

*Aquatic Toxicology*

## EMBRYONIC EXPOSURE TO 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN IMPAIRS PREY CAPTURE BY ZEBRAFISH LARVAE

DANIELLE CHOLLETT, KATHRYN E. PEREZ, and TISHA C. KING-HEIDEN\*

Department of Biology, University of Wisconsin La Crosse, La Crosse, Wisconsin, USA

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**Abstract:** As a ubiquitous, persistent environmental contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has the potential to cause lethal deformities in larval fishes. Few studies have examined its impacts on larval growth and craniofacial development in conjunction with feeding capability. The authors used morphological and behavioral assessments to demonstrate that feeding capability of larvae is impaired even when craniofacial structures are not grossly malformed. Zebrafish embryos were exposed to 25 pg TCDD/mL, 50 pg TCDD/mL, or 100 pg TCDD/mL or <0.1% dimethyl sulfoxide for 1 h at 4 h postfertilization and then raised in clean water for 21 d or 90 d to assess craniofacial morphology, feeding capability, and long-term survival. The lower jaw was 5% smaller in 21-d larvae exposed to  $\geq 50$  pg TCDD/mL, and those larvae caught 10% fewer prey items; survival was reduced by 13% to 23%. The direct cause of TCDD's impacts on feeding capability is not known, but feeding success was correlated with growth, length of lower jaw, and survival. Since low larval mortality rates are key for recruitment, this suggests that exposure to concentrations of TCDD during embryonic development that do not initially cause mortality still has the potential to impact the recruitment success of feral fish. Furthermore, the present work provides additional evidence that behavioral end points are often more sensitive than morphological ones and should be included when assessing the sublethal toxicity of environmental contaminants. *Environ Toxicol Chem* 2014;9999:1–7. © 2013 SETAC

**Keywords:** 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin    Jaw    Prey capture    Zebrafish

## INTRODUCTION

As a ubiquitous and persistent organic pollutant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is considered the most potent halogenated aromatic hydrocarbon [1]. Fish are particularly sensitive to dioxins [2], and exposure to TCDD during critical periods of development reduces individual fitness [3,4] and likely impacts recruitment success of wild fish populations. For example, exposure to dioxin-like compounds is thought to be a contributing factor to the decline of lake trout (*Salvelinus namaycush*) in Lake Ontario, Canada, from 1940 to 1980 [5] as the result of poor recruitment. Exposure to TCDD during embryonic development leads to blue sac syndrome in larval fishes. Blue sac syndrome is a combination of several systemic toxicities including severe pericardial and yolk sac edema, craniofacial malformations, and cardiovascular toxicity, eventually leading to mortality, and is considered a hallmark of TCDD toxicity (see King-Heiden et al. [6]). Susceptibility to TCDD is age-dependent, with embryonic and early developmental life stages being the most sensitive [2].

During early ontogeny, efficient feeding can literally mean the difference between life and death. Two periods of larval development that are critical for growth and survival are when fish transition from endogenous to exogenous feeding and when shifts in dietary requirements occur (e.g., size of prey items) [7–9]. Craniofacial structures of fish determine gape, which, among other factors, plays a crucial role in constraining foraging patterns, growth, and recruitment success [8,10,11]. The efficiency by which larval fishes can capture prey items tends to follow trends in larval growth, and fish tend to select prey items that optimize growth and survival (see Graeb

et al. [8]). Exposure to environmental contaminants could impair recruitment success by limiting growth and survival during this critical stage of development.

Evaluating the potential population-level consequences of exposure to contaminants such as TCDD on feral fish populations requires a careful examination of sublethal exposure during critical periods of growth and development. These types of studies are notoriously difficult to address in wild fish populations because of the inherent complexities of the ecosystem. While laboratory-based feeding studies do not perfectly reflect environmental conditions (e.g., lack competition for prey items, have fluid dynamics that differ from those in the field, and utilize unexposed prey items) and are not often verified by field studies, they can be a sensitive assay to assess sublethal toxicity and predict risk to wild fish populations. Studies in zebrafish can provide an integrative framework for predicting risk to wild fish populations [6].

The developmental toxicity that results from exposure to TCDD has been well studied in zebrafish, and effects on craniofacial development have been fairly well documented. The developing craniofacial structures and jaw are sensitive to TCDD toxicity [12,13], and prolonged exposure causes cranial defects in adults [13,14]. However, few studies have evaluated the physiological implications of these landmark toxic responses following exposure to sublethal concentrations of TCDD. Studies in swim-up rainbow trout (*Oncorhynchus mykiss*) [15] and *Fundulus heteroclitus* larvae [16,17] associated neurotoxicological end points with inability to capture prey just following hatching; however, these studies assessed feeding success in first-feeding larvae and did not provide a careful examination of craniofacial features. King-Heiden et al. [13] demonstrated that zebrafish larvae exposed to sublethal concentrations of TCDD during the earliest stages of development had smaller lower jaws compared with control fish, but careful examination of craniofacial structures and functional

\* Address correspondence to tking-heiden@uwlax.edu.

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analyses were not conducted. In the present study, we provide a detailed analysis of craniofacial structures of zebrafish in first-feeding through late metamorphosis (the first 3 wk of larval development) following embryonic exposure to sublethal concentrations of TCDD, as described by Hernández [18]. We combine these morphological assessments with functional studies by assessing the ability of early metamorphic larvae (21 d postfertilization [dpf]) to capture prey at an age just prior to observed increases in mortality when fish are transitioning to larger food items and entering juvenile stages of development. We show that, in lieu of overt craniofacial malformations, embryonic exposure to TCDD results in an impaired ability to capture prey and leads to increased mortality at later life stages.

## MATERIALS AND METHODS

### *Chemicals, test species, fish husbandry, and TCDD exposures*

The TCDD (>99% purity) was obtained from Chemyn and dissolved in dimethyl sulfoxide (DMSO) for preparation of dosing solutions. The AB strain of zebrafish was used for all experiments, and fish were raised according to Westerfield et al. [19]. Zebrafish exposures occurred in the Peterson/Heideman lab at the University of Wisconsin Madison (WI, USA). Following exposure to <0.1% DMSO or TCDD during early embryonic development, all fish were transferred to our lab at the University of Wisconsin La Crosse and raised in clean zebrafish water (60 mg/L Instant Ocean Salts; Aquatic Ecosystems) at 26 °C to 28 °C on a 14:10-h light:dark light cycle. Densities were maintained as described in King-Heiden et al. [13], and all fish housing and experimental procedures were approved by the Animal Care and Use Committee at the University of Wisconsin La Crosse.

### *Dose-dependent effects on survival through adulthood*

To determine the dose-dependent effects on long-term survival following embryonic exposure to various concentrations of TCDD, zebrafish were exposed to <0.1% DMSO or nominal concentrations of 25 pg TCDD/mL, 50 pg TCDD/mL, or 100 pg TCDD/mL for 1 h beginning at the late blastula/early gastrula stage of development (4–6 h postfertilization [hpf]). There were a total of 3 experimental groups per dose, each containing 50 fish. Two replicate experiments were performed. At 120 hpf, a subsample of fish was used to confirm that zebrafish were responding as previously described [13]. Two fish per group (for a total of 12 fish across replicates) were randomly selected and immobilized in 3% methylcellulose, and lateral images were acquired using an Optronic MicroFire camera mounted on a Leica MZ16 stereomicroscope to evaluate toxicity, as described by King-Heiden et al. [13]. Survival in remaining fish was monitored daily through maturity (90 dpf).

### *Dose-dependent effects on development, growth, and craniofacial toxicity*

In a separate experiment, zebrafish embryos were exposed to <0.1% DMSO or nominal concentrations of 25 pg TCDD/mL, 50 pg TCDD/mL, or 100 pg TCDD/mL for 1 h beginning at 4 hpf to 6 hpf. There were 5 experimental groups per treatment, and 3 replicate experiments were performed ( $n = 15$  groups of 25 fish). Gross morphology and craniofacial structures were assessed in first-feeding larvae and at 2 stages of early metamorphosis (7 dpf, 14 dpf, and 21 dpf, respectively) in subsamples of larvae using a combination of standard and geometric morphometrics. One fish per  $n$  was imaged live as previously described and then

fixed and stained with alcian blue according to Xiong et al. [20] ( $n = 15$ ). Briefly, fish were fixed in 4% paraformaldehyde and dehydrated in a series of graded ethanol solutions. Fish were stained with 0.4% alcian blue overnight at room temperature, neutralized with 4% sodium borate, bleached with 3%  $H_2O_2$ , digested with 0.1% trypsin, and cleared in a graded solution of glycerol in 0.25% KOH. Fish were stored in 100% glycerol at 4 °C until imaged for morphometric analyses.

### *Standard morphometric analysis*

Lateral images of live larvae at 7 dpf, 14 dpf, and 21 dpf were used to measure total length and lower jaw length (normalized to total length). Once stained with alcian blue, larvae (7 dpf, 14 dpf, and 21 dpf) were carefully immobilized in 3% methylcellulose, ensuring that the jaws were in the same orientation and plane. Some samples were damaged during tissue processing and therefore not analyzed. A total of 12 to 15 samples were assessed per treatment group. To assess growth of craniofacial features, ventral photographs of the alcian blue-stained fish were acquired and Scion Image Pro was used to measure the following: the angle of the ceratohyal and the lengths between 1) Meckel cartilage and the palatoquadrate joints, 2) the junctions of the palatoquadrate and hyosymplectic joints, 3) the arch of Meckel cartilage and the basihyal, and 4) the basihyal and the midpoint between the junctions of palatoquadrate, hyosymplectic, and interhyal.

### *Geometric morphometric analysis*

Ventral photographs of craniofacial structures from the above experiments were also analyzed to quantitatively compare variations in shape. Nine type 1 landmarks (Figure 1) were applied to each image using tpsDig2, version 2.17, for 171 individuals [21]. Because the landmarks include the entire jaw from a ventral view, they include object symmetry. Digitized landmarks were used to evaluate interactions between age, treatment, and age  $\times$  treatment and to evaluate symmetry.

### *Dose-dependent effects on ability to capture prey*

Because few studies have examined zebrafish feeding efficiency, we performed pilot studies to determine the density of prey items that would be appropriate for brief prey-capture trials. Our goal was to establish a low enough density that challenged fish to search, pursue, and capture prey but also allowed fish to be fairly efficient at capturing sufficient prey items (control fish captured 30–40% of prey items). At 21 dpf, 1 fish per group ( $n = 15$  fish) from the above experiments was utilized for feeding experiments. Food was withheld the evening prior to and the morning of feeding experiments, and feeding experiments were performed in the afternoon. Fish were placed individually in 50-mL beakers containing 30 mL of zebrafish water. Following 3 h of acclimation, 50 brine shrimp were placed into each beaker. Fish were given 20 min to eat, after which they were removed and the remaining brine shrimp were counted to calculate the proportion of brine shrimp eaten. Following feeding experiments, lateral images of live fish were taken, and fish were fixed and stained as described in the *Dose-dependent effects on development, growth, and craniofacial toxicity* section.

### *Data analysis*

All data except those from survival and geometric morphometric analyses are presented as the mean  $\pm$  standard error of the mean. Cumulative survival rates were determined using Kaplan-Meier survival analysis with a log-rank

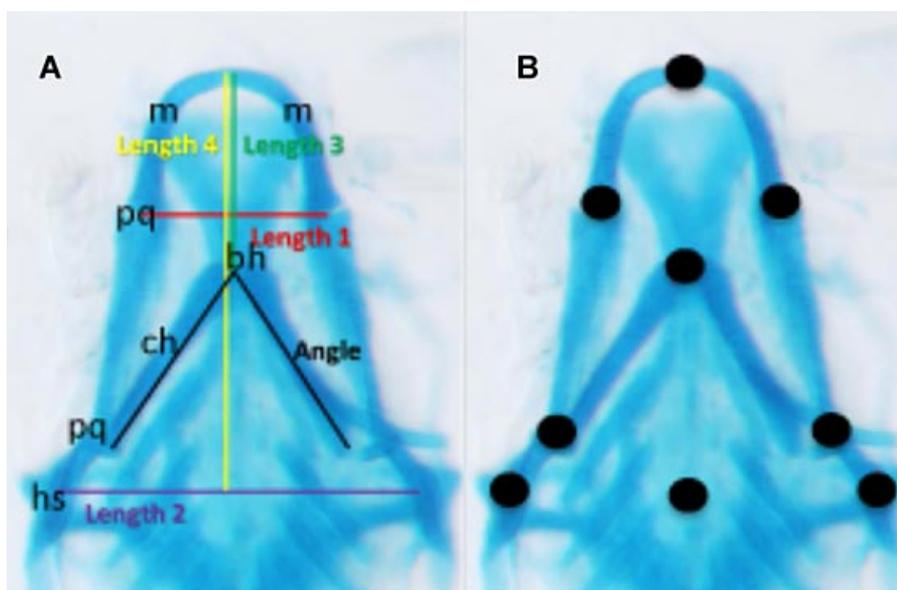


Figure 1. Ventral skeletal elements used to assess craniofacial development. (A) Lengths and angles measured for standard morphometrics. (B) Type 1 landmarks used for geometric morphometrics. Names, abbreviations, and description of the skeletal elements observed are provided in the text. ch = angle of the ceratohyal; m = Meckel cartilage; pq = palatoquadrate joint; hs = hyosymplectic joint; bh = basihyal.

significance test. Data were evaluated for homogeneity of variance (homoscedasticity, Levene's median test) and for normality prior to two-way analysis of variance (ANOVA) to determine treatment-related effects on growth or standard morphometrics or one-way ANOVA to evaluate feeding capability. Pairwise multiple comparisons were conducted using Tukey's post hoc test with significant differences identified at  $p < 0.05$ . Linear regression was performed to establish relationships between 1) craniofacial structures and proportion of brine shrimp captured, 2) total length and proportion of brine shrimp captured, and 3) total length and mortality.

For geometric morphometric analyses, the program rpsRelw, version 1.49, was used to generate relative warp scores for use in SYSTAT 8.0 to perform a multivariate ANOVA to test the effects of age, dose, and age  $\times$  dose on the overall shape of craniofacial structures [22]. We used tpsRegr, version 1.38, to test whether the variation in shape relates to TCDD concentration [23]. This test is basically a multivariate analysis of covariance, assuming that time is continuous, and allows us to examine the trajectories of shape for each dosage over time. Because time is not actually continuous—it is in 7-d, 14-d, and 21-d intervals—this test will be less sensitive to any pattern of shape change over continuous time. Finally, we used MorphoJ, version 1.05f, to examine symmetry [24]. A series of ANOVAs using procrustes distances were used to determine if there was significant asymmetry in any group of fish [25].

## RESULTS

### Impacts on survival and growth

Embryonic exposure to TCDD had no significant effect on hatching success and did not induce blue sac syndrome in 7-dpf zebrafish larvae, confirming that these concentrations are below levels that cause overt morphological malformations. Embryonic exposure to TCDD did not affect survival at 7 dpf, 14 dpf, or 21 dpf; however, exposure to  $\geq 50$  pg TCDD/mL resulted in a 13% to 23% decrease in survival beginning at 28 dpf ( $p < 0.05$ ,

Figure 2A). There was no change in survival for any treatment after 35 dpf.

Size was initially impaired by approximately 5% at 7 dpf following exposure to  $\geq 50$  pg TCDD/mL ( $p < 0.05$ ); however, there was no significant effect on total length at 14 dpf. At 21 dpf, size was highly variable. Although there appears to be a trend for a subtle decrease in total length for surviving larvae, it was not significantly affected (Figure 2B). At 21 dpf, total length was negatively correlated with survival ( $R^2 = 0.989$ ,  $p < 0.05$ ).

### Impacts on craniofacial morphology

Standard morphometrics revealed no significant differences in the size of craniofacial structures following exposure to TCDD until 21 dpf. At 21 dpf, the craniofacial structures are shorter and wider (Figure 3) in larvae exposed to  $\geq 50$  pg TCDD/mL during embryonic development. The overall length of the lower jaw was 5% shorter in larvae exposed to  $\geq 50$  pg TCDD/mL ( $p < 0.05$ , Figure 4A), and the angle of the ceratohyal was approximately 6 degrees wider in fish exposed to  $\geq 25$  pg TCDD/mL ( $p < 0.05$ , Figure 4B). While the size of the jaw was altered, a change in overall shape was not detected by geometric morphometric methods. There was a significant effect of age (Wilks' lambda,  $p = 0.000$ ) and age  $\times$  dose (Wilks' lambda,  $p = 0.002$ ) on the overall shape of craniofacial structures; however, there was no significant difference in shape change by dosage over time or by dosage independent of time (Wilks' lambda,  $p = 0.1375$ ). The data suggest that fish exposed to different concentrations of TCDD do not have a different shape. Control fish and those exposed to 25 pg or 50 pg TCDD/mL did not show significant asymmetry in craniofacial shape, but the procrustes ANOVA revealed significant asymmetry of craniofacial shape in the pooled (all ages) fish exposed to 100 pg TCDD/mL ( $p = 0.0172$ ).

### Impacts on feeding

Twenty-one-day-old larvae exposed to  $\geq 50$  pg TCDD/mL at 4 hpf to 6 hpf captured approximately 10% fewer brine shrimp than control larvae (Figure 5A). This impact on ability to capture

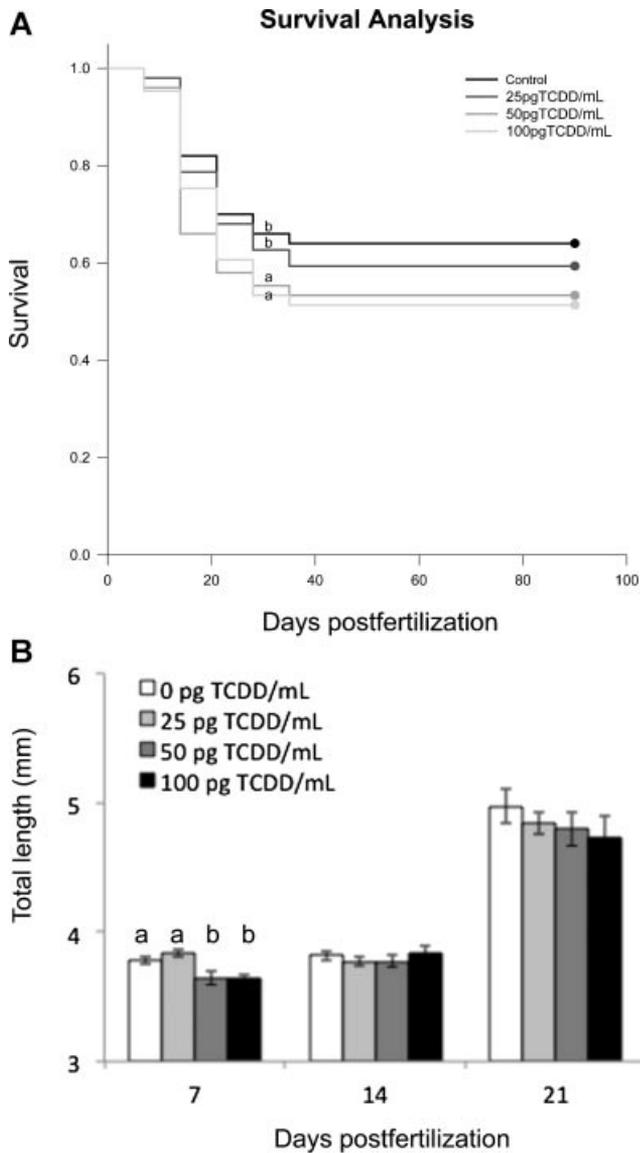


Figure 2. Survival rates and growth through 21 d postfertilization (dpf). (A) Survival through adulthood (90 dpf) following exposure to, 3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 1 h during embryonic development. (B) Growth as measured by total length during early larval stages. Letters denote significant differences ( $p < 0.05$ ).

prey correlates with overall size (Figure 5B,  $R^2 = 0.729$ ,  $p < 0.05$ ) and the reduced length of the lower jaw (Figure 5C,  $R^2 = 0.888$ ,  $p < 0.05$ ).

**DISCUSSION**

*Latent toxicity of TCDD in zebrafish*

In comparison to other fish species, zebrafish are typically considered to be quite resistant to TCDD [2]; however, the overall toxic response remains similar and leads to the development of blue sac syndrome, which is lethal [4,6,26]. Exposure to concentrations of TCDD at levels that do not cause blue sac syndrome can still translate to reductions in survival. Blue sac syndrome, the hallmark of developmental dioxin toxicity, was not seen in first-feeding zebrafish larvae following exposure to  $\leq 100$  pg TCDD/mL; however, cardiovascular function and craniofacial development were affected [13]. We used a combination of morphological and behavioral

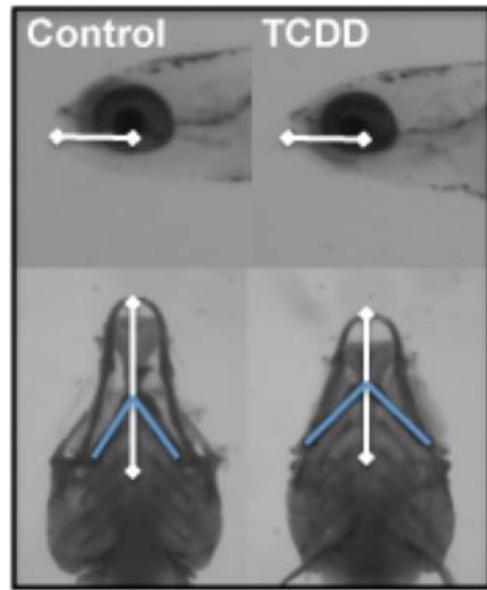


Figure 3. Impact of developmental exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on craniofacial structures. Representative micrographs of 21-d postfertilization larvae. Craniofacial structures were evaluated by gross morphological measurements (top panel) and by measuring specific craniofacial structures (lower panel). Impacted structures are depicted with lines and angles.

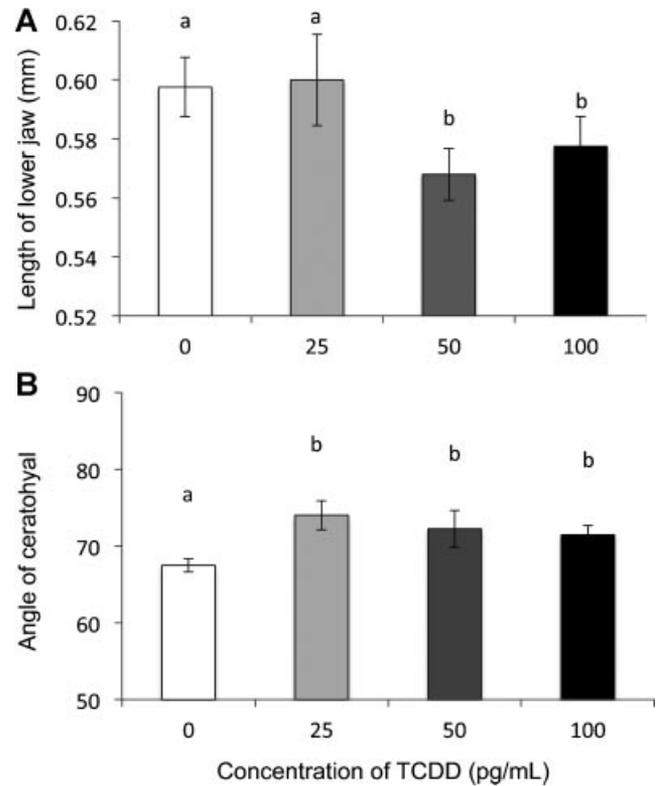


Figure 4. Quantification of craniofacial structures at 21 d postfertilization. (A) Length of the lower jaw as measured by distance between the basihyal and the junctions of the palatoquadrate, hyosymplectic, and interhyal (L4). (B) Angle of the ceratohyal in fish exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Letters denote significance ( $p < 0.05$ ).

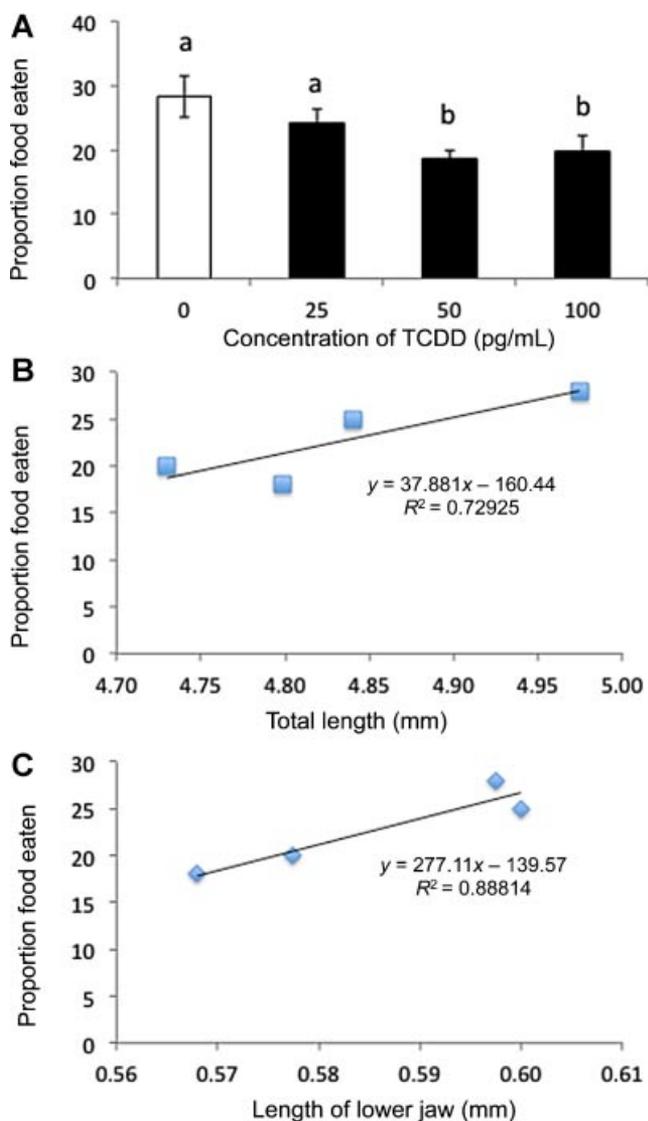


Figure 5. Impacts on ability to capture prey. (A) Proportion of prey captured by zebrafish exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) during embryonic development. Reduced prey capture was correlated with (B) growth (total length) and (C) length of the lower jaw. Letters denote significant differences ( $p < 0.05$ ).

assessments to evaluate larvae at older ages following exposure to TCDD only during the earliest stages of embryonic development. The present study further supports that the no-observed-effect concentration for impacting survival and craniofacial structure and function in zebrafish is  $\leq 25$  pg TCDD/mL and the lowest-observed-effect concentration is 50 pg TCDD/mL.

Embryonic exposure to environmentally relevant concentrations of TCDD has subtle impacts on growth and survival. In larvae exposed to  $\geq 50$  pg TCDD/mL, there was a 13% to 23% increase in mortality as they entered late metamorphic development, just prior to the onset of juvenile development. Survival in our fish exposed to 100 pg TCDD/mL was higher than reported in a recent study in which zebrafish embryos were exposed to TCDD at the same age and duration [14]; however, the 2 studies demonstrate similar survival of zebrafish larvae following exposure to  $\leq 50$  pg TCDD/mL. Since exposures were done in the same laboratory using the same stock solutions and exposure paradigm, it is likely that these differences in survival

and our lack of a dose-response in our 2 highest treatment groups reflect the use of nominal concentrations of TCDD and that this concentration is near the 50% lethal concentration or a threshold concentration for this age of development [13,14,27]. While survival is correlated with growth, we were not able to detect significant impacts on growth for the population, perhaps because growth is so variable during this period of development. A combination of impaired physiological conditions likely contributes to increased mortality during key transitions in larval life history, including impacts on feeding efficiency.

#### Feeding efficiency and craniofacial morphology

Interestingly, feeding capability is reduced following TCDD exposure even when overt malformations of craniofacial structures are not observed. While the overall sizes of the jaws of fish exposed to TCDD were smaller, effects were minimal and overall shape was not altered. Feeding capability was correlated with both total length and jaw length. Similarly, *Fundulus* larvae exposed to TCDD or PCB126 at levels that did not induce blue sac syndrome or substantial craniofacial malformations captured 20% to 40% fewer prey [16,17]. Neither study provided a detailed morphometric analysis of craniofacial structures. While a 10% reduction in ability of zebrafish to capture prey is not as substantial, it is within the range shown for *Fundulus* and could still contribute to the increase in mortality that is seen just after this period (21–28 dpf). In zebrafish, this age marks the initiation of gonad differentiation and significant increases in growth [28]; therefore, our findings suggest that even subtle impacts on the ability to capture prey in conjunction with previously described reductions in cardiac output and inability to inflate the swim bladder [13] can contribute to increased mortality at this critical period. The cause of observed decreases in the efficiency with which zebrafish larvae capture prey is not clear.

Our data suggest that there is a change in the size of craniofacial structures but not overall shape relative to TCDD exposure levels. Exposure to environmentally relevant concentrations of TCDD during the earliest stages of development caused a slight reduction in the length of the ventral jaw (distance between Meckel cartilage and palatoquadrate joints) and an altered angle of the ceratohyal in later stages of development, although the overall shape of craniofacial structures was not significantly altered. King-Heiden et al. [13] and Baker et al. [14] report that these subtle impacts on craniofacial structures and jaw size can persist through adulthood. There is a great deal of individual variation in jaw shape in these larval fish, perhaps resulting from individual variations in growth. The result of significant craniofacial asymmetry in the 100-pg TCDD/mL treatment group would likely contribute negatively to prey capture.

We hypothesize that these subtle changes in jaw morphology impair suction feeding and therefore prey-capture efficiency. Larvae rely on the hyoid and associated branchial arches and ceratohyal for suction feeding [18,29]. In larval zebrafish, the cartilaginous lower jaw is well formed and the ceratohyal (hyoid), interhyal, and associated branchial arches are the most pronounced part of craniofacial features associated with feeding [18,29]. A pronounced depression of the ceratohyal is essential to facilitate suction feeding [29,30]. However, more detailed video analyses would be required to determine whether the ability to detect prey or initiations in feeding strikes/efficiency of feeding strikes accounts for our observed decreased prey-capture efficiency in zebrafish or whether other factors are involved.

Ability of larvae to capture prey can depend on multiple factors such as size and swimming performance. In the present study, the ability of zebrafish to capture prey was also correlated with total length, suggesting that a reduction in growth may also be a factor in the decreased prey-capture ability of zebrafish following embryonic exposure to TCDD. The present findings are similar to data reported for *Fundulus*, which also show that larval feeding capability was negatively correlated with growth [16,17]. Limited data are available on the functional changes in feeding performance associated with allometric growth in larval fishes; however, Hernández [18] provides a detailed analysis of larval zebrafish feeding mechanics, indicating that larval feeding kinematics are greatly affected by total length (feeding capability increases with size). While it is not known whether the subtle differences in size (fractions of 1 mm) across our treatment groups is sufficient to be the leading cause of observed decreases in feeding capability of larval zebrafish exposed to TCDD, overall size is likely a factor. Alternatively, swim performance could be a factor. Marit and Weber [31] show that developmental exposure to sublethal concentrations of TCDD impairs swimming performance of adult zebrafish. While they did not evaluate swimming performance of larvae, inhibited swimming speed or endurance could be an additional explanation for our observed decreased prey-capture ability. Furthermore, we cannot rule out the possibility that subtle neurotoxicity, such as impaired vision as observed in rainbow trout larvae [15], reductions in overall neurons [32], or impacts on learning or brain development as seen in rodents [33,34], is not also involved.

The fact that feeding behaviors of larval zebrafish are affected following exposure to concentrations of environmental contaminants that do not elicit gross morphological malformations or mortality is not surprising. An animal's behavior is an integrated physiological response to its environment, and many behaviors are altered by toxicants at concentrations much lower than traditional toxicity measures such as morphology or mortality [35–37]. Complex behaviors such as feeding can be a sensitive indicator of sublethal stress and can be used as predictors of population-level effects [35,37]. Further, studies that bridge the gap between simple locomotor and avoidance behaviors (e.g., C-start response, swimming velocities, efficiency of feeding strikes) with more ecologically relevant scenarios (e.g., predation studies, competitive foraging studies) are key for predicting ecotoxicological risk [37]. The present study highlights the need for comparative structural, neurobehavioral, and physiological assessments to better understand the impacts and potential risks of toxicants such as TCDD that have multiple organ targets.

#### *Relevance to wild fish populations*

Low larval mortality rates are key for sustaining populations of wild fishes. Even minor alterations in growth or mortality rates of larvae can result in an order of magnitude or greater difference in annual recruitment [9,38,39], and among other factors, foraging is critical for survival [9]. Small larval fish are at greater risk for starvation, and feeding kinematic studies assume that there is a selective advantage for efficient feeding. Survival to reproductive age is highly dependent upon efficient feeding during early ontogenetic stages [10,40]. The present study demonstrates that early exposure to environmentally relevant concentrations of TCDD causes subtle craniofacial malformations that likely contribute to a reduced efficiency of zebrafish larvae ability to capture prey. This, in conjunction with more pronounced impacts on cardiovascular functions and ability to

inflate the swim bladder [13,14], likely contributes to observed subsequent increases in mortality that are sufficient to impact recruitment success.

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