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Effects of methylmercury on the early life stages of an estuarine forage fish using two different dietary sources

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ABSTRACT

Marine fish accumulate methylmercury (MeHg) to elevated concentrations, often higher than in freshwater systems. As a neurotoxic compound, high MeHg tissue concentrations could affect fish behavior which in turn could affect their populations. We examined the sublethal effects of MeHg on larvae of the Sheepshead minnow (*Cyprinodon variegatus*), an estuarine fish, using artificial or natural diets with varying MeHg concentrations (0–4.8 ppm). Larvae were fed control and MeHg-contaminated diets at low or normal (10% of their body mass) daily food rations from 7 to 29 days when they reached juvenile stage. Growth, respiration, swimming activity and prey capture ability were assessed. Food ration affected Hg toxicity in our study. Natural diets containing 3.2 ppm MeHg had no impacts on growth and swimming in fish that were fed normal food rations but depressed growth and swimming at low food rations. MeHg toxicity did not differ between artificial and natural foods, however fish accumulated more MeHg from the former. Artificial food containing 4.8 ppm MeHg only affected prey capture after 21 days of exposure. Sheepshead minnows, a forage fish species occupying a low trophic level in coastal waters, can be MeHg tolerant, especially when food is abundant, and can serve as an enriched Hg source for higher trophic level predators.

1. Introduction

Natural and anthropogenic sources release Hg into the atmosphere, which is deposited in terrestrial and aquatic ecosystems (Sunderland et al., 2009). Once it enters aquatic systems, Hg undergoes many complex biogeochemical processes, including methylation by microbes to form methylmercury (MeHg) (Fitzgerald et al., 2007; Lamborg et al., 2014). MeHg, which is a known neurotoxic compound (Sanfeliu et al., 2003), is the predominant form of Hg found in fish tissues and the principal Hg species that biomagnifies in aquatic food webs (Fitzgerald et al., 2007). It could potentially impact brain function and cause functional and behavioral disorders (Sandheinrich and Wiener, 2011). While studies have suggested that MeHg can affect freshwater fish when their whole-body Hg concentrations are as low as 0.5 ppm wet wt (Sandheinrich and Wiener, 2011), the impacts of MeHg in marine and estuarine fish remain poorly understood (Depew et al., 2012).

Sensitivity to MeHg may vary considerably among different life stages for the same fish species. Previous studies examined the effects of MeHg in juveniles and adults (Friedmann et al., 1996; Lee et al., 2011; Sandheinrich and Drevnick, 2016). The developmental or behavioral effects of MeHg on larvae, which seem to be more sensitive to MeHg than juveniles and adults (Saraf et al., 2018), remain poorly known, especially via dietary exposures. At early life stages, small changes in growth or impaired behavior can lead to increased mortality, potentially creating population-level effects (Alvarez et al., 2006; Saraf et al., 2018). While behavior of marine fish in response to aqueous Hg has been investigated (Weis and Weis, 1995), we are aware of only a few studies (Alvarez et al., 2006; Puga et al., 2016; Ye and Fisher 2020) that have examined the effects of dietary MeHg on marine fish behavior. Two of these studies assessed the swimming behavior of larvae exposed to MeHg via maternal transfer and found that swimming behavior was affected in larvae after hatching from MeHg-fed mothers. Aside from exposure to MeHg by maternal transfer, larvae are also exposed to MeHg-contaminated food directly in the wild. As the extent of any behavioral impairments after dietary MeHg exposures is still unclear in marine and estuarine fish in general, further assessments are needed. This study provides the first assessment of the potential effects of dietary MeHg exposure to larvae of an estuarine fish species.

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Prey capture ability is crucial for larval fish (Mayer and Wahl, 1997). Changes in prey capture ability have effects on larval growth and survival, since larvae have a limited ability to withstand starvation (Zhou et al., 2001), and such effects could ultimately affect populations (Weis, 2014). The direct result of impaired prey capture ability is reduced feeding rate; many aspects of prey capture ability can be affected, such as reduced search efficiencies (Weis et al., 2001), reduced strike frequencies (Brown et al., 1987),decreased capture rates and efficiencies (Little et al., 1990), and increased prey handling time (Weis et al., 2001).

Furthermore, few dietary exposure studies have assessed how food composition affects MeHg toxicity (Leaner and Mason, 2004). In nearly all prior dietary exposure studies, artificial food consisting of commercial fish flakes was used to simplify the feeding of the fish; only a few studies have used natural food (live or dead prey) in dietary MeHg experiments. The Hg form in artificial food is MeHg chloride (MeHgCl), while in natural food it is mostly MeHg-cysteine (MeHgCys), since the Hg forms in these two food types are different, the bioavailable Hg may be different for the fish (Leaner and Mason, 2002; George et al., 2008; Depew et al., 2012).

In this study, we examined the effects of dietary MeHg exposure to an estuarine forage fish, the sheepshead minnow (*Cyprinodon variegatus*) at their larval stage. The Sheepshead minnow was chosen because it is a commonly used species in toxicity experiments and is easily maintained in the lab. Moreover, Sheepshead minnows are important in US East coast ecosystems and are a major prey item for predatory fish and seabirds. We tested whether: 1) food rations affect fish bioaccumulation of, or sensitivity to, MeHg; 2) MeHg bioaccumulation or toxicological effects differ between artificial and natural foods; and 3) dietary MeHg affects fish behavior such as prey capture ability.

2. Methods

2.1. Fish maintenance and acclimation

All experimental protocols used in this study and fish rearing procedures were approved by Stony Brook University Institutional Animal Care and Use Committee (789199-1). Sheepshead minnow larvae were purchased from Aquatic Biosystems Inc. (Colorado, USA) at 2 days post hatch. All fish were held together and fed newly hatched *Artemia* and acclimated to laboratory conditions until 7 days post hatch prior to the start of experiments. Fish were maintained at 24 ± 0.5 °C under a 14:10-h light-dark cycle throughout the acclimation and experiments.

2.2. Experimental design

The general experimental approach was to have larvae fed diets comprised of either artificial food or natural food with varying MeHg concentrations. Fish were reared on Hg-free or contaminated food for their entire larval stage (i.e. from age 7 days post hatch to 29 days) until they reached the juvenile stage. At each sampling time, growth, respiration (i.e. oxygen consumption rate), routine swimming behavior, and prey capture ability were assessed. Specifically, the experiments used a nested design. Each experiment had three or four time points during which samples were taken; at each time point there were two or four MeHg treatments; for each MeHg treatment there were four replicate jars with each jar containing 5 fish. Thus, for each parameter (e.g., swimming speed, growth, etc.) measurements of 20 fish were taken (except fish weight data, where n = 4). Four jars for each treatment were destructively sampled at each time point. A schematic overview of all three experiments conducted is given in Fig. S1. Details regarding numbers of replicates and sample sizes are given in Fig. S2-4.

After acclimation, larvae were randomly distributed into glass jars (100 mL) at a density of five larvae per jar. All fish were kept in 100 mL of 0.2 μm filtered Southampton surface seawater (SHSW, salinity = 35, pH = 7.9, collected 8 km off Southampton, NY). Experimental containers received continuous and gentle bubble aeration and 50% of the water was changed every other day to maintain water quality. Fish were daily exposed to diets with and without MeHg from day 7 (exposure day 0) to day 29. On exposure days 0, 7, 14, and 21, routine swimming behavior was tested. Immediately following swimming behavior tests, fish respiration was tested (i.e. on days 0, 7, and 14 only). For the prey capture experiment, fish were only tested on day 0, 7, and 21.

Four jars containing a total of 20 fish for each treatment were randomly and destructively sampled at each time point for bioassays. After completing all behavioral tests on a given day, the fish were humanely euthanized, and rinsed with deionized water in preparation for Hg analysis. Fish lengths were measured individually. The total wet and dry weights of five fish from the same jar were recorded before being digested with acids for further Hg analysis (details below).

Overall, sequential experiments assessed MeHg effects using natural food at low and normal food rations; compared artificial and natural foods at normal food rations; and determined the impact of MeHg on the ability of larval fish to capture prey.

2.3. Diet preparation

Artemia spp. (the "natural food") and flake food (the "artificial food"; TetraMin Tropical) were used in this study. To produce MeHg-contaminated natural food, the marine diatom (*Thalassiosira pseudonana*) was grown in 500 mL of 0.2-μm filtered SHSW for 5 days to reach a cell density of 1×10^6 mL^{-1}. The diatoms were then exposed to MeHgCl at two treatment levels (1000 ng L^{-1} or 1500 ng L^{-1}) for a 2-day period; for controls, diatoms were not exposed to MeHg. Newly hatched *Artemia* were then added to the algal cultures and fed on diatoms for 48 h with aeration. The *Artemia* were then filtered onto a 100-μm mesh, rinsed with MeHg-free water to remove excess MeHg from the carapace, and added to 100 mL filtered SHSW to make an *Artemia* slurry. MeHg in *Artemia* was either incorporated into tissues or left in the digestive tract as undigested diatoms. After counting the density of *Artemia* in the slurry, aliquots of the *Artemia* slurry were frozen at −81 °C until they were used for fish feeding. *Artemia* controls were prepared identically but using the control diatom cultures.

Artificial food was prepared by incorporating MeHg into flake food via an agar/gelatin matrix as described in Stefansson et al. (2013) and stored at 4 °C to minimize degradation. Measured concentrations of Hg in both natural and flake food are shown in Table S1. The dietary MeHg concentrations can range from 0.05 to 0.14 ppm wet wt in coastal ecosystems (Fitzgerald et al., 2007; Francesconi and Lenanton, 1992). Based on preliminary experiments, the MeHg concentrations were chosen to produce MeHg levels in fish that would be comparable to MeHg concentrations of fish from contaminated sites. In the diet comparison experiment, the target MeHg in diets was 2.5 ppm; the actual MeHg concentrations in artificial food was 2.9 ppm and 2.4 ppm in natural food. Very little (<2%) MeHg degradation was observed during storage. Artificial food and natural food were fed to fish at either 5% (low food ration) or 10% (normal food ration) of fish body weight d^{-1} (EPA, 1996). For artificial food, 10% body weight food consisted of a mixture of 5% treatment food and 5% MeHg-free flakes. For natural food, 10% body weight food consisted of a mixture of 5% treatment food and 5% newly hatched *Artemia* (not exposed to MeHg). The 1:1 mixing of food with and without MeHg for the normal food ration treatments allowed assessment of food rations without affecting the total MeHg exposure.

2.4. Swimming behavior

Quantification of routine swimming behavior was conducted using video recordings. Fish were gently transferred from exposure jars to 6-well plates with one fish per well. Each well was 35 mm in diameter. Three freestanding video stages were used to serve as platforms for experiments, each stage being equipped with a single high definition video camera (Sony Model HDR camcorder) mounted overhead. Since the camera could only record the horizontal movement of the fish, only 4 mL of SHSW was added in each well to reduce any vertical movement. After 30 min of acclimation to the well, fish were recorded for 30 min. To help avoid experimental artifacts, only the middle 20 min of videos were used for data analysis by using LoliTrack v.4 software (Loligo Systems). Mean speed (cm s^{-1}), mean acceleration (cm s^{-2}), total time active (s), and total distance swum (cm) were measured for individual fish. Specific details of video system design, video processing, and data analysis are described in Rountos et al. (2017).

2.5. Respirometry

Immediately after swimming behavior was assessed, fish respiration was measured using a SDR SensorDish Reader set (PreSens). Fish were carefully and individually transferred to the sensor dish and oxygen levels in each well were measured every 15 s. When fish were settled in the well, the oxygen consumption rates were calculated by taking the slope of the line (10 min for exposure on day 0, 5 min for exposure at days 7 and 14 when rates were higher). Oxygen levels used in calculations were all above 5 mg L^{-1} and temperature did not change during the entire experimental period. Further details of the oxygen measurements are described in Kielland et al. (2017).

2.6. Prey capture

Prey capture ability after dietary exposure was recorded with video cameras. The time limit set for the feeding experiment was based on the preliminary experiment with the goal that some, but not all of the prey would be consumed. Ten *Artemia* were introduced in a 35 mm diameter well with 4 mL SHSW. After the *Artemia* introduction, individual fish were placed in the center of the well with a modified transfer pipet and their feeding behavior was recorded for 5 min. Total attempts, total captures, and the time to capture each *Artemia* were recorded by analyzing the videos. Capture efficiency was calculated by dividing the total captures by the total attempts. Camera recordings of two side-by-side wells were made for control and MeHg-treated fish simultaneously. All prey capture tests were conducted between 9 a.m. and 12 noon on each recording day to avoid complications relating to circadian rhythm effects.

2.7. Hg analysis

Baseline MeHg in SHSW was measured by a Tekran 2700 automated MeHg analysis system (Tekran Instruments Co.; detection limit at 0.004 ng L^{-1}). Total Hg concentrations in artificial and natural fish food were determined by a DMA-80 direct mercury analyzer (Milestone Inc.; detection limit at 10 ng L^{-1} for a 1 ml sample). Given that all the food was prepared by using methylmercury chloride and total baseline Hg in control food was relatively negligible, the total Hg values measured by the DMA-80 were considered to represent MeHg concentrations. MeHg levels in larvae were measured by a LUMINA 3300 atomic fluorescence spectrometer (AFS) (Aurora Biomed Inc), which is 10 times more sensitive than the DMA-80 (detection limit 1 ng L^{-1}) since the MeHg levels in larvae were too low to reliably measure with the DMA-80. Euthanized fish were digested by acids prior to Hg analysis. In brief, five individual fish from the same jar were kept in 1 mL trace metal grade nitric acid overnight in a 15 mL metal-free tube. After adding 1 mL trace metal grade hydrogen peroxide, the sample was digested at 100 °C for 5 h. After cooling down, 10% HCl was added into tubes to 15 mL in total. Total Hg in digest was then determined by AFS. Since over 90% of Hg in fish is in the methylated form (Marrugo-Negrete et al., 2008), the total Hg value was assumed to represent MeHg. The AFS was calibrated with Hg standard solutions and calibration checks were performed every 10 samples in order to monitor the stability of the AFS. Certified reference material of fish tissue, DORM-4 (NRCC), was used to validate the Hg recovery either using DMA80 or AFS. The certified value of total Hg in DORM-4 is 0.412 ± 0.036 ppm and the measured value in this study was 0.432 ± 0.037 ppm (n = 15).

Hg uptake efficiency in the fish was calculated as:

$$\text{Hg uptake efficiency } (\%) = \frac{\text{Hg mass in fish}}{\text{Hg concentration in diet} \times \text{total mass of food eaten}} \times 100\%$$

2.8. Statistical analyses

Statistical analyses were conducted using R (Version 3.4.3). The experiments followed a nested (hierarchical) and balanced experimental design. In the natural food at 3.2 ppm test, fish lengths and swimming behavior were analyzed using mixed effects models considering time, food ration, and treatments as fixed effects, jars as a random effect. Weight data were analyzed using a three-way analysis of variance (ANOVA) considering time, food ration and treatments as three factors since weight data were gathered by pooled fish from the same jar. Hg concentrations were analyzed using a two-way ANOVA considering time and food ration as two factors.

For the artificial food and natural food containing around 2.5 ppm MeHg, fish lengths and swimming behavior were analyzed using mixed effects models considering time and treatments as fixed effects, jar as a random effect. Weight data and respiration data were analyzed using a two-way ANOVA considering time and treatments as two factors since weight data were gathered by pooled fish from the same jar and the respiration data were based on the weight data. MeHg concentrations in fish were analyzed using a one-way ANOVA considering time as a factor.

Prey capture data were analyzed using a mixed effects model with jar as a random factor; specifically, time to capture each *Artemia* was analyzed by survival analysis using mixed effects cox model (coxme). Total attempts and total capture of fish were analyzed using a generalized linear mixed model (glmer) fitting a Poisson distribution since they were count data; capture efficiencies were analyzed using glmer fitting a

Table 1
Hg concentrations in fish tissues (ppm dry wt) ± 1 SD (n = 4) under different treatments. Food rations of 5% body wt d^{-1} and 10% body wt d^{-1} were compared for natural food. The asterisk denotes significance ($P < 0.05$) between different food rations or different food types at that time.

Day	Food ration test		Food type test		Prey capture test
	Natural 5% 3.2 ppm	Natural 10% 3.2 ppm	Artificial 10% 2.9 ppm	Natural 10% 2.4 ppm	Artificial 10% 4.8 ppm
7	1.64 ± 0.31	1.28 ± 0.11	0.53 ± 0.01	1 ± 0.09	3.99 ± 0.68
14	3.19 ± 0.64	1.68 ± 0.45 *	2.02 ± 0.39	1.56 ± 0.37	NA
21	2.9 ± 0.63	2.07 ± 0.1 *	3.27 ± 0.47	1.28 ± 0.43 *	11.4 ± 1.48

binomial distribution since they were ratio data. Correlation analysis for Hg concentrations and prey capture ability were determined by Spearman correlation coefficient. Assumptions of normality and homoscedasticity were tested before each analysis and data were transformed if necessary. A statistical significance level was set at 0.05. When significant differences were detected, least-square means with p-values adjusted as per Tukey's method were used for multiples comparisons after mixed effects models and Tukey's multiple pairwise comparisons were used after ANOVAs.

3. Results

3.1. Hg accumulation

Survival across all treatments and experiments was 90% or greater. Baseline MeHg concentrations in SHSW were below the detection limit. Hg levels in all control fish were also below the detection limit throughout all experiments. Since very little MeHg degradation was observed in the food, we assume there was no aqueous MeHg exposure and that fish obtained MeHg only from their diet. Fish accumulated Hg from their diets as the exposure time increased. When exposed to natural food (3.2 ppm treatment), the MeHg level in fish fed the low ration reached 2.9 ppm at 21 d, while in fish fed the normal ration it reached 2.1 ppm (Table 1). MeHg body burdens in fish fed diets containing about 2.5 ppm MeHg were comparable for artificial food and natural foods during the first 14 d, but after 21 d, fish that consumed artificial food accumulated about 2.5 times more MeHg in their bodies ($P = 0.006$, Fig. 1 A and Table 1). There were measurement errors in Hg mass in fish and Hg concentrations in diets, but probably the largest uncertainty was in the total mass of food eaten for the artificial food. The consumption of the powder was hard to quantify, unlike with the natural food where discreet *Artemia* could be observed and thus food consumption was easy to detect. It was also assumed that individual fish ate equal amounts of food within the same jar. Thus, a calculated Hg uptake efficiency of 100 ± 20% was found for the artificial diet treatments. Hg uptake efficiency decreased as the exposure time increased for all natural food treatments; while MeHg uptake efficiency using artificial food increased with exposure time (Fig. 1 B). For the experiment assessing prey capture, Hg levels in the control group fish were also below the detection limit throughout the dosing period, while fish Hg levels in the 4.8 ppm exposure group increased significantly (i.e., from 0 to 15 ppm; Table 1 and Fig. S 7).

3.2. Effects of mercury using natural foods at different food rations

The data indicated that, in general, food rations affected fish growth and swimming activity while MeHg had no significant effects on growth or swimming behavior when fed normal food rations but did have significant effects when fish were fed low rations. Fish weight and length (Fig. 2 A and B) were significantly different among days ($P < 0.001$) and affected by different food rations ($P < 0.001$) and MeHg treatments ($P \leq 0.01$). There were also significant interactions between days and

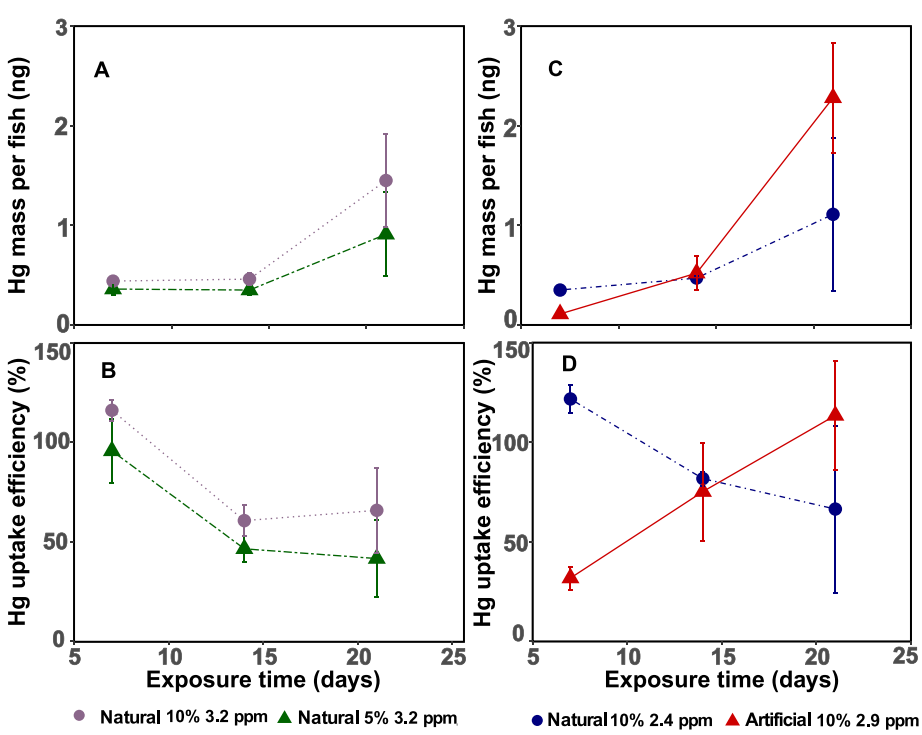

Fig. 1. Panel A and B display mean Hg mass per fish ±1 SD (n = 4) and Hg uptake efficiency ± 1 SD (n = 4) for low food ration (5% body wt d^{-1}) or normal rations (10% body wt d^{-1}) for MeHg-treated natural food. Panel C and D display mean Hg mass per fish ±1 SD (n = 4) and Hg uptake efficiency ± 1 SD (n = 4) for either MeHg-treated artificial food or natural food for normal food rations. All control treatments showed no detectible Hg in fish throughout the experiments.

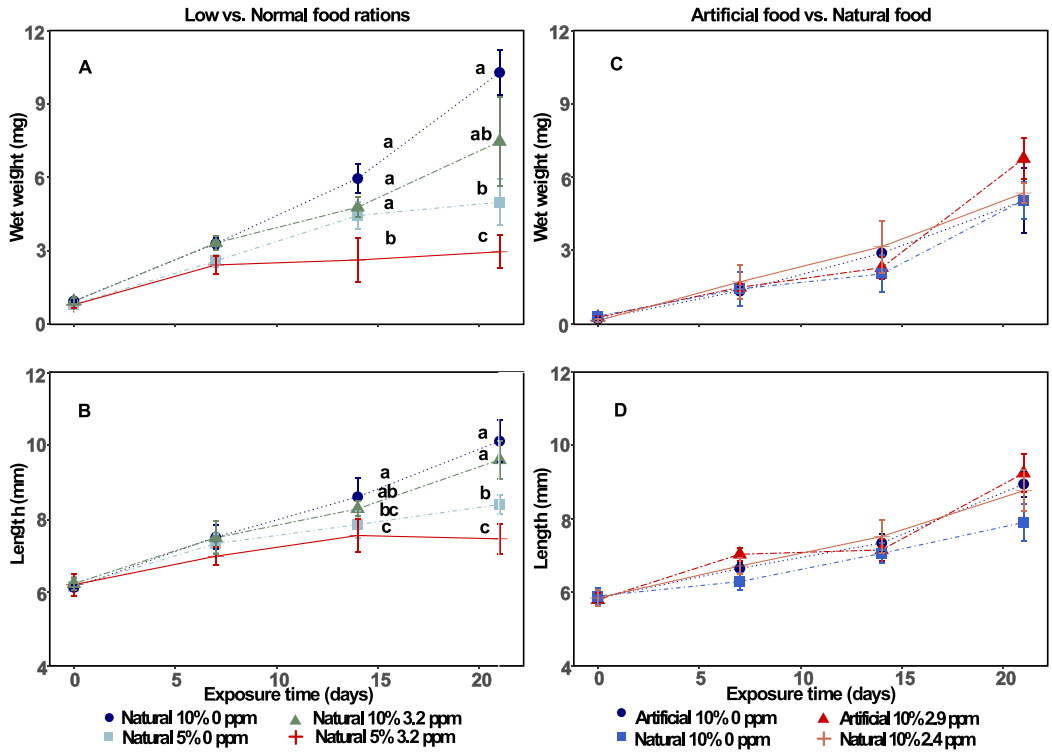

Fig. 2. Panel A and B display mean wet wt and length ±1 SD (n = 4) for each treatment over the 21 days dosing period for low rations (5% body wt d^{-1}) or normal rations (10% body wt d^{-1}) for controls or MeHg-treated natural food (3.2 ppm). Letters denote significance (P < 0.05) among treatments at that experimental time point. There was no significant difference in fish Hg concentrations between normal and low rations before day 7. Panel C and D display mean wet wt and length ±1 SD (n = 4) for either artificial food or natural food for controls or MeHg-treated food. There was no significant difference in fish wet wt or length between control and MeHg treatment either using artificial food or natural food at 2.5 ppm.

Table 2
Increase in fish biomass and length ±1 SD over a 3-week exposure period using natural food at different food rations. Low food ration was set at 5% of fish body wt d^{-1}, normal food ration set at 10% of fish body wt d^{-1}. MeHg concentrations in diet was 0 ppm for controls and 3.2 ppm for the MeHg treatments. Statistical comparisons between treatments are given in Fig. 2.

Days of exposure	Low food ration control	Low food ration MeHg	Normal food ration control	Normal food ration MeHg
	Wet wt (mg d^{-1})			
0–7	0.252 ± 0.05	0.233 ± 0.07	0.335 ± 0.04	0.341 ± 0.07
7–14	0.265 ± 0.11	0.029 ± 0.18	0.379 ± 0.12	0.209 ± 0.10
14–21	0.078 ± 0.21	0.048 ± 0.22	0.619 ± 0.22	0.383 ± 0.31
	Length (mm d^{-1})			
0–7	0.166 ± 0.07	0.111 ± 0.07	0.195 ± 0.06	0.179 ± 0.08
7–14	0.074 ± 0.08	0.081 ± 0.10	0.157 ± 0.12	0.112 ± 0.09
14–21	0.078 ± 0.08	−0.013 ± 0.12	0.217 ± 0.16	0.191 ± 0.10

treatments (P < 0.001). Fish fed the low food ration with 3.2 ppm MeHg had significantly lower wet weights than fish fed the low food ration with 0 ppm MeHg (P < 0.001), or both the normal food ration with 3.2 ppm MeHg (P < 0.001) and the normal food ration with 0 ppm MeHg (P < 0.001). Fish fed the low food ration with 0 ppm MeHg also had significantly lower wet weight than fish fed a normal food ration with 0 ppm (P < 0.001) or 3.2 ppm MeHg (P = 0.005). Fish fed the low food rations with 3.2 ppm MeHg also had shorter lengths compared to fish fed the normal food rations with 3.2 ppm MeHg (P = 0.01) or 0 ppm (P = 0.04). When fed low food rations with MeHg, fish grew slowly between 7 and 21 days compared to the control fish at this food ration. Increases in biomass in the second and third weeks were significantly lower (9.1 and 1.6 times lower, respectively) in these fish compared with controls fed the low food ration (Table 2, Fig. 2).

Swimming speeds (Fig. 3 A) were significantly different among days (P = 0.016), food ration (P < 0.001) and MeHg treatments (P < 0.001). Swimming speeds in fish fed low food rations with 3.2 ppm MeHg were significantly lower than swimming speeds in fish fed low food rations with 0 ppm MeHg (P = 0.039), normal food rations with 3.2 ppm MeHg (P = 0.0002) and normal food rations with 0 ppm MeHg (P = 0.0001). Swimming acceleration (Fig. 3 B), active time (Fig. 3 C) and total distance swum (Fig. 3 D) displayed similar patterns (Table S 2).

3.3. Effects of MeHg using different food types at 2.5 ppm

The data indicated no significant effects of MeHg (around 2.5 ppm) on fish growth, overall swimming behavior, and fish respiration either using artificial food or natural food (Table S 3). No significant differences were found for fish weight (Fig. 2 C) and length (Fig. 2 D) between MeHg treatments. Fish did show significantly increased weight (P < 0.001) and length (P < 0.001) among days.

For artificial food, there was no significant difference among days and between MeHg treatments in swimming speed (Fig. 4). A significant difference was observed among days (P < 0.001) but not between MeHg treatments in swimming acceleration (Fig. S 5). For total time active (Fig. S 5), significant differences were found among days (P < 0.001) and between MeHg treatments (P = 0.04). There were also significant interactions between days and MeHg treatments (P = 0.003) for total active time. Fish were more active after 21 days of MeHg exposure compared to the control treatment. Total distance swum (Fig. S 5) was not significantly different among days but it was between MeHg treatments (P = 0.02). The interaction between days and MeHg treatments was also significant (P = 0.001) for distance swum. Fish swam longer distances after 21 days of MeHg exposure. Mass-specific respiration (Fig. 5 B) was significantly different among days (P < 0.001) but not

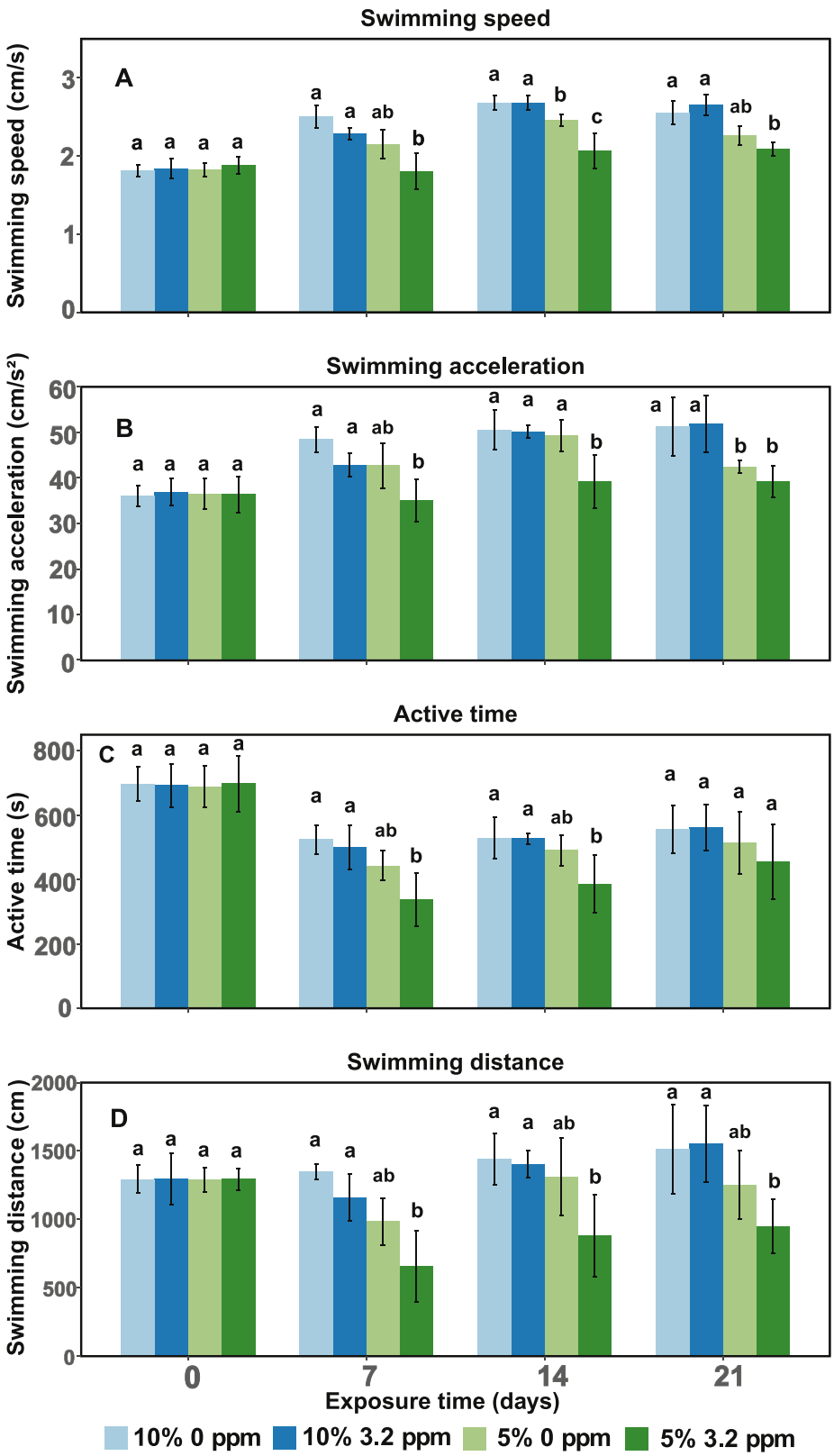

Fig. 3. Effects of natural food at 3.2 ppm on (A) swimming speed, (B) swimming acceleration, (C) total time active, and (D) total distance swum. Error bars represent ±1 SD. Letters denote significance ($P < 0.05$) among treatments at that experimental time point.

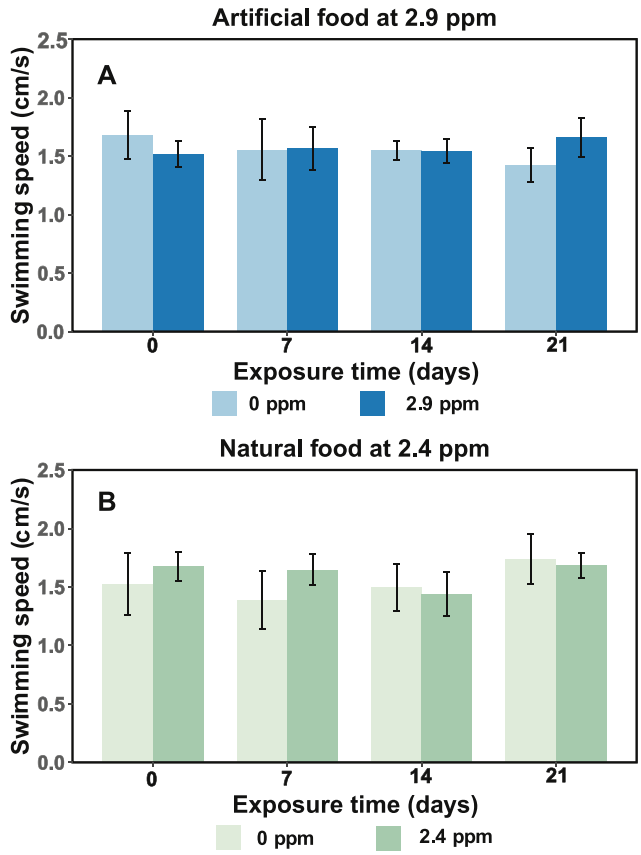

Fig. 4. Effects of MeHg obtained from artificial or natural food on fish swimming speed. Error bars represent ±1 SD. No significant differences were observed between treatments for either diet.

between MeHg treatments.

For natural food, no changes were observed in swimming speed (Fig. 4B), swimming acceleration (Fig. S 5) and total distance swum (Fig. S 5) among days and between MeHg treatments. Total time active (Fig. S5) and mass-specific respiration (Fig. 5 D) were significantly different among days ($P < 0.001$) but not between MeHg treatments.

3.4. Effects of MeHg on prey capture

No significant differences were observed in the consumption of *Artemia* by larvae after 0 d (Fig. 6 d (Fig. 6 B) between the 0 ppm and 4.8 ppm MeHg treatments, while there was a marginally significant difference after 21 d exposure ($P = 0.051$, Fig. 6 C). Fish from the 4.8 ppm treatment took longer to consume *Artemia* than fish in the control treatment. For example, after 21 d exposure, fish from the control group took less than 50 s to consume 50% of *Artemia*, while fish from the MeHg group took around 90 s (Fig. 6 C). MeHg did not affect the number of total attempts, total captures and capture efficiencies on earlier days (i.e. day 0 and day 7), but on day 21, reduced total attempts ($P = 0.008$), total captures ($P < 0.001$) and capture efficiencies ($P = 0.001$) were observed (Fig. 6).

Positive correlations were observed between the total attempts and total captures ($P < 0.001$, $r = 0.46$), but there is not enough evidence to conclude that there was a correlation between Hg levels in fish and the total attempts. Hg levels in the fish were also not significantly correlated with total captures or capture efficiency. These relationships are shown in Fig. S 8 and a summary of the statistical details is shown in Table S 4.

4. Discussion

4.1. Survival, growth, and respiration

Our study found that dietary MeHg exposure did not affect larval survival over a 21 day period but did have sublethal effects. In fish that fed on low food rations of natural food and displayed lower growth rates, the MeHg was higher than in fish which were fed normal food rations and had faster growth rates due to greater growth dilution of the tissue Hg concentrations in the latter group. This result was comparable to many other studies which suggest that rapid growth can reduce Hg concentrations in wild fish and aquatic invertebrates and potentially reduce the trophic transfer of Hg in marine food webs (Karimi et al., 2007, 2010; Dang and Wang, 2012). Fish in the low food ration MeHg treatment also were smaller than in low ration control fish after 21 days dosing, suggesting that the reduced growth was not only a direct effect of the reduced food intake but also an effect of MeHg exposure. Thus, sheepshead minnow larvae that do not have access to sufficient amounts of food may be more sensitive to dietary MeHg. This observation is consistent with other studies (Brynhildsen et al., 1988; Hashemi et al., 2008a) that showed starved or malnourished larvae are more vulnerable to other chemical stressors, including copper, zinc or cadmium. Potential mechanisms for negative impacts of MeHg on fish growth at low food rations include decreased levels of cortisol and thyroid function, decreased nutrient absorption and increased energy needs associated with maintaining homeostasis under stressful conditions (Berg et al., 2010; Berntssen et al., 2003; Houck and Cech, 2004).

Previous studies have found different Hg sensitivity between freshwater and marine fish; generally, Hg toxicity to freshwater fish occurs at notably lower concentrations than in marine fish. In their review, Depew et al. (2012) reported that freshwater fish often display sensitivity to tissue concentrations of Hg when concentrations exceed 0.5 ppm on a wet wt basis, whereas it is not uncommon that some marine fish species have tissue Hg concentrations that exceed this level, even in unpolluted waters (Karimi et al., 2012). As an example, Friedmann et al. (1996) found that a diet with only 0.1 ppm wet wt of MeHg reduced length, weight and gonadosomatic index in the freshwater walleye (*Stizostedion vitreum*). In our experiments, diets with about 3 ppm wet wt MeHg did not affect fish growth when fish were fed on a normal food ration. In comparison, Lee et al. (2011) found that dietary MeHg had a significant effect on growth of the adult San Francisco Bay sturgeon (*Acipenser medirostris* and *A. transmontanus*), but the lowest treatment they used in the study was a concentration of 25 ppm MeHg (dry wt basis) in the diet which is much higher than naturally occurring levels which typically range from 0.02 to 0.1 ppm wet wt in coastal ecosystems (Fitzgerald et al., 2007). Consistent with our experimental results, Stefansson et al. (2013) found that sheepshead minnow juveniles (28 d old post-hatch before dosing started) have a high tolerance for MeHg, and that reduced growth was not observed when fish fed on a diet containing 3 ppm MeHg (dry wt), however a diet of 7 and 14 ppm dry wt affected these fish.

Previous studies found that fish exposed to pollutants have a higher metabolic activity, possibly indicating reallocation of energy to detoxification (Beyers et al., 1999). It is reasonable to expect a metabolic cost associated with cellular or tissue repair mechanisms as the fish attempts to maintain or re-establish normal respiration (Houck and Cech, 2004). Nevertheless, after eating either artificial or natural food containing around 2.5 ppm MeHg, the mass-specific respiration in the fish was not affected by MeHg. It is presumed that higher MeHg concentrations would have affected respiration rates, but concentrations of MeHg higher than those used here, and much higher than those found in natural waters, were not tested.

4.2. Swimming behavior

As the sheepshead minnow larvae grew older, they became more

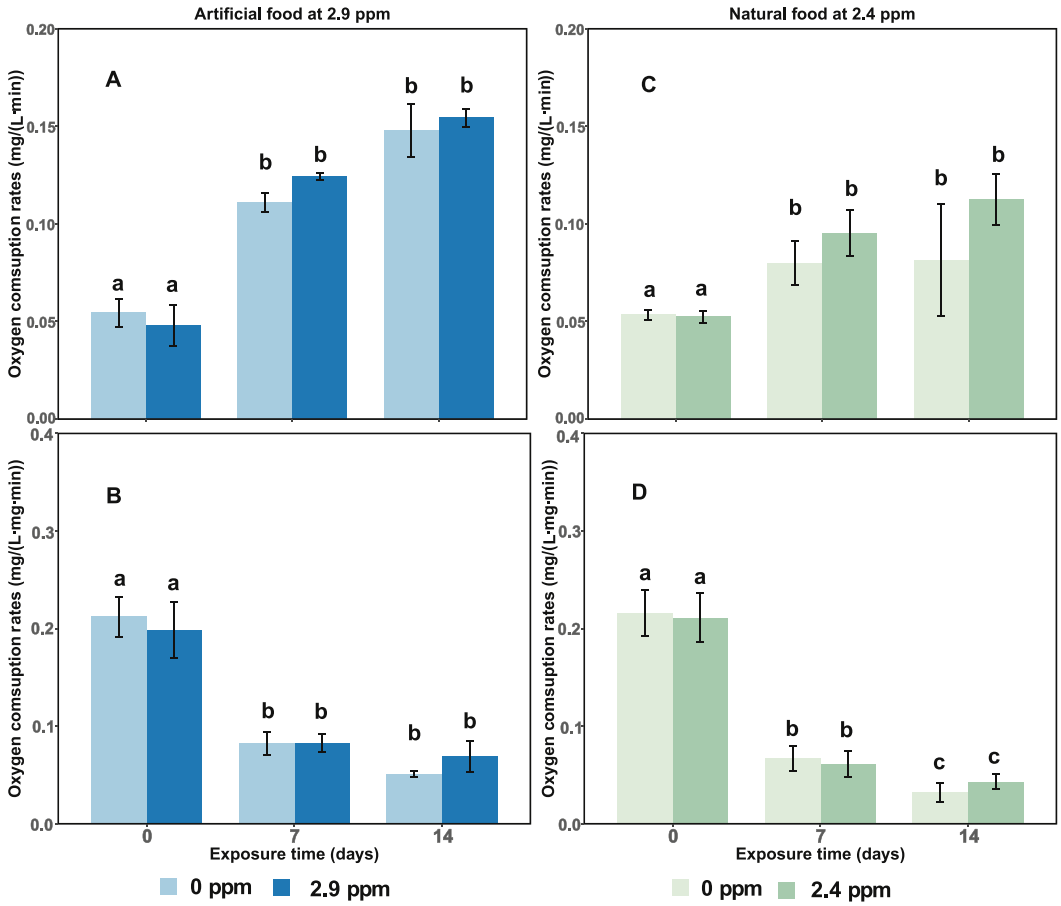

Fig. 5. Comparison between artificial food and natural food on fish respiration. Panels A and C display the direct measurement of oxygen consumption rates; panels B and D the mass-specific respiration measured as oxygen consumption rates per fish wet wt. Mass-specific respiration differed among days ($P < 0.001$) but not between MeHg treatments for artificial food or natural food. Error bars represent ±1 SD. Letters denote significance ($P < 0.05$) among days and between treatments.

active in general but MeHg did not impact their routine swimming behavior in our study when fed normal food rations. As a neurotoxic compound, MeHg exposure is often linked to behavioral abnormalities in fish (Sandheinrich and Wiener, 2011). Potential mechanisms involve MeHg interference with neurotransmitter production, receptors, or cell signaling pathways (Depew et al., 2012; Weis, 2009). There are few studies that have considered the impacts of dietary MeHg on marine or estuarine fish behavior. In our study, when fed a normal ration of natural food, MeHg had no significant impact on fish swimming but at a low ration swimming was affected by the MeHg. Previous studies suggested that the nutritional state or condition of an organism may affect its sensitivity to metals (Brynhildsen et al., 1988; Hashemi et al., 2008a, 2008b). In our experiments, the normal food ration diet contained greater quantities of nutritious newly hatched *Artemia*, which provided extra calories of a high quality diet. It has been suggested that toxic metals can cause decreased glycogen levels, but extra food can supply extra energy to the fish, reducing such a decrease in glycogen levels (CiCiK and Engin, 2005; Kramer et al., 1992). This might explain the negligible effects of dietary Hg in the fish fed normal food rations. In the low feeding ration MeHg treatment, fish need to balance normal activity (e.g. growth, routine behavior) and detoxification processes using the limited energy resources available to them with this diet. An imbalance between energy intake and energy expenditure may have occurred, leading to minimal fish growth after 7 days exposure. Previous studies suggest that starvation might increase the Hg burden in fish tissues (Cizdziel et al., 2002; Drevnick et al., 2008). For very undernourished fish, in addition to lack of growth dilution, the hypothesized mechanisms are that MeHg is distributed among the fish tissues as these fish catabolize muscle tissue for energy (Cizdziel et al., 2003). Fish livers, which shrink in size and mass due to starvation, increased most dramatically in MeHg concentration and may have diminished detoxification capability (Drevnick et al., 2008). Fish blood, which is the conduit for internal Hg distribution to other organs, also increased in Hg levels in starving fish (Cizdziel et al., 2002). Thus, starvation might increase the Hg burden in many tissues, including the brain, the ultimate site for toxic neurological effects. This might help explain why the swimming behavior of fish fed low rations was affected by MeHg. These findings are comparable to those of another study (Puga et al., 2016), which found that MeHg efficiently accumulated in juvenile white seabream's (*Diplodus sargus*) brain and led to a decrease in total swimming time. Another possible explanation for the effects of MeHg on fish swimming observed at low food rations is that growth was depressed, and smaller individuals typically swim more slowly than larger ones (Fig. S 6; Bainbridge, 1958). Importantly, reduced swimming activity can decrease a fish's ability to capture food and escape from predators (Vieira et al., 2009).

4.3. Comparisons between artificial food and natural food

Using artificial food to simplify the dosing process may lead to higher MeHg accumulation in fish as indicated in our study and Leaner and Mason (2004). However, Bowling et al. (2011) found higher accumulation of MeHg in largemouth bass (*Micropterus salmoides*) from natural food than from artificial food. Furthermore, there appears to be insufficient evidence to conclude that one form of dietary MeHg exerts more, less or the same toxicity than the other. Leaner and Mason (2004) found

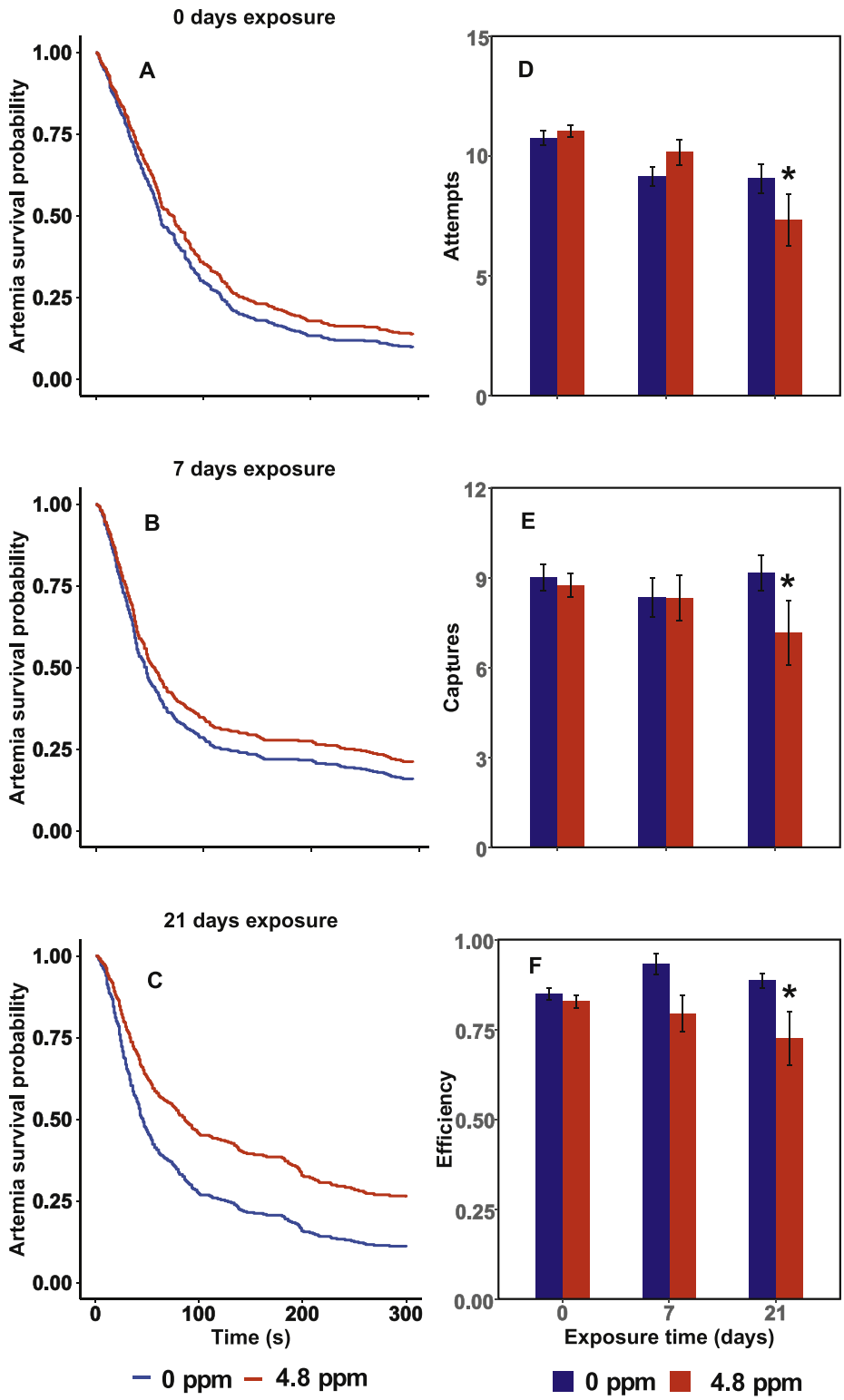

Fig. 6. Prey capture ability of sheepshead minnow. Panel A, B and C display the *Artemia* survivorship. Time to capture each *Artemia* was recorded and applied in survival analysis to represent fish's prey capture ability. After the 21 day exposure period, there was a small difference between controls and the Hg treatment (P = 0.051). Panels D, E, and G display fish's total attempts, total capture and capture efficiency, respectively. The error bars represent ±1 SD. The asterisk denotes significance (P < 0.05) between control and Hg treatment at that time.

that after a single feeding on natural or artificial food, MeHg in fish reached 0.1 ± 0.02 ppm and 0.41 ± 0.02 ppm wet wt, respectively; they did not evaluate the toxicity of the MeHg by two different diets. We are unaware of studies that have assessed the toxicity of MeHg to marine fish - adults or larvae - comparing artificial versus natural dietary routes. In our study, the lack of MeHg toxicity from either artificial or natural diet at 2.5 ppm dry wt might also be explained by the normal food rations, which, as noted above, provided extra energy for the fish. The Hg level in the artificial food treatment was slightly higher than in the natural food treatment, but the normalized Hg uptake efficiency using artificial food was still higher than when using natural food after 21 days exposure; this may be explained by MeHgCl being taken up more easily than MeHgCys (Depew et al., 2012; Leaner and Mason, 2002). The decrease in Hg uptake efficiency over time in all the natural food treatments may have resulted from increasingly greater Hg excretion from the fish than following uptake from the artificial diet.

4.4. Prey capture ability

Feeding behavior of the sheepshead minnows was mostly affected after 21 days of dietary MeHg uptake. In previous studies (Weis et al., 2001; Zhou et al., 2001), it was found that fish had impaired prey capture ability, but instead of having poor coordination to capture prey, fish made fewer attempts to capture prey. However, in those studies, only embryos were exposed to aqueous Hg or MeHg while in our study we fed larvae dietary MeHg. We also found fish displayed fewer capture attempts. Moreover, the studies of Weis et al. (2001) also provide evidence that the toxic effects of MeHg were transitory and diminished over time after exposure. They suggested that the neurotoxic effects of Hg may have been a retardation of neurological development or a depression of a neurochemical process rather than a result of tissue damage. Instead of temporary effects, we found that the impaired prey capture ability in *C. variegatus* was exposure-time dependent. The fish did not show a pronounced effect after 7 d of exposure, but clear impacts were evident by 21 d of exposure. A lack of direct correlation between Hg body burdens and prey capture may be the result of Hg body burdens in the first 2 weeks being below a certain threshold for eliciting a toxic effect. Prey capture ability has obvious effects on fish growth and survival. The larval stages of fish are especially sensitive because of their limited tolerance of starvation (Weis, 2014). Impaired feeding may reduce growth and prolong larval development, which may in turn increase predation risks and decrease survival (Weis, 2009).

4.5. Environmental relevance and ecological impacts

In contaminated coastal marine food webs, adult forage fish can reach 0.46 ppm wet wt MeHg and piscivorous fish can reach 2.3 ppm wet wt MeHg (Francesconi and Lenanton, 1992). For regions that are impacted less by atmospheric deposition and fluvial sources of Hg, fish at lower trophic levels typically have $\leq$0.15 ppm wet wt MeHg (Baeyens et al., 2003; Hammerschmidt and Fitzgerald, 2006). In our experiments, the highest Hg concentration in fish fed artificial food containing 4.8 ppm MeHg was 11 ppm dry wt, whereas the highest Hg concentration in fish fed natural food containing 3.2 ppm MeHg was 3 ppm dry wt. Given that the wet/dry ratio of sheepshead minnow larvae is about 7.5 $\pm$ 1.8, the MeHg concentrations would be 1.5 ppm wet wt and 0.4 ppm wet wt from artificial and natural foods respectively, within the range of forage fish concentrations in contaminated sites in the wild. The MeHg exposure in this study covers the entire larval stage and suggests that sheepshead minnows supplied with abundant food have a high tolerance for dietary MeHg exposure. Sheepshead minnows may therefore be able to survive in many contaminated ecosystems and transfer their MeHg to predatory fish which can assimilate MeHg efficiently from their prey (Reinfelder et al., 1998; Pickhardt et al., 2006).

Fish sensitivity to MeHg exposure may be greater in the wild where other stresses such as food limitation (Boyce et al., 2010), modified temperature ranges (Maulvault et al., 2017), lower dissolved oxygen concentrations and the presence of other contaminants can affect toxicity (Dasgupta et al., 2015; DePasquale et al., 2015; Gobler and Baumann, 2016).

5. Conclusion

This is the first study using natural diets and a food chain approach to study the impact of MeHg on larval estuarine fish. Experiments evaluated the impacts of MeHg to the whole larval stage of sheepshead minnow, using both artificial and natural food sources, providing a fuller assessment of sublethal outcomes. MeHg in fish at concentrations of 0.4–1.5 ppm wet wt from artificial food or natural food had no effects on fish growth or swimming when presented with normal food rations. When fed natural food, MeHg in fish at 0.4 ppm wet wt depressed growth and swimming under low food rations. Sheepshead minnows survived a high MeHg burden and could possibly serve as enriched Hg sources for higher trophic level predators. Ultimately, fish sensitivity to MeHg will likely vary in natural ecosystems based on food availability and probably other environmental stressors. Additional studies should determine whether findings using artificial food can be extrapolated to waters containing diverse natural diets.

CRediT authorship contribution statement

Xiayan Ye: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Konstantine J. Rountos:** Methodology, Writing - review & editing. **Cheng-Shiuan Lee:** Methodology, Writing - review & editing. **Nicholas S. Fisher:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2020.105240.

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