. They are both euryhaline and eurythermal which enables them to thrive in these highly variable environments\textsuperscript{22,23}. *F. heteroclitus* have a limited home range, spending most of their lives within the same estuary. Mature females will lay between 100 and 400 eggs in a spawning season\textsuperscript{23}. This large clutch size is an advantage in laboratory studies, allowing multiple pairings of adults and randomized treatment to minimize genetic bias. The chorion of the developing embryo is clear allowing for observation throughout their two-week developmental period. It is also possible to stimulate hatching of *F. heteroclitus* embryos to ensure that all juveniles are in a similar developmental stage during subsequent experiments.

*F. heteroclitus* has been extensively studied as a model to understand adaptations to natural environmental variables such as temperature and evolved tolerance to anthropogenic chemicals\textsuperscript{24,25}. Research has also shown that *F. heteroclitus* can develop tolerance to high levels of polychlorinated biphenyls (PCBs), polycyclic hydrocarbons (PAHs), and other dioxin-like compounds (DLCs) with some costs of fitness\textsuperscript{25-31}. Exposure to DLCs impact essential biological processes, including development, reproduction, and immune function, resulting in population-
level consequences\textsuperscript{32}. Superfund sites have been extensively studied, including Newark Bay, NJ (2,3,7,8 – tetrachlorodibenzo-p-dioxin, TCDD), the Elizabeth River, VA, (creosote-derived PAHs) and New Bedford Harbor (NBH), MA, (PCBs). The resistant phenotype observed in NBH is characteristic of that observed in \textit{F. heteroclitus} in other DCL contaminated sites. Notable is the poor expression of the classic biomarker CYP1A\textsuperscript{33}. These effects of DLCs are heritable through at least 2 generations, consistent with genetic adaptation\textsuperscript{33}.

The mechanism underlying this tolerance has been linked to genetic diversity and differential expression of multiple aryl hydrocarbon receptor (AHR) genes\textsuperscript{34}. The AHR is a ligand-activated transcription factor with high affinity for TCDD and some DLCs. Activation of this pathway regulates expression of a large set of genes in the toxic response to DLC exposure. \textit{F. heteroclitus} express four AHR paralogs (AHR1a, AHR1b, AHR2a and AHR2b), the products of distinct loci\textsuperscript{32}. Allelic variation at one or more proteins in the AHR pathway underlie many of the differences observed among species in the sensitivity of their response to DLC.

In other studies, mummichogs have been shown to develop tolerance to metal contaminants. Shaw \textit{et al.} reported increased expression of detoxification proteins following repeated laboratory exposures to arsenic\textsuperscript{35}. This adaptation was not shown to be inherited by following generations\textsuperscript{35}. Mummichogs have also been shown to acquire tolerance to copper and zinc, allowing them to live in highly toxic environments\textsuperscript{36}. The ability of \textit{F. heteroclitus} to adapt to highly contaminated environments may provide insight into the molecular mechanisms by which natural populations adapt to multi-generational environmental exposures\textsuperscript{32}.
1.3. Behavior and toxicology

Environmental effects on behavior have been widely documented in different fish species: Biskop-tandkarpe *Brachyrhaphis episcopi*, African cichlids *Astatotilapia burtoni*, zebrafish *D. rerio*, rainbow trout *Oncorhynchus mykiss*[^37^], white seabream *Diplodus sargus*[^38^] and mummichogs, *F. heteroclitus*[^39^]. Looking at the toxicological effects on behavior allows researchers to assess impacts on a whole organism level. Behavioral responses can be related both to the mechanisms of toxicity and to whole population affects[^40^]. Common behaviors in fish assessed in toxicological studies include swimming patterns, feeding behavior, predator response, response to novel objects, scototaxis (light avoidance), and thigmotaxis[^40^] (open space avoidance). These behaviors can be broken down into three types: cognitive, sensorimotor, and basic motor response. Cognitive behaviors are those related to learning and memory. In fish, these are often assessed by navigating mazes or performing specific tasks to receive food[^40^]. Sensorimotor responses, behavior involving the senses such as sight and smell, include responses to a predator’s olfactory cue, or visual cues such as moving shadows or color differentiation. Finally, basic motor response refers to a fish’s locomotive behavior, such as swimming patterns, speed or total distance traveled in a certain amount of time[^40^]. These layers of behavior build on each other and can help determine the overall effect of different stressors.
Chapter 2

IMPACTS OF ARSENIC ON SPONTANEOUS MOVEMENT, LIGHT/DARK PREFERENCE, AND NOVEL OBJECT INTERACTION

2.1. Abstract

The behavior of mummichog (*F. heteroclitus*) juveniles was examined following arsenic exposure during embryonic development. Fish were collected from two reference sites, Scorton Creek (SC), Massachusetts, and Wells (WE), Maine, and one contaminated site, Callahan Mines (CM), Maine. Embryos were exposed to 0, 10, 50, or 500 ppb sodium arsenite from four-days post fertilization (dpf) until hatch, ~14dpf. Juveniles were tested between 5- and 14-days post hatch using an Open Field Test, a Light/Dark Preference Test, and a Novel Object Test. While there was no dose-dependent response, juveniles of fish from different locations showed different behavioral responses. The Open Field Test showed no treatment effect except for the WE fish at the highest exposure level (500 ppb). Results of the Light/Dark Test showed no effect of treatment for fish from any location. All fish spent more than half of the test duration in the light. Fish from WE exposed to 500 ppb arsenic spent more time in the light than the WE control fish. WE fish exposed to 10 ppb arsenic spent more time in the light than any other group of fish tested. In the Novel Object Test, fish from SC exposed to 500 ppb arsenic traveled farther after the introduction of the novel object than other SC fish. Overall, these data suggest that the behaviors measured showed little response to environmentally relevant, low doses of arsenic and that the juvenile fish may be inheriting some genetic differences from their parents related to the environment of the parental populations that are in turn, influencing behavior.
2.2. Introduction

Three different populations of parental fish were used for this experiment: Wells National Estuarine Research Reserve (WE), Maine, Scorton Creek (SC), Massachusetts, and Callahan Mine (CM) in Brooksville, Maine (Fig. 2.1). Both WE and SC have been used as ‘clean’ reference sites in numerous environmental studies\textsuperscript{41,42}. SC has an average salinity range of 24-31ppt, and an average annual temperature range of approximately 4-20°C. The Wells National Estuarine Research Reserve has background levels of lead below the EPA drinking water standards in the sediment linked to historical uses in industry\textsuperscript{43}. WE has an average salinity range of 28-32ppt\textsuperscript{43} and an average annual temperature range of 3-18°C. The substrate at the SC sampling site contains muddy sediments; similarly, WE can be characterized by muddy sediments with some areas that are predominantly sandy.
Figure 2.1 Locations of all adult populations used in the studies described in both chapters 2 and 3. A) Callahan Mine in Brooksville, ME; B) Wells Harbor in Wells, ME; C) Scorton Creek in Barnstable, MA; D) New Bedford Harbor in New Bedford, MA; and E) Block Island, RI.

<table>
<thead>
<tr>
<th>Location</th>
<th>Annual Temperature Range (°C)</th>
<th>Salinity Range (ppt)</th>
<th>Bottom Type at sample site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callahan Mines</td>
<td>3-14</td>
<td>10-24</td>
<td>rocky</td>
</tr>
<tr>
<td>Wells Harbor</td>
<td>3-18</td>
<td>28-32</td>
<td>muddy/sandy</td>
</tr>
<tr>
<td>Scorton Creek</td>
<td>4-20</td>
<td>24-31</td>
<td>muddy</td>
</tr>
<tr>
<td>New Bedford Harbor</td>
<td>5-20</td>
<td>10-30</td>
<td>rocky</td>
</tr>
<tr>
<td>Block Island</td>
<td>7-20</td>
<td>31-33</td>
<td>rocky</td>
</tr>
</tbody>
</table>

Table 2.1 Physical characteristics of the locations of fish populations used in this study. 

36,41-44
Callahan Mine is located on Goose Pond, a tidal estuary fed by Marsh Creek and emptying into Penobscot Bay. In 1972 the former copper/zinc, open pit mine was closed and flooded. In 2002 the site was labeled as a Superfund site by the EPA. The average salinity range of CM is 10-24ppt\textsuperscript{36} and the yearly average annual temperature range is approximately 3-14°C. Historically levels of cadmium, copper, iron, lead, manganese, molybdenum, and zinc at the Callahan Mine site exceed regulation levels and low levels of arsenic are present\textsuperscript{45} (Table 2.2). Clean-up efforts are currently underway with the EPA completing their second 5-year review in April 2021. Considerable improvements have been made in the reduction of metal contaminant levels at this site, but many contaminants are still present (Table 2.2). The substrate in the CM estuary is predominantly gravel and rock around the area where mining took place, becoming a more typical muddy bottom as you move away from the mouth of the pond. A large and well-studied population of \textit{F. heteroclitus} is easily accessible at CM\textsuperscript{36}. 


<table>
<thead>
<tr>
<th>Metals</th>
<th>EPA Regulations or Recommendation</th>
<th>Concentration (ppb) 2005</th>
<th>Concentration (ppb) 2020</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminum</td>
<td>50</td>
<td>380</td>
<td>4.238</td>
</tr>
<tr>
<td>Antimony</td>
<td>6</td>
<td>ND</td>
<td>0.558</td>
</tr>
<tr>
<td>Arsenic</td>
<td>10</td>
<td>1.9</td>
<td>2.326</td>
</tr>
<tr>
<td>Barium</td>
<td>2000</td>
<td>7.4</td>
<td>ND</td>
</tr>
<tr>
<td>Cadmium</td>
<td>5</td>
<td>18</td>
<td>1.130</td>
</tr>
<tr>
<td>Chromium</td>
<td>100</td>
<td>ND</td>
<td>0.312</td>
</tr>
<tr>
<td>Cobalt</td>
<td>NA</td>
<td>1.3</td>
<td>0.108</td>
</tr>
<tr>
<td>Copper</td>
<td>1000</td>
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<td>4.366</td>
</tr>
<tr>
<td>Iron</td>
<td>300</td>
<td>386</td>
<td>14.348</td>
</tr>
<tr>
<td>Lead</td>
<td>0</td>
<td>50</td>
<td>0.280</td>
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<td>Manganese</td>
<td>50</td>
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</tr>
<tr>
<td>Zinc</td>
<td>5</td>
<td>6500</td>
<td>37.726</td>
</tr>
</tbody>
</table>

Table 2.2 Levels of metal contaminants present in the surface water of Goose Pond in 2005 and/or 2020 compared to current EPA drinking water regulations or recommendations. Concentrations from 2005 taken from King & Hathaway. Concentrations from 2020 taken from unfiltered seawater collected in Summer 2020. All units are in parts per billion (ppb). ND = not detected, NA = not applicable, EPA does not have a drinking water standard for cobalt, only a food standard.

The chapter aims to explore the effect that embryonic exposure of environmentally relevant levels of arsenic has on mummichog locomotive, sensorimotor, and cognitive behaviors. We are also exploring how parental exposure to arsenic and other metal contaminants may impact those effects. This study used adults from both clean sites (SC and WE) and a site known to contain arsenic and other metals (CM). We examined the behavior of offspring using the Open Field, Light/Dark Preference, and Novel Object Test. These three tests were used to explore each of the three levels of Tierney’s behavioral hierarchy.
We hypothesized that arsenic exposure would negatively impact juvenile fish movement, scototaxis, and exploration of a novel object. Finding potential links between fish behavior and arsenic exposure could prove mummichogs a useful model for understanding how early-life environmental exposures may impact later behaviors.

2.3. Methods

2.3.1. Parent population collection and husbandry

Adults from the CM and WE populations were caught in the field using wire-mesh minnow traps (Gee’s, Tackle Factory, Fillmore, NY). The adults from CM used in this study were collected from the gravel and rock-based areas (Fig. 2.2). To check for potential year-to-year differences in each population, mummichogs were collected from each location over multiple breeding seasons. Fish were collected from Callahan Mine in the summers of 2015, 2017, and 2018; from Wells in 2016 and 2018. Embryos were received from Scorton Creek adults in 2015, 2016, 2017 and 2018. Weather prevented sampling in Wells in 2017. In 2018, we observed low egg production in adult female fish and high mortality rates in embryos from Wells. This left us with too few fish to test. The Novel Object Test was added in 2017; therefore, fish from Wells were not included in this test. Data from locations obtained in multiple years were tested for homogeneity and pooled when not significantly different.
Figure 2.2 Callahan Mine (CM) sampling sites. Adults were collected along the eastern side of Dyer Cove and the Southwestern edge of Goose Pond; collection points indicated by yellow stars. Map modified from King and Hathaway\textsuperscript{46}.

Fish (~20 males and 20 females) were housed in an 80-gallon tank containing ~30 ppt artificial seawater (Instant Ocean\textsuperscript{™}) at room temperature for two weeks to spawn. Embryos were collected daily via ‘egg baskets’, mesh-covered cylinders placed at the bottom of the tank. Embryos from the SC population were generously provided by Dr. Diane Nacci at the US EPA National Health and Environmental Effects Research Laboratory (NHEERL), Atlantic Ecology Division, Narragansett, RI. Adults from SC were collected in 2010 and 2014 and maintained in the US EPA lab in flow-through, natural filtered seawater and serve as a well-established reference population.
2.3.2. Fish husbandry and arsenic exposure

Embryos were placed into individual wells of a 24-well plate (Falcon, Corning Life Sciences, Tewksbury MA) and kept at 28°C on a 14:10 light/dark cycle. Each well contained 2 mL of 30ppt saltwater with a 50% water changes daily. Four treatment levels were used: 0, 10, 50, and 500 ppb AsNaO₂ (Sigma). These levels represent a control (0 ppb), the current EPA standard for drinking water (10 ppb), the previous EPA standard (50 ppb), and a high exposure (500 ppb), that is still considered environmentally relevant in New England. Embryos were screened for viability daily for four days. Exposure began four days post fertilization with three replicate 24-well plates for a total of 72 embryos per treatment. After hatching, fish were moved into new 24-well plates with 2 mL of clean 30 ppt saltwater (no arsenic). Fish were fed newly hatched Artemia (Brine Shrimp Direct; Ogden, Utah) daily with 50% water changes daily.

Behavior tests were performed at 5-days post hatch (dph; Fig. 2.3). This allowed time for the fish to darken in color ensuring more consistent video tracking. The testing arena was a 5cm diameter, 20 mL petri dish (Falcon, Corning Life Sciences; Tewksbury, MA), filled with approximately 13 mL of 30 ppt saltwater. All behavior tests were recorded using a Nikon DSLR 3400 camera (Melville, NY) at 30 frames per second. Tests were performed in order of least- to- most stressful to minimize stress bias.
Figure 2.3 Timeline showing exposure of experimental embryos and timing of behavior tests.
2.3.3. Water sample analysis for contaminants

Unfiltered water samples were collected from CM in the summer of 2019 and sent to Dartmouth Trace Elemental Analysis Core for metal analysis using inductively coupled plasma mass spectrometry (ICP-MS).

2.3.4. Open Field Test

![Figure 2.4 Set up for three different behavior tests. A) Open Field Test; B) Light/Dark Preference Test; and C) Novel Object Test. All test arenas were created using 5cm diameter, 20 mL petri dishes (Falcon, Corning Life Sciences; Tewksbury, MA).](image)

Fish were first tested for spontaneous movement in an Open Field Test. Individuals were placed randomly in an empty, open arena and allowed to swim freely for 3 minutes (Fig 2.4A). Fish were recorded for the initial 2-minute acclimation time and the following 3-minute test period. Location of fish, total distance traveled, and time spent in specific areas were recorded. Total distance traveled was used as a proxy for activity\textsuperscript{47}. The center of the arena (defined as a...
center circle) and outer ring that were of equal area (Fig 2.4A). Time spent in the outer ring of the arena was used as a measure of anxiety\textsuperscript{47}. Fish displaying exploratory behaviors are expected to move away from the edge of the arena and into open water\textsuperscript{48}.

**2.3.5. Light/Dark Preference Test**

The second test was a Light/Dark Preference Test (scototaxis test) which placed exploratory behavior at direct odds with hiding behavior. Individuals were placed in the light portion (uncovered) of a partially covered arena and allowed to swim freely in a one-minute acclimation period and then recorded for an additional 2 minutes (Fig. 2.4B) Time spent in the covered and uncovered portion of the dish was recorded for both the acclimation period and the duration of the test.

**2.3.6. Novel Object Test**

The final test was a Novel Object Test, which measured how fish reacted to a new object being introduced to their environment. Differences in how fish react to novel objects in their environments have been related to differences in cognitive behavior, or indications of changes in cognitive abilities\textsuperscript{49}. Individuals were able to swim freely in an open arena (5cm diameter petri dish) for 5 minutes to acclimate to their environment, after which a novel object (5mm diameter steel bead) was introduced (Fig. 2.4C). Reaction to the novel object was recorded for 5 minutes following its introduction. Videos were analyzed for the distance traveled after the introduction of the novel object, and the average distance between the fish and the object\textsuperscript{40,50}. 20
2.3.7. Statistical analysis

Fish were tracked using idTracker software\textsuperscript{51} and data analyzed using MATLAB. Statistics were done in SPSS Statistics 24 (IBM; Armonk, NY). A one-way ANOVA was used to test for significance among treatments.

Fish were sacrificed at the completion of testing in accordance with University of Maine IACUC approved protocols (protocol numbers A2013-07-03 and A2017-05-05).

2.4. Results

2.4.1. Open Field Test

Fish from both Callahan Mine (CM) and Scorton Creek (SC) showed no effect of treatment on total distance traveled in the Open Field Test (Fig. 2.5). All SC treatment groups, however, traveled significantly farther than all CM groups (p<0.05; Fig. 2.5). Fish from the Wells (WE) site demonstrated increased movement that correlated with increased arsenic exposure. The 500ppb arsenic exposed WE fish traveled significantly farther than those from any other treatment group (p <0.05; Fig. 2.5).
Figure 2.5 Fish from CM traveled a shorter total distance in the Open Field Test than fish from either SC or WE, regardless of treatment; response to arsenic treatment was observed only in WE fish. The total distance traveled during a 3-minute Open Field Test was averaged among each treatment for each location. Each individual fish was tested once. Letters denote significant differences. Error bars represent ± standard error. n = 81-99 (CM), n = 62-68 (SC), n = 42-49 (WE).

The amount of time fish from CM or WE spent in the center of the arena was not correlated with arsenic exposure (p>0.05; Fig. 2.6). Fish from SC exposed to 50 and 500ppb of arsenic spent less time in the center of the arena than did the controls and the 10ppb exposed fish and any other group from CM and WE (p<0.05; Fig. 2.6).
Figure 2.6 The total average time spent in the center of the arena during a 3-minute Open Field Test. Letters denote significant differences. Error bars represent ± standard error. n = 81-99 (CM), n = 62-68 (SC), n = 42-49 (WE).

2.4.2. Light/Dark Preference Test

Regardless of location or treatment, all fish spent more than 50% of the testing period in the light (Fig. 2.7). Embryonic arsenic exposure did not appear to influence the light/dark preference of fish from CM or SC (Fig. 2.7). Fish from WE exposed to 10ppb arsenic showed a strong preference for the light compared to controls (Fig. 2.7). Fish from WE exposed to high levels of arsenic (500ppb) also showed a stronger preference for the light compared to controls, but less than fish exposed to the low dose (10ppb; Fig. 2.7).
Figure 2.7 The Light/Dark Preference Test revealed a preference for the light half of the arena. Percent of time spent in the light half of an arena was averaged over the 2-minute test. The dashed line marks the 50% threshold. Error bars represent ± standard error. Letters denote significant differences. n = 113-131 (CM); n = 94-103 (SC); n = 48-54 (WE).

2.4.3. Novel Object Test

Fish from both CM and SC traveled similar distances after introduction of the novel object. Fish from SC exposed to 500ppb arsenic, traveled farther after the introduction of the novel object when compared to the control fish from SC (Fig. 2.8). There was a similar trend for increased movement in the high exposure group from CM (p>0.05; Fig. 2.8).
Figure 2.8 High exposure groups from both CM and SC swam farther than control groups after novel object introduction. The distance traveled over five minutes, after the introduction of the novel object, was averaged within treatments. Error bars represent ± standard error. Letters denote significant differences. n = 25-44 (CM). n = 29-42 (SC).

2.5. Discussion

Overall, there is little evidence of an effect of embryonic arsenic exposure on *F. heteroclitus* behavior over the levels of arsenic and the range of behaviors that were tested. This study was focused on the response to environmentally relevant levels of arsenic which may have been too low to elicit significant behavioral responses in the juvenile fish using these assessment tools. Studies on *F. heteroclitus*, using higher doses of arsenic between 800ppb and 25ppm (parts per million) have found that embryonic exposure can impact the formation of muscle fibers and affect growth\(^{47,52}\). Another confounding factor is the natural resilience that is well documented in *F. heteroclitus*\(^{26,27,36,57}\). It is possible that *F. heteroclitus* are not affected by these low doses of arsenic. The tests used in this study may also not be sensitive to any impact arsenic may be having on these fish. This study also focuses on a limited period. The effects of embryonic
arsenic exposure can be seen for months after exposure\textsuperscript{39}. It is possible that the embryonic arsenic exposure in this study has lasting impacts we do not see in this timeframe.

The differences we see in behavior among these populations appear to be influenced by location, the home of the parental breeding stock. Callahan Mine is a low-energy system with a predominantly gravel bottom at the collection site. Scorton Creek and Wells are high-energy systems with muddy or sandy bottoms. The variation in substrate is likely to affect food availability or predation levels in these habitats that in turn may be affecting the behavior of the populations living there\textsuperscript{37}. Fish living in SC may have to adopt a ‘movers’ strategy, actively hunting food\textsuperscript{37}. In the slower moving waters at CM, fish may be able to adopt a ‘stayers’ strategy and use ambush tactics to find food\textsuperscript{37}. If those behavioral traits are heritable, may explain the differences we see in activity levels during the Open Field Test (Fig. 2.5). Fish from SC in our experiment may be more inclined to exhibit exploratory behaviors, moving around their environment, in search of food, increasing their activity levels. While fish from CM may only exhibit exploratory behaviors while assessing the new environment of the arena, then adopting the ‘stayer’ strategy of their parents, decreasing their overall activity levels. Arsenic has been shown to cause heritable epigenetic changes in \textit{D. rerio}. Arsenic was also linked to increased anxiety-like behaviors even two generations after exposure stopped\textsuperscript{53}.

We see no effect of arsenic on CM fish behavior except for the high dose exposure group during the Novel Object Test. In the Open Field Test, we see that fish from CM are less active than fish from SC. This decrease in activity is not related to any freezing behavior as we see that fish from
CM swim more slowly than fish from SC and maintain a consistent speed throughout the Open Field Test (data not shown). This supports the idea that the population from CM may have adapted to different foraging styles that fish from SC\textsuperscript{37}. Fish from CM show no changes in thigmotactic behavior during the Open Field Test, again showing little to no anxiety-like behaviors. Fish from CM showed no impact of arsenic exposure on their behavior in the Light/Dark Preference test, where fish spent more than half of their time in the light. \textit{F. heteroclitus} are a diurnal species, so they are more active during the day\textsuperscript{22}. Many studies support the idea that fish will preferentially seek out shelter in dark areas of their environment\textsuperscript{54,55}. Recent studies, however, show that the opposite behavior may be displayed by juvenile fish\textsuperscript{56,57}. Our study supports that idea. In adult mummichogs, melanophores are distributed along their backs which suggests that they seek dark areas to minimize the chance of being seen by a predator\textsuperscript{54}. During the juvenile stage mummichogs have a small amount of coloration but not nearly as much as their adult counterparts. This may contribute to why juveniles have a light preference while adults have a dark preference.

Scorton Creek has been used as a reference site for numerous studies of \textit{F. heteroclitus}\textsuperscript{25,41,42}. Fish from SC showed a response to arsenic treatment in the Open Field Test and the Novel Object Test but also only at high doses. This suggests that low-dose exposures do not affect these fish. Arsenic has been shown to impact the ability of \textit{D. rerio} to form long-term memories even at concentrations as low as 1ppb\textsuperscript{58}. The high-dose exposure group from SC showed an increase in thigmotaxis in the Open Field Test. This increase in anxiety-like behavior in response to arsenic has also been shown in beta fish (\textit{Beta splendins}). In this study, adult female beta fish
were exposed to a 100ppb arsenic solution for 96-hours. These females spent more time in
dark than the unexposed control fish, suggesting increased anxiety for this species.\textsuperscript{59}

Thigmotaxis has been shown to be increased by exposure to 500ppb of arsenic in zebrafish, but
only in juveniles and adults after chronic exposure.\textsuperscript{60} Increased anxiety-like behaviors have
been linked with decreased exploratory behaviors in zebrafish\textsuperscript{61} which in turn could lead to
decreased foraging abilities and shoaling behaviors.\textsuperscript{62}

Fish from WE show no response to arsenic treatment apart from the total distance traveled in
the Open Field Test and only at the high levels. This again supports the idea that the lower dose
exposures are not affecting these fish and/or that our detection methods are not sensitive
enough to detect responses. Fish from WE displayed the same light preference as both the SC
and CM fish.
CHAPTER 3

IMPACTS OF ARSENIC ON SOCIABILITY AND LIGHT/DARK STARTLE RESPONSE

3.1. Abstract

Fundulus heteroclitus from 3 different locations were exposed to arsenic during embryogenesis and their behavior post exposure was monitored. Adult fish were collected by Dr. Nacci’s lab group from two reference sites Scorton Creek, MA (SC), Block Island, RI (BLOC), and one contaminated site, New Bedford Harbor, MA (NBH). Embryos were exposed to 0, 10, or 500ppb sodium arsenic from 24 hours post fertilization until hatch, ~14 days post fertilization. Heart rate and heart morphology were assessed during development. Growth rate was assessed weekly during the first three weeks post hatch. Mortality data suggest that fish from BLOC have less successful fertilization and/or increased mortality during development. This does not seem to be related to arsenic exposure. Fish from NBH exposed to 10ppb arsenic had a decreased angle between the heart and eyes when compared to control fish from NBH. Sociability and Light/Dark Startle Response Tests were performed at 5-25 days post hatch to assess fish behavior. In the Sociability Test, all fish showed a preference to associate with conspecifics, with no noticeable impact of arsenic exposure or location. Fish did respond to light stimulus in the Light/Dark Test, but the reaction was not affected by arsenic or by location. Overall, these data suggest that the behaviors measured showed little response to environmentally relevant, low doses of arsenic.
3.2. Introduction

Following the previous experiments, we wanted to examine more complex behaviors. The Sociability Test was modeled after the test designed for adult mosquito fish (*Gambusia holbrooki*) scaled down for juvenile fish\(^{63}\). On Tierney's scale of behavioral hierarchy this test fell between cognitive and sensorimotor\(^{40}\). The Sociability Test requires fish to be able to sense, either visually or olfactorily, conspecifics and make decisions about being in association with those conspecifics. The Light/Dark Startle Response Test monitors the fish's reaction to a visual stimulus and activity levels. This would encompass both the sensorimotor and locomotor levels of the hierarchy of behavior.

A collaboration with Dr. Nacci also allowed us access to new populations and new techniques in husbandry, including keeping embryos in scintillation vials and maintaining embryos on damp filter paper to help stimulate hatching. Block Island is located off the coast of Rhode Island and the mummichog population from here has been used as a reference population\(^{27}\). New Bedford Harbor, Massachusetts, has been a registered Superfund site since 1983.

This chapter aims to explore the effect that embryonic exposure of environmentally relevant levels of arsenic has on mummichog cognitive and sensorimotor behaviors using the Sociability Test and Light/Dark Startle Response Test, respectively. We hypothesize that arsenic exposure will negatively impact juvenile fish movement, decrease the time fish spend socializing, and the startle response. Finding potential links between fish behavior and arsenic exposure could
prove mummichogs a useful model for understanding how early–life environmental exposures may impact later behaviors.

3.3. Methods

3.3.1. Collection and maintenance of adult fish

Adult mummichogs had been previously collected from three different locations: Block Island, RI (BLOC) in 2014 and 2018, Scorton Creek, MA (SCO) in 2015, 2016, and 2017, and New Bedford Harbor, MA (NBH) in 2015, 2016, and 2017. These breeding populations were housed at the US EPA National Health and Environmental Effects Research Laboratory (NHEERL), Atlantic Ecology Division, Narragansett, RI in flow-through, filtered natural seawater at ambient temperatures, and fed TetraMin (Blacksburg, VA) flake food daily. Over one hundred males and females from each location were spawned during the summer of 2019; embryos randomly selected from each collection cohort.

3.3.2. Embryo collection and exposure

Fish were manually spawned the day before the full or new moon between June and August of 2019. Embryos were incubated for 24 hours in natural seawater, at 23°C on a 14:10 light cycle. After this time embryos were screened for successful fertilization. Embryos that showed the beginnings of development, usually a thin line demonstrating the start of the spine, were transferred to 20mL glass scintillation vials, and randomly assigned to treatment groups. The control group vials contained 10mL of 25ppt seawater. The two treatment groups contained 10mL of either 10ppb or 500ppb sodium arsenite (AsNaO₂ Sigma) in 25ppt seawater.
Embryos were checked daily, and any dead embryos were removed. After 7 days post fertilization (dpf) embryos were moved into individual wells of a 24-well plate (Falcon, Corning Life Sciences, Tewksbury MA). Each well contained a filter paper (Restek cellulose filter; Fisher, Waltham, MA), which was kept damp using the same incubation solution. Embryos were kept at 28°C on a 14:10 light/dark cycle and checked daily to ensure that the filter paper and embryo remained damp (Fig. 3.1).
Figure 3.1 Timeline showing the exposure time and the collection time of experimental endpoints.
3.3.3. Survivability

Mortality as a percent of initial embryos exposed was calculated at three timepoints during development: 10dpf, 5dph, and at the end of the experiment (>20 dph; Fig. 3.1). Total number of surviving fish was determined at 10dpf when heart rate data were collected. Mortality was assessed at 5 days post hatching, as this indicated that fish had successfully hatched and began feeding. The total number of surviving fish was taken at the end of the experiment (over 20 dph).

3.3.4. Heart rate and morphology

At 10dpf, embryos were screened for heart rate and were given a heart morphology score. These were used as indicators of normal fish development. Individual embryos were observed visually under a dissecting microscope. Embryos were acclimated for up to 3 minutes until heart rate appeared stable. Heart rate was then recorded for 1 minute using a mounted phone camera (DROID Turbo main camera, GoSky-Optics cellphone adapter mount). Heart rate was calculated by counting the number of heart beats during the 1 min video. Two methods were used to assess heart morphology: heart morphology scoring and the angle between the heart and eyes. The heart morphology score outlined by Matson gives a broad overview of how heart morphology is assessed (Fig. 3.2). Fish with low heart scores (0 or 1) tend to survive to hatch. We also examined the angle created by two heart chambers, the ventricle and sinus venosus, and the pupils of the eyes (Fig. 3.3). A large angle would indicate that the heart was becoming elongated, with the heart chambers becoming perpendicular (closer to 90°) to the
eyes instead of near parallel\textsuperscript{65} (between 0° and 15°). As the angle increases the chances of the embryo surviving to hatching decreases\textsuperscript{65}.

Figure 3.2 Representative images taken from the heartrate video (top) with outlines of the heart underneath in red (middle), with the associated heart morphology score (bottom). A score of 0 represents a healthy, functioning heart; a score of 1 indicates that the heart is slightly elongated and not all chambers are clearly defined; a score of 2 means that the heart is elongated, and chambers are hard to identify. The three chambers of the heart are labeled: sinus venosus (SV), atrium (A), and ventricle (V).
3.3.5. Hatching and juvenile fish care

At 14dpf the wells containing the embryos were filled with 2mL of 30ppt artificial seawater (Instant Ocean) and the filter paper removed. Flooded plates were gently agitated overnight at room temperature to stimulate hatching. Any embryos that died or failed to hatch after 2 days were removed from the plate. After hatching, a photo was taken to capture the initial size of the fish. Photos were also taken at 7- and 14-days post hatch to track the growth rate. Juvenile fish were checked daily. Fifty percent water changes were performed daily. After 3 days post hatch, fish were fed live *Artemia* (Brine Shrimp Direct; Ogden, Utah) daily following water changes.

3.3.6. Sociability Test

Behavior tests were performed beginning at 5 days post hatch. The first test, the Sociability Test, was modeled after the test designed by Bertram for mosquito fish (*Gambusia holbrooki*) scaled down for juvenile fish. The Sociability Test demonstrates the tendency of fish to
associate with conspecifics. Preliminary data on swimming activity and distance traveled by these juvenile fish were collected to inform an appropriately sized arena (data not shown). The arena was designed so that the fish could easily cross it multiple times within the testing period. The sociability apparatus consisted of three chambers: two small chambers, one on the left and one on the right of a large central area (Fig. 3.4).

![Figure 3.4 Sociability test arena. Area colored white represents the area in which the focal fish may freely swim. Stimulus fish are randomly assigned either to the left or right chamber (grey) to prevent side bias. White and grey areas are separated by a clear, plexiglass wall each containing four 1cm diameter holes covered in 0.5mm mesh. This allows for the focal fish to pick up visual and chemical cues from the stimulus fish and vice versa without physical interaction. These chambers were separated by a plexiglass wall with 4 small holes covered by 0.5mm plastic mesh, to allow for visual and olfactory cues to pass between chambers while preventing the focal fish in the central chamber from directly interacting with stimulus fish in the outer chambers. These stimulus fish were from the same cohort as the focal fish and raised under the same conditions as the experimental control fish. For each test, a group of 20 stimulus fish was placed randomly in either the left or right outer chamber. After allowing 20 minutes for the stimulus fish to acclimate to the arena, a single focal fish was placed in the central chamber.}
The movement of the focal fish was then recorded for 20 minutes which included the time it took for the focal fish to acclimate to the arena. Time spent in association with the stimulus fish was determined by analysis of the video. Association was defined as the focal fish being within 1cm (or ~2 body lengths at time of testing) of the stimulus group. These videos were also analyzed for the total distance traveled to assess the activity levels of the focal fish.

3.3.7. Light/Dark Startle Response

The second test examined the Light/Dark Startle Response of the juvenile fish using the DanioVision box (Noldus). Each well in a six-well plate was filled with 7mL of 25ppt saltwater. Up to six focal fish were placed into individual wells. Fish were acclimated in the DanioVision box in the dark for 5 minutes; a light stimulus was introduced for another 5 minutes (Fig. 3.5). This cycle of dark and light was repeated a second time, ending the test with another 5-minute dark cycle. This created a 25-minute video which was then analyzed for the distance traveled during each of the 5-minute light or dark segments. Special attention was paid to the first 5 seconds after changes in the light stimulus. Alterations in light stimulate the startle response, which is a rapid darting motion or change in activity before returning to normal. The first 5 seconds was determined to be the window for the startle response by looking at when fish activity returned to stable levels that were consistent for the remainder of the 5-minute light or dark cycle.

After completion of all experiments all fish were euthanized in accordance with IACUC protocol (A2017-05-05).
Figure 3.5 Infographic summary of Light/Dark Startle Response Test demonstrates the changing light stimulus throughout the test. Each five-minute cycle allows for the fish to acclimate to the light stimulus. Test was performed via the use of the DanioVision box (Nodulus).

3.3.8. Data analysis

Fish were tracked in videos using idTracker software and data analyzed using MATLAB. Statistics were done in JMP Pro 15 (SAS; Cary, NC). Data were tested for normality and homogeneity of variance and a general linear model (GLM) was used to test for significance among locations and treatments. When using GLM, location and treatment were included as fixed factors. In addition, when analyzing the sociability data the cohort number was included in the analysis to check for any influence of the stimulus group.

3.3.9. Animal care and use

The care and use of experimental animals complied with University of Maine Animal Welfare Laws, guidelines and policies as approved by University of Maine Institutional Animal Care and Use Committee (protocol number A2017-05-05).
3.4. Results

3.4.1. Mortality rate

Arsenic exposure had no impact on the mortality rate or hatching success of *F. heteroclitus*.

Location, however, correlated with mortality with fish from BLOC having a lower number of surviving embryos starting at 10dpf and continuing throughout the experiment (Table 3.1; p<0.05).

<table>
<thead>
<tr>
<th>Location</th>
<th>Treatment</th>
<th>10 dpf</th>
<th>19 dpf (5dph)</th>
<th>34dpf (20dph)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLOC</td>
<td>0ppb</td>
<td>48.3 ± 13.7</td>
<td>39.2 ± 13.4</td>
<td>35.8 ± 11.7</td>
</tr>
<tr>
<td></td>
<td>10ppb</td>
<td>50.0 ± 16.1</td>
<td>44.2 ± 15.2</td>
<td>38.3 ± 12.4</td>
</tr>
<tr>
<td></td>
<td>500ppb</td>
<td>55.0 ± 17.3</td>
<td>37.5 ± 15.0</td>
<td>38.3 ± 14.5</td>
</tr>
<tr>
<td>SCO</td>
<td>0ppb</td>
<td>70.6 ± 11.7</td>
<td>63.9 ± 5.9</td>
<td>60.3 ± 6.7</td>
</tr>
<tr>
<td></td>
<td>10ppb</td>
<td>79.4 ± 10.3</td>
<td>70.6 ± 7.5</td>
<td>54.7 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>500ppb</td>
<td>81.1 ± 11.6</td>
<td>63.1 ± 19.3</td>
<td>70.3 ± 5.3</td>
</tr>
<tr>
<td>NBH</td>
<td>0ppb</td>
<td>68.0 ± 5.0</td>
<td>48.9 ± 14.1</td>
<td>52.7 ± 12.3</td>
</tr>
<tr>
<td></td>
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<td>40.0 ± 12.5</td>
<td>50.0 ± 16.4</td>
</tr>
<tr>
<td></td>
<td>500ppb</td>
<td>60.7 ± 9.4</td>
<td>42.7 ± 18.8</td>
<td>48.7 ± 17.5</td>
</tr>
</tbody>
</table>

Table 3.1 Embryos/juveniles from BLOC parental fish had higher mortality than either SCO or NBH (p<0.05). Data are shown as percent of fish alive at given timepoint ± standard error. Initial number of embryos are BLOC n=120, SCO n=110, and NBH n=150.

3.4.2. Heart rate and morphology

Arsenic exposure had little impact on the angle between the ventricle/sinus venosus and the eyes (Fig. 3.6), heart morphology score (Fig. 3.7), or heart rate (Fig. 3.8). We did see a decrease in the average angle between the heart and the eyes of fish from NBH that had been exposed to 10ppb arsenic when compared to the control fish from NBH. Fish from NBH exposed to 10ppb arsenic also demonstrated an increase in heart morphology score. This increase in score
may reflect the decreased angle indicated in Fig. 3.6, exhibited by the same group fish. Fish exposed to 500ppb arsenic did have a lower heart rate than controls, but it is not statistically significant (p>0.05).

Figure 3.6 Arsenic exposure had little to no impact on heart alignment at 10 days post fertilization. Fish from NBH exposed to 10ppb arsenic had a significant decrease in the angle between the heart and eyes. Error bars represent ± standard error. n = 38-43 (BLOC), n = 50-62 (SCO), n = 53-76 (NBH). A ‘*’ indicates significant difference (p<0.05).

Figure 3.7 Arsenic has little to no effect on the heart morphology scores of exposed embryos. Fish from BLOC exposed to 500ppb had the highest percentage of fish receiving a score of 2. A score of 0 represents a healthy, functioning heart, a score of 1 indicates that the heart is slightly elongated and not all chambers are clearly defined, a score of 2 means that the heart is elongated, and chambers are hard to identify. n = 38-43 (BLOC), n = 50-62 (SCO), n = 53-76 (NBH).
Figure 3.8 Arsenic had no significant impact on heart rate at 10 days post fertilization. Error bars represent ± standard error. n = 50-62 (SCO), n = 38-43 (BLOC), n = 53-76 (NBH).

### 3.4.3. Growth rate

We detected no significant changes in the growth rate of *F. heteroclitus* after exposure to arsenic throughout the duration of the experiment (Fig. 3.9). Fish from both BLOC and SCO exposed to 500ppb arsenic were slightly smaller than fish in other treatments at 14dph. Further exploration would be needed to confirm if these fish from BLOC would continue to have a decreased growth rate or if they would recover to a growth rate similar to the control fish.
Figure 3.9 Arsenic showed little to no impact on the growth of *F. heteroclitus* after embryonic exposure during the two weeks post hatch. Fish from both Scorton Creek (SCO) and Block Island (BLOC) that were exposed to 500ppb arsenic showed a slight decrease in average standard length at 14 days post hatch (dph). 0ppb treatment group (dotted line), 10ppb treatment group (dashed lines), and 500ppb treatment (solid line). Error bars represent ± standard error. n = 60 (SCO), n = 39 (BLOC), n = 20 (NBH).
3.4.4. Sociability

*F. heteroclitus* is a shoaling species, with fish from similar year classes or similar sizes typically swimming in loose aggregates\(^2\). We were able to confirm that this sociability arena allows for the juvenile fish to associate with conspecifics. Fish from all locations regardless of treatment spent more time in association with fish from their cohort than in other areas of the arena. If movement were random, we would have expected to see an equal amount of time spent associating with both sides of the arena (colored grey in Fig. 3.4), regardless of where the stimulus fish were. Prior exposure to arsenic appeared to have no effect on the amount of time fish spent in association with other juvenile fish (Fig. 3.10).

![Figure 3.10 Arsenic had no impact on the amount of time focal fish spent in association with conspecifics. Error bars represent ± standard error. n = 45-55 (BLOC), n = 67-79 (SCO), n = 60-72 (NBH).](image)
These videos were also analyzed for activity levels to compare to the Startle Response tests. Neither arsenic exposure nor parental location appeared to impact activity (Fig. 3.11). Due to the large size of the arena, we also analyzed for any changes in the tendency for fish to remain near the edges of an arena (thigmotaxis). Again, there was no effect of exposure or location (Fig. 3.12).

![Graph showing dist traveled (cm) across different locations and arsenic concentrations.](image)

Figure 3.11 Arsenic had no impact on the activity level of fish during the Sociability Test. Error bars represent ± standard error. n = 45-55 (BLOC), n = 67-79 (SCO), n = 60-72 (NBH).
Figure 3.12 Arsenic had no impact on thigmotaxis during the Sociability Test. Center was determined as more than two body lengths away from the edge of the Sociability Test arena (Fig. 1). Error bars represent ± standard error. n = 45-55 (BLOC), n = 67-79 (SCO), n = 60-72 (NBH).

3.4.5. Light/Dark Startle Response

Distance traveled was used as an indicator of activity during each of the light/dark cycles. The first three seconds of each cycle were examined to look at the startle response of the fish. Fish responded to the light stimulus with a lower average distance traveled during the light phases (Fig. 3.13). In most treatment groups there was a trend of decreasing startle responses over time (Fig. 3.14). There does not appear to be any effect of arsenic or location on activity level or startle response. This activity level is consistent with what we saw in the Sociability Test.
Figure 3.13 Arsenic had no effect on activity level in the light or dark phase of the Light/Dark Startle Response Test. Figure shows the average distance traveled within the full 5 minutes of each cycle by fish from BLOC (A), SCO (B), and NBH (C). Error bars represent ± standard error. n = 35-39 (BLOC), n = 50-55 (SCO), n = 39-47 (NBH).
Figure 3.14 Arsenic had no effect on startle response in the light or dark phase of the Light/Dark Startle Response Test. Figure shows the average distance traveled within the first 3 seconds of each cycle by fish from BLOC (A), SCO (B), and NBH (C). Error bars represent ± standard error. n = 35-39 (BLOC), n = 50-55 (SCO), n = 39-47 (NBH).
3.5. Discussion

3.5.1. Mortality

Embryonic arsenic exposure did not have an impact on mortality. Lower initial survival, however, was observed in fish from BLOC at all exposure levels relative to fish from other locations. Mortality was tracked throughout the experiment at 10dpf, 5dph, and at the end of the experiment (>20dph). Lower initial survival observed in fish from BLOC may have a genetic/epigenetic basis underlying naturally occurring defects in development, or greater population-specific sensitivity to arsenic exposure.

3.5.2. Heart Rate and Morphology

We saw inconsistent patterns of response to arsenic exposure among the three locations examined. The most significant influence of arsenic and location is in the NBH population exposed to 10ppb arsenic. This group showed an increased angle between the heart and eyes but only when compared to controls. Unlike what we have seen in mummichogs, zebrafish juveniles showed a significant increase in heart rate after developmental exposure to as little as 50ppb arsenic66. Adult carp (Cyprinus carpio) exposed to 2ppm arsenic trioxide via food for two weeks had significant damage to their heart tissues. This damage was linked to oxidative stress caused by arsenic exposure triggering apoptotic pathways67. Developmental exposure to 10ppb PAHs led to the development of significant heart defects and higher heart morphology scores in F. heteroclitus28. The lack of impact on heart rate or morphology seen in the current study may be due several underlying causes, including exposure levels being below the response
threshold. Increasing the arsenic exposure may induce heart defects in *F. heteroclitus* that are seen in other species.

### 3.5.3. Growth Rate

We saw little to no impact of arsenic on growth rate. There was, however, a trend for fish from BLOC exposed to 500ppb to be smaller than other fish from BLOC. Studies have shown that exposure to arsenic can affect the formation and growth of muscle fibers which can impact growth\(^47\). Exposure to up to 25ppm arsenic during development is related to reduced expression of genes related to muscle fiber formation and growth\(^47\). Previous studies did show that *F. heteroclitus* exposed to 50-200ppb arsenic experience stunted growth at 56dph. However, after 280dph both exposed and control fish were similar lengths\(^68\). *F. heteroclitus* exposed to up to 800ppb arsenic during development have increased expression of IGF-1 for up to a year after exposure\(^52\). This is believed to be a compensatory mechanism to combat growth impairment due to arsenic exposure\(^52,68\). In this study we may not have tracked growth for long enough or exposed fish to a high enough dose of arsenic to capture any significant impact on growth.

### 3.5.4. Sociability

*F. heteroclitus* will naturally form a shoal. We saw all groups of fish in this experiment forming those loose aggregates in the sociability arena. We did not see any effect of arsenic on the focal fish’s tendency to associate with conspecifics. Other studies have also shown the tendency of mummichogs to shoal\(^22\). Exposure of several fish species to other contaminants have been shown to impact shoaling. Adult female mosquitofish (*Gambusia holbrooki*) exposed to the
metabolite 17β-trenbolone for 21 days show a reduced tendency to form shoals. These exposed females spent more time exploring their arena individually than in association with other members of their species. Adult banded killifish (Fundulus diaphanous) ceased shoaling behavior after an acute exposure to 4-nonylphenol. Male mice (F1) exposed to 85ppm arsenic in utero displayed impaired social behavior at ~10 months old, resulting in affected mice spending less time in association with conspecifics. This behavior was even present in male offspring of the next generation (F2). The social impairment seen in these mice was linked to decreased serotonin and dopamine receptor expression.

3.5.5. Light/Dark Startle Response

In studies examining a startle response it is common to see a decreasing response after multiple instances of the stimulus as fish begin to acclimate to the alternating light/dark periods. We see a similar trend in our light/dark startle response. If this test had been carried out for further cycles of light and dark, we would have expected to see a continuing decrease in the startle response until the activity level remained constant throughout the entire test regardless of the introduction of the light stimulus. In zebrafish exposed during development to ~8ppb of lead, startle response was diminished, and upon repeated introduction of the stimulus, exposed fish stopped responding earlier than did control fish. Zebrafish exposed to 50ppb arsenic throughout the larval, juvenile, and adult stages showed a decreased startle response when compared to control fish. This was attributed to sensory cells on the lateral line being damaged due to arsenic exposure. We did not see similar responses in the mummichogs suggesting that zebrafish may be more sensitive to arsenic toxicity.
3.6. Conclusions

Overall, we did not see any significant effect of location or arsenic exposure. There are several possible reasons why *F. heteroclitus* may not be affected. It is well documented that *F. heteroclitus* are resilient to a variety of contaminants\(^{25,31,35,36}\). It is possible that *F. heteroclitus* is resilient to the environmentally relevant, low doses of arsenic exposure were used in this study. Fish exposed to arsenic may have been able to recover from negative impacts after exposure ceased. Research has shown that effects of arsenic can be ameliorated post exposure. Fish that had decreased growth due to arsenic recovered and were comparable to controls after 280dph\(^{68}\). The recovery time in this experiment was shorter, up to \(~30\)dph. Exposure was stopped after hatching to prevent water containing arsenic from contamination of the testing arena as fish were transferred into and out of the arena. Another possibility is that the tests used here were not sensitive enough to demonstrate an impact of arsenic.

CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

Overall, behaviors were not well correlated with arsenic treatment level. There are a variety of factors that may have played into this. The levels of arsenic we used in this experiment are all environmentally relevant to New England and may be below the response threshold of the mummichog juveniles in the behavior tests used here. Arsenic can readily pass through the chorion at high doses\(^{47}\) (5-25ppm). It is unclear what percent of the environmentally relevant doses used in this study can pass through the chorion. Further exploration may uncover how arsenic passes through the chorion and how much is stored in the bodies of developing
mummichogs. *F. heteroclitus* has been well known to adapt to a wide variety of contaminants in their environments and may be resistant to acute, low-dose arsenic exposures.

Continuation of this research would benefit from examining the impacts of an increased arsenic dose exposure, longer duration of observations, repeated behavior tests, and connection to underlying toxicity mechanisms. Multiple studies have documented that early life exposure to arsenic has long lasting effects even into adulthood. Multigenerational studies have yielded significant results. Research in zebrafish has shown that the impact of arsenic can be seen even two generations after the initial exposure\(^7\). Exposing *F. heteroclitus* to higher doses of arsenic may impact their behaviors as is documented in other social species\(^7\). Exposing mosquitofish (*Gambusia holbrooki*) to arsenic through their diet resulted in increased aggression and food guarding behaviors\(^7\). Research may also include looking for alterations in genes directly impacted by arsenic. *F. heteroclitus* may also rely on increasing the level of arsenic detoxification proteins such as glutathione and AsMT3 (arsenic methyltransferase) to tolerate arsenic exposure\(^3\).

In conclusion, environmentally relevant levels of arsenic do not have a significant impact on the juvenile behavior of *Fundulus heteroclitus*.
REFERENCES


BIOGRAPHY OF THE AUTHOR

Torey J. Bowser was born in Ashtabula, Ohio where she graduated from Geneva High School in 2011. She graduated from the University of Maine with a Bachelor of Marine Science in 2015 and stayed on to attend graduate school. Torey was a teaching assistant for the School of Marine Sciences and took great joy in gaining valuable teaching experience under several School of Marine Science faculty members. Torey is a candidate for the Master of Science degree in Marine Biology from The University of Maine in August 2021.