The Effects of Diatom-derived Polyunsaturated Aldehydes on Embryonic and Larval Surf Smelt (*Hypomesus pretiosus*) Fitness

By

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Master's Thesis

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Jiho Kim

November 30, 2024

The Effects of Diatom-derived Polyunsaturated Aldehydes on Embryonic and Larval Surf Smelt (*Hypomesus pretiosus*) Fitness

A Thesis
Presented to
The Faculty of
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Master of Science

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Abstract

Polyunsaturated aldehydes (PUAs) are secondary oxylipins produced by some diatoms. PUAs are produced at a greater rate when diatom cells are damaged, suggesting that they may act as chemical grazing deterrents. Past studies showed the deleterious effects of particulate PUAs on diatom consumers like copepods and marine invertebrates. However, to date, very few studies have explored the potential for diatom-derived PUAs to affect marine vertebrates, such as forage fishes. Forage fishes are a foundational functional group in marine ecosystems whose early life history stages are often sympatric with diatoms due to their nearshore spawning behavior and planktivorous diet. In this study, I addressed the question of whether PUAs detrimentally affect a common Salish Sea forage fish, the surf smelt (*Hypomesus pretiosus*; Girard 1854). The project focused on determining whether PUAs affect the development and physiology of surf smelt embryos and larvae. This was done by measuring survival and hatch success rates, embryonic heart rates, usage of endogenous energy reserves, and morphological features at hatch. Higher concentrations of PUAs resulted in higher mortality and lower hatch success rates of embryonic surf smelt. Embryonic heart rates were equivalent among treatments when embryos were exposed to PUAs soon after fertilization, suggesting that surf smelt embryos can acclimate to PUAs if exposed during early development. However, higher concentrations of PUAs significantly lowered the heart rates of embryos that were exposed to PUAs days after fertilization. Exposure to PUAs diminished the consumption rate of endogenous energy reserves, and the overall size of surf smelt at hatch was reduced. Our results indicate that exposure to dissolved PUAs could impair the fitness of ecologically foundational forage fish early life history stages. Negative effects that manifest into low adult population sizes will have cascading effects on marine ecosystems.

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Table of Contents

List of Figures and Tables

Introduction

Diatoms

Diatoms are unicellular, photosynthetic eukaryotes that are ubiquitous in freshwater and marine environments (Malviya et al. 2016). They are the most diverse group of phytoplankton, comprising about 200,000 species (Armbrust 2009). Diatoms play a significant role in global biogeochemical cycles, including those of carbon, nitrogen, and biogenic silica (Benoiston et al. 2017, Malviya et al. 2016). They account for about 40% of ocean primary production and 20% of total primary production on Earth, generating as much oxygen as all terrestrial rainforests combined (Armbrust 2009, Harvey et al. 2019, Tréguer et al. 2018). The organic carbon produced by diatoms is consumed by higher trophic organisms. As the organic detritus matter sinks from the surface, it transports resources to regions outside the range of effective photosynthesis. The silica frustules encasing diatoms add ballast to the cells, thus allowing diatoms to contribute disproportionately to the biological pump (Agusti et al. 2015, Benoiston et al. 2017, Tréguer et al. 2018).

Diatoms are extraordinarily productive in nutrient-rich environments with sufficient light, such as well-mixed coastal and upwelling regions like the Salish Sea (Armbrust 2009, Benoiston et al. 2017, Harvey et al. 2019, Malviya et al. 2016). Diatoms grow rapidly, increasing their abundance exponentially and quickly producing extensive algal blooms when conditions become favorable (Armbrust 2009, Franzè et al. 2018, Tréguer et al. 2018). Given their high productivity and standing stock biomass, diatoms were historically considered optimal prey for a wide consortium of zooplankton predators, particularly copepods (Clarke 1939). Copepods dominate zooplankton communities and are a crucial link in transferring primary production to higher

trophic levels in marine food webs (Ban et al. 1997, Clarke 1939), and their diatom-packed fecal pellets contribute to the biological pump (Agusti et al. 2015, Tréguer et al. 2018).

Traditionally, copepod fecundity, or reproductive output, was thought to depend upon diatom biomass (Ban et al. 1997, Clarke 1939). However, this paradigm began to shift in the 1990s when studies found that despite the fact that diatoms support copepod somatic growth and development, they also suppress copepod reproductive success (Ban et al. 1997). Many studies have demonstrated that diatom-fed copepods experienced reductions in egg viability, lower hatching success, impaired embryogenesis, and an increase in the number of malformed copepod nauplii (Ianora et al. 1996, Ianora et al. 2004, Miralto et al. 1999, Poulet et al. 1994). These deleterious effects were shown to be diatom density-dependent; higher diatom concentrations caused greater inhibition of hatching over a shorter time of induction (Ban et al. 1997, Chaudron et al. 1996). Miralto et al. (1990) isolated the compounds responsible for the reductions in consumer fecundity from pelagic diatoms and identified them as polyunsaturated aldehydes (PUAs). Since the discovery that diatom-derived PUAs retard embryogenesis, other studies have explored the negative effects of PUAs on other marine invertebrates. Caldwell et al. (2002 & 2004) found that PUA exposure inhibited sperm motility and fertilization success in multiple benthic invertebrate species. Ruocco et al. (2019) showed that sea urchin embryos exhibited malformations and lower survival rates when exposed to either binary or ternary mixtures of dissolved PUAs. Additionally, mixtures of different PUA compounds expressed stronger effects on sea urchin larvae than individual PUAs (Ruocco et al. 2019, Van Donk et al. 2011).

Polyunsaturated Aldehydes

PUAs are oxylipins, bioactive lipids derived from lipoxidation of polyunsaturated fatty acids (PUFAs; Bartual et al. 2020, Fontana et al. 2007). The lipoxidation of PUA precursors occurs within seconds of algal cell damage, triggering activation of lipoxygenase enzymes in the algal cells for approximately 40 minutes (Fontana et al. 2007). PUAs exist in varying molecular forms and concentrations within diatoms, depending on diatom species and growth phases (Pezzolesi et al. 2017, Wichard et al. 2005) and inorganic nutrient concentrations (Pezzolesi et al. 2017, Ribalet et al. 2014). PUAs have been found in a large number of pelagic diatom species (Ianora et al. 2004, Wichard et al. 2005, Vidoudez et al. 2011). Although only a few studies have explored the production of PUAs by benthic diatoms (Ruocco et al. 2018, Pezzolesi et al. 2017), such species are thought to be widespread. Johnson (2023) surveyed common benthic diatoms from the Salish Sea and found that PUAs were produced in all eight species examined.

Various roles for diatom-derived PUAs have been suggested, including allelochemicals that impair the growth and performance of smaller phytoplankton species (Ianora & Miralto 2010), intraspecific infochemicals that induce diatom bloom termination (Ianora & Miralto 2010, Leflaive & Ten-Hage 2009, Van Donk et al. 2011), and pheromone signals to attract gametes during sexual reproduction (Ianora and Miralto 2010, Van Donk et al. 2011). Because PUAs are produced when diatom cells are damaged upon grazing, and there appears to be a consistent negative effect of PUAs on different grazer functional groups, it has been suggested that the fundamental role of PUA production is to act as inducible chemical defenses (Pohnert 2000, Van Donk et al. 2011). Inducible defenses are activated only after encounters with consumers and thus are energetically favorable in comparison to constitutive defenses. The inducible nature of PUA production allows diatoms to highly concentrate aldehydes over short periods (Pohnert et

al. 2002). Once formed after cell damage, PUAs accumulate in the cell membranes of organisms that ingest the algal cells or are dispersed in surrounding water (Bartual et al. 2020, Ribalet et al. 2014, Van Donk et al. 2011).

To date, investigations into the effects of PUAs on marine organisms have been largely limited to examining zooplankton and other invertebrates that both directly and indirectly consume diatoms. One notable exception was a study that explored the effects of dissolved PUAs on zebrafish (*Danio rerio*; Hamilton 1822), a freshwater model organism used extensively in developmental studies (Raymer 2023). Zebrafish embryos were exposed to three different concentrations of PUA mixtures, and zebrafish morphometrics, heart rate, and survival rate were monitored over time. Zebrafish fitness significantly decreased under higher PUA concentrations, suggesting that PUAs may affect vertebrates and other higher-order organisms that are sympatric with PUA-producing diatoms.

Forage Fishes

Aspects of forage fish life history indicate that many species are likely to come into close contact with PUA-producing diatoms and multiple life stages. Forage fishes are schooling fishes that play a critical role in temperate marine ecosystems and economies (Alder et al. 2008, Cury et al. 2000, Penttila 2007). Forage fishes are planktivorous, feeding on both phytoplankton and zooplankton, thus acting as a primary conduit of energy transfer from lower to upper trophic levels in marine food webs. They are primarily composed of small to intermediate-sized species (2.5 to 76 cm; Alder et al. 2008, Rountos 2016), and all of their life history stages serve as resources for a variety of marine predators, including seabirds, marine mammals, and larger fishes such as salmon (Alder et al. 2008, Cury et al. 2000, Penttila 2007). In addition to their

ecological importance, forage fishes support a 16-billion-dollar fishery, making up about 50% of global fishery economics (Alder et al. 2008, Greene et al. 2015, Pikitch et al. 2014).

The common forage fishes found in the North Pacific ecosystem, including the Salish Sea, are Pacific herring (*Clupea pallasii*; Valenciennes 1847), Pacific sand lance (*Ammodytes hexapterus*; Pallas 1814), and surf smelt (Bodtker et al. 2017, Greene et al. 2015, Penttila 2007, Therriault et al. 2009). The abundant aquatic vegetation, numerous shallow estuaries, and gravel beaches of the Salish Sea provide breeding habitats and nursery grounds for these species (Bodtker et al. 2017, Greene et al. 2015, Penttila 2007, Rice 2006).

Of the three most common forage fishes in the Salish Sea, comparatively little is known about the ecology and population health of surf smelt (Penttila 2007, Russell et al. 2022). Surf smelt are obligate beach spawners and mate throughout the year in the Salish Sea, with peak spawning occurring in the summer months (Bodtker et al. 2017, Penttila 2007, Rice 2006). Females deposit their eggs on sand-gravel substrates that range between 1 and 7 mm in diameter at tidal heights seven feet (2.13 m) or above mean low low water (Middaugh et al. 1987, Quinn et al. 2012, Russell et al. 2022). Males follow females and broadcast their milt to fertilize eggs. Once fertilized, surf smelt eggs attach to gravels using their zona radiata membrane (Middaugh et al. 1987). Developing embryos are periodically submerged and exposed by tides for approximately 10-14 days before hatching (Boldt et al. 2018, Penttila 2007, Rice 2006). During surf smelt embryonic and early larval stages, they utilize maternally provided endogenous energy stored as yolk and oil globules (Boldt et al. 2018, Russell et al. 2022, Yúfera & Darias 2007). After hatching, surf smelt larvae continue to rely upon these endogenous energy reserves for several days post-hatch to continue embryogenesis, including the development of functional mouth parts and a digestive system. During this critical period, surf smelt transition from

endogenous to planktivorous exogenous feeding (Boldt et al. 2018, Penttila 2007, Yúfera & Darias 2007).

Surf smelt spawning ecology makes them sympatric with nearshore aquatic algae, including the rich and abundant consortium of benthic and pelagic diatoms. As such, surf smelt eggs and embryos are likely exposed to diatom-derived dissolved PUAs. Further, as planktivores, all free-feeding life stages of surf smelt also directly and indirectly ingest PUA-producing diatoms. If PUAs are toxic to surf smelt, as found in zebrafish (Raymer 2023), then these chemicals may have an important effect on surf smelt abundance.

As a next step in addressing the question of whether PUAs detrimentally affect higher-order marine organisms, I investigated how PUAs affect surf smelt embryonic development and physiology. Given their ecological and economic importance, any negative impact on their population demographics will cause cascading effects on marine ecosystems and coastal economies (Cury et al. 2000, Penttila 2007, Pikitch et al. 2014, Therriault et al. 2009). Forage fishes also act as indicators of marine system health, as they require clean, cold water, and unaltered shorelines for high fecundity and population abundance (Bodtker et al. 2017, Greene et al. 2015, Russell et al. 2022). This work was intended to support marine conservation and protection efforts by determining whether PUAs impair the early development of this ecologically important coastal species.

Methods

Overview

To explore the effects of diatom-derived PUAs on the developmental and physiological rates of early life history stages of surf smelt, multiple experiments were performed with surf smelt embryos and larvae. Gametes were stripped from wild surf smelt during spawning and fertilized *in vitro*, and surf smelt embryos and larvae were reared in the laboratory. Experiments were set to test the following questions and hypotheses:

Questions and Hypotheses:

1. Do dissolved PUAs decrease the survival of embryonic and larval surf smelt?

   H_0: Exposure to dissolved PUAs does not decrease the survival of embryos or larvae.
   H_A: Exposure to dissolved PUAs decreases the survival of embryos or larvae.

2. Do dissolved PUAs decrease the hatching success of embryonic surf smelt?

   H_0: Exposure of surf smelt embryos to dissolved PUAs does not decrease the hatching success.
   H_A: Exposure of surf smelt embryos to dissolved PUAs decreases the hatching success.

3. Do dissolved PUAs elevate the heart rate of embryonic surf smelt?

   H_0: Exposure to dissolved PUAs does not elevate the heart rate of surf smelt embryos.
   H_A: Exposure to dissolved PUAs elevates the heart rate of surf smelt embryos.

4. Do surf smelt consume endogenous energy reserves faster when exposed to dissolved PUAs?

   H_0: Embryonic surf smelt do not consume endogenous energy reserves faster when exposed to dissolved PUAs.
   H_A: Embryonic surf smelt consume endogenous energy reserves faster when exposed to dissolved PUAs.

5. Do dissolved PUAs decrease the size of surf smelt at hatch?

   H_0: Exposure of embryonic surf smelt to dissolved PUAs does not decrease the size at hatch.
   H_A: Exposure of embryonic surf smelt to dissolved PUAs decreases the size at hatch.

Sample Collection and Preparation

Surf smelt gametes were collected from Utsalady Bay, Camano Island, WA (48° 15' 12.676"N, 122° 29' 52.915"W) in August of 2023 on two occasions (23 days apart) and manually spawned using the methods of Tagal (2022). Gravid adults were caught in fish traps during spawning events. Immediately upon capture, eggs were harvested from females by gently hand-stripping their abdomen and collecting eggs in 50mL sterile polypropylene centrifuge tubes. To this, males were stripped in the same fashion, and several drops of sperm were added to the centrifuge tubes containing eggs. Eggs and sperm were gently mixed with ~50mL seawater to facilitate fertilization, stored in a cooler on ice, immediately transferred to the Shannon Point Marine Center in Anacortes, WA, and stored in a refrigerator overnight. Gametes were harvested from four females and four males in the first collection and ten females and six males in the second collection.

About 15 hours post-fertilization (hpf), the embryos were rinsed with autoclaved filtered seawater (AFSW) to remove any debris. Embryos were then parsed out into multiple Petri dishes and fully submerged in AFSW for 24 hours. The following day, living embryos were again parsed out into Petri dishes, with 100 embryos in each dish, to start experiments.

Treatments

PUA mixtures show synergistic effects and cause greater negative effects when combined than individual PUAs (Ruocco et al. 2019). Moreover, they typically exist in mixtures within species and populations in nature (Johnson et al. 2024). We therefore examined the effects of PUA mixtures over a range of concentrations. Mixtures of the commonly naturally occurring trans, trans-2,4-Heptadienal (Lot No. AWNA1-LQ from TCI), 2E,4E-Octa-2,4-dienal (Lot No. A157811-005 from Ambeed), and trans, trans-2,4-Decadienal (Lot No. PCMQM-CY from TCI)

were used to make the PUA treatments. Hereinafter, these PUAs will be referred to as heptadienal, octadienal, and decadienal, respectively. The concentrations were chosen based on Raymer (2023), and they ranged from 1X to 10X (Table 1). To make PUAs miscible in seawater, PUAs were dissolved in methanol. Methanol concentration was no greater than 1%. Seawater (SW) and seawater mixed with 1% methanol solution (M) were used to control for PUA and methanol effects on surf smelt.

Experimental Design

The first experiment (Experiment 1) was conducted using 1X, 5X, and 10X concentrations of PUA mixtures and SW and SW+M control (Table 2). Each treatment had four replicate Petri dishes and as mentioned, ~100 embryos in each dish. These embryos were exposed to, and reared in, treatment water from 3 dpf until the termination of the experiment (termed chronically). All embryos from the two highest PUA treatment concentrations died before measurements were made. This led to conducting a second experiment (Experiment 2), where PUA concentrations were reduced to minimize mortality. Experiment 2 PUA concentrations were 1X, 2X, 3X, 4X, and 5X, and the two controls (Table 1). Each treatment in Experiment 2 had three replicate Petri dishes with ~100 embryos in each. As in Experiment 1, the embryos were chronically exposed and reared in treatment water from 3 dpf until the termination of the experiment. One replicate from treatment 1X was lost due to experimenter error. The developmental and physiological metrics measured in Experiments 1 and 2 are in Table 2.

Two additional experiments (Exps. 3 & 4) were conducted with embryos and larvae that had only been reared in AFSW and not exposed to PUAs until later in development, prior to experimentation (termed naïve embryos and larvae; Table 2). In Experiment 3, once all naïve

embryos developed beating hearts after nine days post-fertilization (dpf), ten haphazardly selected embryos from each of the three replicate Petri dishes were acutely exposed to 1X, 3X, 5X, and 10X PUA concentrations, and the two controls for five hours. After the five-hour incubation period, heartbeats were monitored. In Experiment 4, naïve larvae that had just hatched were exposed to 1X, 5X, and 10X PUA concentrations and the two controls, and larval survival was monitored. All treatment levels had three replicates with 25 hatched larvae in each. Experiment 4 lasted until all larvae in all treatments expired. For the entire duration of the experiment, treatment water was changed daily.

Experimental Setup and Daily Routine

All experimental Petri dishes containing embryos or larvae were placed in an environmental incubator set at 15.5ºC, the average ambient seawater temperature of the summer spawning season (Rice 2006), under a 14:10 h light:dark cycle. To mimic tidal exchanges that embryos are exposed to in nature, fresh treatment/control water was added at the same time each day, and embryos were inundated for six hours to replicate the daily submersion in the high intertidal zone. Water was then removed from the dishes by pipetting, leaving just enough water to prevent the desiccation of embryos. If applicable, measurements were made as the water was removed, and the dishes were placed back in the incubator when done. The cycle was repeated for the respective duration of the experiments (Table 2).

Data Collection and Statistical Analysis

Before conducting any statistical analysis, each data set for all measurements was assessed for normality and homoscedasticity. A Shapiro-Wilk normality test was performed using the ‘shapiro.test’ function from the ‘stats’ R package (R Core Team 2023). Homoscedasticity was assessed using the ‘leveneTest’ function of Levene’s test for homogeneity

of variance from the 'car' package in R (Fox & Weisberg 2019). If data did not violate any assumptions, a linear model or a linear mixed model (LMM) was used to model, predict, and compare the results across treatments and controls. A linear model was created by generalized least squares (GLS) using 'gls' function, and a LMM was produced by 'lme' function both from the 'nlme' R package (Pinheiro et al. 2024). If data violated the assumption of normality, a generalized linear mixed model (GLMM) was chosen over LMM to represent the data using the 'glmmTMB' function from the 'glmmTMB' R package (Mollie et al. 2017). If the assumption of homoscedasticity was violated, either the variances were weighted, or the dispersion of the residuals was allowed to vary based on the factor that drove the heteroscedasticity the most.

Unless noted, all models included treatment and time as fixed factors. Models were produced from the simplest to most complex, including Petri dish and individuals as random factors, and Akaike Information Criterion (AIC) values were used to select the most parsimonious model with the best fit. Excluding the survival and hatch success rate analysis, the results across treatments and controls were compared through pairwise comparison of marginal means using the 'emmeans' function from the 'emmeans' R package (Lenth 2024).

Embryo and Larval Survival + Hatch Success Rate

To explore the effects of PUA exposure on the survival of embryonic surf smelt, embryos were exposed to PUA mixtures 24 hpf until 100% mortality was reached, or until the experiment was terminated due to unhatched embryos surviving, but not hatching, well past natural predicted hatching times based on incubation temperature. Survival data was collected by counting the number of dead embryos (Exps. 1 & 2) or larvae (Exp. 4) in each Petri dish. Before placing the dishes back in the incubator, dead embryos (distinguished by their cloudy and opaque color) were counted and removed from the dishes. In addition, any time hatched larvae were observed,

the number of hatches was recorded, and hatched larvae were removed from the dishes. Removed larvae were preserved in buffered formalin for morphometric and endogenous energy reserve measurements.

During the courses of Experiments 1 and 2, embryos experienced one of the four events: death, hatch, death after hatch within 24 hours, or developmental arrest (not hatching nor dying). The probabilities of each event happening are not independent of one another as one event may preclude the other from occurring, making them competing events (Wolbers et al. 2014). To account for the presence of these competing risks, the survival and hatch success rates were analyzed together. Because the question of interest was whether a surf smelt embryo successfully matured into the developmental stage of hatching under exposure to PUAs, the larvae that died within 24 hours of hatching were only counted towards the hatching event, and the embryos that experienced developmental arrests were counted as censored individuals in the "time-to-event" analysis.

To investigate whether survival and hatch success differ across a range of PUA concentrations in the presence of competing risks, the cumulative incidence function (CIF) was used. The CIF is a non-traditional type of survival or time-to-event analysis that estimates the occurrence of an event of interest while accommodating competing risks (Austin et al. 2016). The function is defined as:

$$CIF_k(t) = \Pr(T \leq t, D = k)$$

where:

Pr = probability

T = time from baseline time until the occurrence of the event of interest

t = specific point in time at which the instantaneous hazard is being calculated

D = type of event that occurred

The CIF at time t for the event k denotes the probability of event k occurring anytime between baseline T and time t and before a different event occurs (Austin et al. 2016). In the presence of competing risks, one of the two models for regression is used along with the CIF: the cause-specific and the subdistribution hazard model or the Fine-Gray model as introduced by Fine and Gray (Austin & Fine 2017, Gray 1988). The hazard function describes the instantaneous risk of the event of interest in subjects. The main difference between the two models is that the cause-specific hazard function calculates the risk in subjects who are event-free, while the subdistribution hazard function includes those who are event-free as well as those who have experienced a competing event (Austin et al. 2016, Wolbers et al. 2014). The subdistribution hazard function is as follows:

$$\lambda_k^{sd}(t) = \lim_{\Delta t \to 0} \frac{\Pr\left(t \leq T < t + \Delta t, D = k \middle| T \geq t \cup (T < t \cap K \neq k)\right)}{\Delta t}$$

where:

Δt = small time increment

$t + \Delta t$ = time slightly after t

In this study, a death event can preclude the hatching from occurring, and a hatching event can alter the probability of embryonic mortality. Thus, the CIF was used with the Fine-Gray model of competing risk analysis, which has a one-to-one relationship with CIF for interpretation (Austin and Fine 2017). The model was created using 'crr' function from the 'tidycmprsk' R package (Sjoberg 2024) and included treatment and dish as factors and time and event as dependent variables. The CIF plot was generated using the 'cuminc' function from the same R package.

For Experiment 4, where survival of hatched larvae exposed to a range of PUAs was monitored without any competing risk events, Cox proportional hazard regression, or Cox regression, was used to assess the effects of PUAs on newly hatched larvae. Cox regression analysis is based on a hazard function that accounts for the effects of multiple covariates and yields a hazard ratio (HR). The HR can be interpreted as a relative risk of the event of interest occurring using the following equation (Schober & Vetter 2018, Van Dijk et al. 2008):

$$h(t) = h_0(t) * exp^{\Sigma \beta_k x_k}$$

where:

$h_0(t)$ = baseline hazard at time t

β_k = regression coefficient(s)

x_k = covariate(s)

Cox regression is a semiparametric analysis because there isn't a distribution assumption. Instead, it has a proportionality assumption where the effects of covariates are assumed to be constant and additive over time (Abd ElHafeez et al. 2021, Schober & Vetter 2018). The HR is used to model and create the survival curve instead of using survival probabilities, where an HR > 1 is associated with an increased risk of event occurrence, and an HR < 1 is associated with a decreased risk of event occurrence (Schober & Vetter 2018, Van Dijk et al. 2008).

Using the hazard function with PUA concentration and time as the fixed factors and Petri dish replicate as the random factor, the HR for the entire duration of the experiment was calculated and modeled. Cox regression model was produced using 'coxme' function from the 'coxme' R package (Therneau 2024) which provides the HR along with 95% confidence intervals and p-values. The survival curve was created using 'ggadjustedcurves' function from the 'survminer' package in R (Kassambara et al. 2021).

Heart Rate

To determine whether PUAs act as a stressor that elevates surf smelt metabolism, embryonic heart rates were compared across PUA treatments. The 10 haphazardly chosen embryos were videoed for 10 seconds under PUA exposure. Videos were taken using an Olympus SZ-CTV dissecting microscope mounted to a FLIR Blackfly USB3 Vision camera running the Micro-Manager 2.0.0 program. Because chorions are translucent, it is possible to visualize heart contractions. Heartbeats from each embryo in all PUA treatments were recorded over the 10-second videos. The recorded heartbeats were multiplied by 6 to get heart rates in beats per minute (bpm).

GLMMs were used to predict the heart rates of surf smelt embryos exposed to different PUA treatments in all three experiments (Table 2). The models were produced with the assumption of having a negative binomial distribution family because the variances were larger than the means in all three experiments, exhibiting data overdispersion. The models included treatment as the fixed factor and the dish as the random factor to account for the variance within treatments. For Experiments 1 and 2, the variances were weighted in the models to further address data heteroscedasticity.

Endogenous Energy Reserve Consumption

To test whether PUAs affect the metabolism of surf smelt embryos and thus lead to faster consumption of endogenous energy reserves, yolk and oil globule areas were measured from each larva at hatch. A lateral view image of each larva was taken using an Olympus SZ-CTV dissecting microscope networked to a FLIR Blackfly USB3 vision camera. The areas of yolk and oil globules were determined by manually tracing the yolk and oil globule using the FIJI Image J imaging processing package at a magnification of 15X (Figure 1).

Prior to statistical analysis, the data set was trimmed to only include up to 14 dpf in both Experiments 1 and 2. According to Penttila (2007) and Rice (2006), the typical incubation time is approximately two weeks for surf smelt before they hatch during the summer spawning. In addition, in both experiments, the total number of hatches peaked at 13 dpf, where the number of hatches dropped 89.5% in Experiment 1 and 73.5% in Experiment 2 from 13 to 14 dpf (Table 3).

An LMM including Petri dish as a random factor was used to model yolk usage of embryos in Experiment 1. Because the samples became opaque before oil globule measurements were made, the oil globule data was lost in Experiment 1. In Experiment 2, a linear model without a random factor produced the best fit to model the yolk usage of embryos. A GLMM with dish as a random factor and allowing dispersion of the residuals to vary for each day was used to model the oil globule measurements in Experiment 2.

Morphometrics

The reallocation of endogenous energy to metabolism as a stress response may negatively affect early life development, which may be apparent in larval size at hatch. To investigate this hypothesis, three different morphological features were measured from the larvae at hatch: standard length (SL), from the tip of the snout to the posterior end of the caudal peduncle, deepest body depth (BD1), and body depth at the anus (BD2) (Figure 2). The image processing protocol was the same as described for the yolk and oil globule measurements.

As in endogenous energy reserve measurements, only measurements up to 14 dpf were included in the analysis. For Experiment 1, SL measurements were modeled using a GLMM to compensate for the violation of the assumption of normality, with Petri dish as a random factor and variances weighted. BD1 data did not violate any assumptions; hence, an LMM with Petri dish as a random effect and residual variance modeled to vary according to each day was used.

Because BD2 data did not meet the assumption of normality, a GLMM with individual as a random effect was used. All three morphological measurements taken from larvae in Experiment 2 violated the assumption of normality. SL was modeled with a GLMM with Petri dish as a random effect and variance weighted. BD1 was also modeled with a GLMM and included individual as a random effect while allowing the residual dispersion to vary based on individuals. Lastly, BD2 was compared using a GLMM with Petri dish as a random effect.

Sensory Perception

The development of sight and hearing are critical to survival. The size of the eye and otic capsule in larvae from Experiment 2 were measured (Figure 3). To visualize these features, preserved larvae previously measured from Experiment 2 were cleared and stained following Summers (2015). Eye and otic capsule area in the cleared and stained larvae were imaged and processed using the imaging process described above for yolk and oil globule area. From each larva, the area of a single eye and otic capsule were taken from either side of the fish.

Both eye and otic capsule data sets expressed overdispersion. To account for this, the Gamma distribution family was used with GLMMs. The model for eye measurement included Petri dish as a random effect while allowing the residual variance to vary with treatments, while the model for otic capsule measurement included individual as a random effect while allowing each day to have different levels of variability.

Results

Embryonic and Larval Survival

In Experiment 1, exposure to PUAs significantly reduced the survival of embryonic and larval surf smelt. Embryos in SW, SW+M, and the 1X treatment survived until 14 dpf, while embryos exposed to 5X and 10X treatments expired by 6 dpf (Figure 4). Embryos in the SW+M and 1X treatments had a higher likelihood of death compared to the SW control, with an HR = 1.33 ($p = 0.002$; Table 4). Embryos in 5X had an HR of 7.03 ($p < 0.001$), while 10X had an HR of 12.7 ($p < 0.001$) compared to the SW control. Embryos in 5X had an HR of 5.27 ($p < 0.001$) relative to 1X treatment (Table S4), and 10X had an HR of 1.81 ($p < 0.001$) relative to 5X treatment (Table S5), suggesting a PUA concentration-dependent effect on embryonic surf smelt mortality.

Surf smelt embryos in Experiment 2 exhibited a similar pattern where higher PUA concentrations resulted in lower survival (Figure 5). Embryos in SW+M control had a similar increase in risk of death compared to SW (HR = 1.10, $p = 0.35$). Individuals in 1X – 3X PUA treatments had HRs greater than 1 relative to SW. However, the pattern of survival was not stepwise with PUA concentration. The 1X treatment had HR = 2.06 ($p < 0.001$), and the 2X treatment experienced HR = 1.49 ($p < 0.001$). The 3X treatment showed an HR of 1.09, but this increase in likelihood of death was not significantly different from the SW control ($p = 0.40$). The 4X and 5X treatments had similar effects on embryonic survival ($p = 0.60$; Table S11), where 4X had an HR of 3.16 and 5X an HR of 3.55 ($p < 0.001$; Table 5). Unlike in Experiment 1, embryos in 5X PUA treatment did not completely expire until the termination of the experiment after 21 dpf.

Naïve larvae in Experiment 4 experienced greater mortality under higher PUA concentrations (Figure 6). Larvae in the two controls and 1X treatment survived until 6 dph, while larvae in the two highest PUA treatments survived only for 2 dph. Larvae in 5X treatment showed an HR of 150.97 ($p < 0.001$), and 10X showed an HR of 179.67 ($p < 0.001$) relative to SW control. SW+M and 1X treatments showed HRs similar to the SW control (Table 6).

Hatch Success Rate

In Experiments 1 and 2, embryos in SW and SW+M began hatching two and one day before the embryos in high PUA treatments, respectively. The total number of hatches peaked at 13 dpf in all controls and PUA treatments in both experiments (Table 3). In Experiment 1, hatch success rates in the SW+M (HR = 0.45) and 1X treatments (HR = 0.57) were similar, showing ~50% lower hatching than the SW control ($p < 0.001$; Figure 4; Table 7). There was 100% embryonic mortality by 6 dpf in 5X and 10X PUA treatments, resulting in hatch success rates of zero for these two high PUA concentrations (HR = 0.00, $p < 0.001$).

Unlike in Experiment 1, embryos in the SW+M control in Experiment 2 hatched as successfully as embryos in the SW control (HR = 0.87, $p = 0.31$). Except for the embryos in the 1X treatments, where the HR was 0.37 ($p < 0.001$) for hatching, embryos from the 2X – 5X treatments demonstrated a stepwise decrease in hatching HR with increasing PUA concentration (Figure 5; Table 8). However, these HRs were insignificant from each other, and only on average for the 1X – 3X treatments (Table S22 – S24). The 4X and 5X were significantly lower than the 1X – 3X treatments, but insignificant from each other (Table S25 & S26). Because few embryos in the 5X treatment in Experiment 2 survived until the end of the experiment, the HR for hatching was 0.08, representing a 92% decrease in hatch relative to the SW control ($p < 0.001$).

Heart Rate

Consistent exposure to PUAs from the early developmental stages did not affect heart rates in surf smelt embryos (Exps. 1 & 2; Figure 7 & 8; Table 9 & 10). In contrast, exposure of naïve embryos to the highest PUA concentrations significantly decreased heart rates (Exp. 3; Figure 9). Embryos in SW+M and 1X and 3X PUA treatments expressed heart rates that weren't significantly different from SW. However, average embryo heart rate in the 5X treatment dropped by 74.3 bpm (42% decrease) ($p < 0.001$), and average embryo heart rate in 10X dropped by 132.7 bpm (77% decrease) ($p < 0.001$), compared to the SW control (Table 11 & S32). The reduction in heart rate for embryos in the 10X treatment was significantly lower than for embryos in the 5X treatment ($p = 0.0003$; Table S31). In addition, the average heart rates of naïve embryos exposed to PUAs were higher than those that were chronically exposed to PUAs (Table S28, S30, & S32).

Endogenous Energy Reserve Consumption

As would be expected with embryo development, yolk and oil globule sizes decreased over time in surf smelt embryos in all treatments and controls ($p < 0.001$). However, PUA exposure slowed the consumption rate of these endogenous energy reserves compared to the controls (Figure 10 & 11). The yolk sizes in embryos from the 1X – 5X treatments were larger than those of the controls but the average yolk sizes were similar across PUA treatments ($p > 0.05$; Table S35). Oil globule size diminished over time in all treatments and controls ($p < 0.001$). The rate at which oil globule size diminished, however, was in general slower with increasing PUA concentration. The exception to this was from embryos in 5X, which produced a smaller globule than the 4X treatment (Figure 12; Table 14 & S38).

Morphometrics

The standard length of surf smelt at hatch was reduced under PUA exposure in Experiments 1 & 2. Hatch time had a significant effect on larval SL, with later hatching larvae showing longer SL ($p < 0.001$). In all PUA treatments larvae expressed SLs that were shorter than the controls ($p < 0.05$; Figure 13 & 14; Table 15 & 16). A concentration-dependent effect on SL was not observed, as SLs of larvae were similar across PUA treatments (Table S41).

In Experiments 1 and 2, BD1 was, on average, deeper in all PUA treatments in comparison to controls ($P < 0.05$; Figure 15 & 16; Table 17 & 18). However, the high within-treatment variability in BD1 sizes made it difficult to discern statistical differences across PUA treatments (Table S45 & S46). In both experiments, BD2 increased with time in all PUA treatments and controls ($p < 0.001$). Other than a time effect, there was no effect of PUAs on BD2 (Figures 17 & 18; Tables 19 & 20).

Sensory Perception

The size of surf smelt eyes increased over time in all treatments and controls ($p < 0.001$). Higher PUA concentrations reduced the size of embryo eyes, with significantly smaller eyes being observed in the 2X – 5X treatment groups compared to controls ($p < 0.001$; Figure 19; Table 21 & S52). However, eye sizes were similar in the 2X – 5X PUA treatments (Table S51). Otic capsules increased in size with embryo age ($p < 0.001$), but no PUA effect was observed (Figure 20; Table 22).

Discussion

Surf smelt are one of the key forage fishes in the Pacific Northwest that are sympatric with PUA-producing diatoms with known toxicity to zooplankton and invertebrates. To date, it remains unclear whether PUAs are toxic to higher trophic organisms. This study was conducted to fill that knowledge gap. I found that PUAs deleteriously affect several aspects early surf smelt development, typically in a concentration-dependent manner. This comprises the first study exploring the effect of mixtures of dissolved PUAs on a marine vertebrate.

Hatch Success Rate

PUAs significantly reduced surf smelt hatch success in a concentration-dependent manner. Embryos in controls began hatching two days earlier than embryos in PUA treatments. Additionally, many PUA-treated embryos continued to hatch well past typical embryo incubation times at the experimental temperatures used here (Table S1; Penttila 2007, Rice 2006). My data suggests that PUAs act to disrupt normal surf smelt incubation times. Fish eggshell is comprised of a protein barrier called the chorion that varies in pore size and thickness across fish species (Cherr et al. 2017). This barrier is broken by chorionase, an enzyme that disintegrates the chorion and allows hatching. Several studies have found that copper and cadmium inhibit chorionase activity and, therefore, impair the hatchability of fishes. Norrgren and Degerman (1993) showed that the hatching success of Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) was lower in acidic water due to high metal concentration than in limed water (restored pH). The interior part of the chorions of embryos in acidic water were intact, whereas those in limed water were dissolved, suggesting low activity of chorionase in acidic conditions (Norrgren & Degerman 1993). Chorionase is produced in hatching glands that embryos develop (Cherr et al. 2017, Jezierska et al. 2009, Norrgren & Degerman 1993). Any impediment to the development

of the hatching glands may, therefore, interrupt the chorionase activity. When exposed to zinc or copper, common carp (*Cyprinus carpio*) developed smaller hatching glands, resulting in low chorionase activity. Brook trout (*Salvelinus fontinalis*) and zebrafish (*Danio rerio*) experienced delayed hatching when exposed to aluminum, copper and nickel (Jezierska et al. 2009). Given that many embryos in the PUA treatments were seemingly developed yet incapable of hatching, it suggests that PUAs may disrupt hatching glands from developing to full efficiency. PUAs may also make embryos too lethargic or weak to break through their chorions, as hatching is a product of biochemical (chorionase activity) and behavioral processes of embryos twisting and freeing themselves out of the chorion (Jezierska et al. 2009).

Embryonic and Larval Survival

High PUA concentrations were lethal to surf smelt at early life history stages. Surf smelt embryos and larvae exposed to high PUA concentrations had higher mortality than those in low treatments and controls. The ecological significance of these findings, however, depends upon whether the high concentrations used in this study are relevant to those encountered in the wild. Ribalet et al. (2007) estimated the release of dissolved PUAs from *Thalassiosira rotula*, one of the most abundant spring bloom-forming diatom species in temperate marine ecosystems, based on the PUA production survey done by Wichard et al. (2005). Release estimates of dissolved mixtures of PUAs were 46.9, 4.7, and 0.5 $\mu mol/L$ per diatom cell soon after cell lysis at 1, 10, and 100 μm distances, respectively. These concentrations are comparable to those used in this study. For example, achieving the 5X concentration used here would require the release of PUAs from only two diatom cells at a 1 μm distance. Given that surf smelt, and other nearshore spawning forage fishes, deposit their eggs on substrates covered with benthic diatoms, it stands

to reason that surf smelt embryos are likely exposed to PUAs in concentrations well in excess of those used here.

Heart Rate

Surf smelt embryos reared in PUA-free filtered seawater before being briefly exposed to PUAs after heart development exhibited depressed heart rates. In comparison, embryos reared in PUA-treated water from the beginning of embryogenesis had similar heart rates across PUA treatments. These results suggest that surf smelt embryos possess the ability to acclimate to sub-lethal PUA stress when exposed early in their life history stages, whereas acute PUA exposure acts as a stress and reduces heart rates. Sokolova (2013) found that high exposure to metals decreased the heart rates of some aquatic ectotherms due to damage and impairment of mitochondrial function. European green crab, *Carcinus maenas*, had depressed heart rates when acutely exposed to high copper, and the marine gastropod, *Hemifusus tuba*, experienced reduced heart rates when exposed to arsenic in high temperatures (Sokolova and Lannig 2008). Acute PUA exposure may act similarly to these heavy metals and damage surf smelt embryo mitochondrial function, lowering their heart rates.

The heart rates of embryos that were chronically exposed to PUAs were lower across all PUA treatments than the heart rates of those acutely exposed to PUAs. Relatively lower heart rates suggest that surf smelt embryos may depress their metabolic rate to reduce basal maintenance costs under stress. Metabolic rate depression suppresses the demand for, and supply of, energy by limiting oxygen uptake. Metabolic rate depression is known to have evolved in species from environments with variable environmental conditions, such as an intertidal zone, to protect organisms from stress (Sokolova 2013). This strategy, in turn, reduces energy for reproduction and growth, as organisms withhold available energy that otherwise could be used

for somatic growth and development (Sokolova & Lannig 2008). Reduced size at hatch was observed in some of our morphological results, suggesting that surf smelt growth and development are underdeveloped under PUA exposure, likely due to metabolic suppression and slowed energy consumption.

Morphometrics

Exposure to PUAs during embryological development resulted in smaller standard lengths of larvae. Shorter body lengths at hatch can reduce the survival of surf smelt larvae. Predation and starvation are the two main drivers for high larval fish mortality (Nunn et al. 2012, Paradis et al. 1996, Taylor & Dunn 2017). Smaller organisms experience high water viscosities as they occupy low Reynolds numbers where viscosity dominates over inertia. As fish grow and develop, their relationship to their hydrodynamic environment shifts as they attain higher Reynolds numbers with increasing size. PUA exposure reduces the size of larval surf smelt at hatch, which will force them to operate in an even lower Reynolds number and thus experience higher viscosities. Operating in a viscous environment is energetically more expensive (Yavno & Holzman 2018). When an aquatic organism swims, friction between its body and water creates drag. At high Reynolds numbers, this drag force is proportional to the square of the organism's speed, while at low Reynolds numbers, the viscosity of the water primarily influences the drag force's strength, making it hard to build momentum to accelerate (Schmidt-Nielsen 1972). Thus, for an organism at a low Reynolds number to move similarly to a high Reynolds number organism, they need to propel themselves with proportionally greater force to overcome the drag force, and as such, expend more of their pre-feeding energy reserves (Yavno & Holzman 2018). In addition, surf smelt larvae switch to exogenous feeding after exhaustion of their endogenous energy reserves. Because they exist in a viscous environment, exogenous-feeding surf smelt

larvae expend more energy foraging, swimming (Baily & Duffy-Anderson 2001, Nunn et al. 2012, Yavno & Holzman 2018), and creating suction forces to draw food inward in comparison to larger larvae (Yavno & Holzman 2018). Rapid growth allows them to escape this energetically costly life stage.

Along with smaller sized larvae having higher energy expenditure, smaller size correlates with high predation mortality in the plankton. Scharf et al. (2003) explored the vulnerability of a few marine forage fishes to piscivory and found that the capture success of forage fish predators declined with increasing prey size. According to Paradis et al. (1996), the susceptibility of ichthyoplankton to predation decreases when their sizes are greater or equal to 10% of their predator's size. The predation rates significantly increase with smaller prey sizes because they are easier to handle, and smaller prey escape their predators less efficiently (Baily & Duffy-Anderson 2001, Nunn et al. 2012, Scharf et al. 2002, Taylor & Dunn 2017).

Unlike standard length, the deepest body depths of PUA-exposed larvae were larger than controls, likely owing to reduced consumption of yolk and oil globules. Surf smelt use their endogenous energy reserves to fuel embryogenesis. Exposure to PUAs decelerated the consumption rate, thus adding depth to this body dimension. This finding is incongruent with endogenous energy consumption under thermal stress. Surf smelt embryos exhibited faster consumption rates of their yolk and oil globules under thermal stress to maintain homeostasis (Russell et al. 2022). As mentioned earlier, surf smelt embryos may depress their metabolic rates in response to stress in order to extend their survival. Metabolic suppression would reduce the rate of endogenous energy consumption needed for growth, resulting in larger yolk and oil globules and shorter standard lengths, as observed in this study (Sokolova 2013, Sokolova & Lannig 2008). Metabolic suppression also reduces an organism's aerobic scope, thus limiting

organismal activity and growth potential (Sokolova 2013). For example, cadmium-exposed Atlantic herring embryos showed repressed activity, which resulted in lower energy requirements. This led to cadmium-exposed larvae having larger yolk sacs compared to larvae from controls (Westernhagen et al. 1974).

PUA-exposed embryos hatched with smaller eyes. This will likely reduce larval fitness because successful exogenous feeding requires the development of sensory structures, including eyes for prey localization (Nunn et al. 2012, Yúfera & Darias 2007). Marine fish larvae are mainly visual feeders (Yúfera & Darias 2007), whose visual acuity is important in prey detection (Caves et al. 2017). Several studies found that larger eyes positively correlated with better visual acuity for a wide range of animal species, which increased the survival of organisms (Beston & Walsh 2019, Caves et al. 2017). Reduced eye area of surf smelt larvae may, therefore, affect their foraging abilities. This, in turn, can delay metamorphosis and decrease survival.

Variability

While my results show definitive PUA effects on the fitness of early life history stages of surf smelt, a common theme observed throughout all analyses was high within-treatment variability. This variability limited my ability to detect treatment differences in a statistically robust manner. One explanation for this high variability is maternal effects, i.e. introduced variability in results as a consequence of fitness differences in mothers. Multiple studies of different species observed that the offspring of mothers who were reared in elevated temperatures exhibited greater thermal tolerance (Pankhurst & Munday 2011). A study exploring the Atlantic silverside (*Menidia menidia*) showed that offspring sensitivity to CO_2 was correlated with maternal provisioning, suggesting that juvenile stress sensitivity was, in part, a reflection of paternal investment in offspring stress tolerance (Snyder et al. 2018). Gagliano and McCormick

(2009) suggested that differences in stress tolerance or adaptability to stress in offspring can arise from the amount of mother-transmitted stress hormone, cortisol. Increased cortisol levels transferred from mother Ambon damselfish (*Pomacentrus amboinensis*) to offspring affected the hatching time and success rate, and elevated offspring heart rates (Gagliano & McCormick 2009). In addition to influencing stress sensitivity, mothers can affect offspring size. Mothers of the bryozoan, *Bugula neritina*, who experienced competition produced larger offspring (Allen et al. 2008). Larger bicolor damselfish (*Stegastes partitus*) produced larvae with greater size at hatch. This specific phenotype was associated with better swimming ability, which is highly likely to influence survival (Johnson et al. 2011). These studies suggest that by pooling gametes from many mothers and fathers, an inadvertent source of variability may have been introduced into this study. Future studies of fish development should take maternal effects into consideration.

Conclusion

Dissolved PUAs may compromise the developmental and physiological fitness of marine vertebrates through sympatry with diatoms in nature. PUAs have the potential to affect surf smelt survival directly via toxicity and indirectly by reducing their ability to swim and capture food. This, coupled with reduced hatching success, could significantly affect recruitment into adult spawning populations, which is a key factor in establishing the long-term health of Salish Sea forage fish populations (Boldt et al. 2018, Therriault et al. 2009). Any reductions in adult forage fish population sizes will have cascading effects on higher trophic organisms and will negatively affect marine ecosystem functioning.

Placing these results in appropriate context will require more thorough explorations of natural PUA concentrations at known surf smelt spawning locations, across a spectrum of

environmental conditions. The actual PUA concentrations that can be found in nature vary depending on factors like temperatures (Bartual & Ortega 2013), nutrient availability (Cózar et al. 2018, Ribalet et al. 2009), and the sizes and species composition of PUA-producing diatoms (Vidoudez et al. 2011, Wichard et al. 2005). Furthermore, this study focused on the effects of exposure of a marine organism to dissolved PUAs. As surf smelt feed directly on PUA-producing diatoms and indirectly on diatom consumers like zooplankton, the effects of exposure to particulate PUAs should also be explored.

Additionally, future studies should explore synergistic stressors with PUA toxicity. For example, future studies could usefully include measures of temperature and PUA toxicity. Many studies show synergistic effects of metals and temperature stress, where the toxicity of metals is sometimes repressed or enhanced depending on temperature (Jezierska et al. 2009, Sokolova 2013, Sokolova & Lannig 2008). Additionally, Bartual and Ortega (2013) showed that temperature variation alters the persistence of different PUA molecules in seawater, which could affect the magnitude of PUA effects on organisms.

Lastly, it remains to be seen if PUAs can be transferred from surf smelt mothers to offspring. Because this is known to happen in copepod zooplankton and results in embryonic developmental arrest (Poulet et al. 1994) and apoptosis (Romano et al. 2003), it would be useful to determine if such effects are seen in vertebrates as well. If PUAs accumulate in mothers and are transmitted to surf smelt offspring, this may amplify the effects on embryos from natural PUA exposure alone.

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Figures and Tables

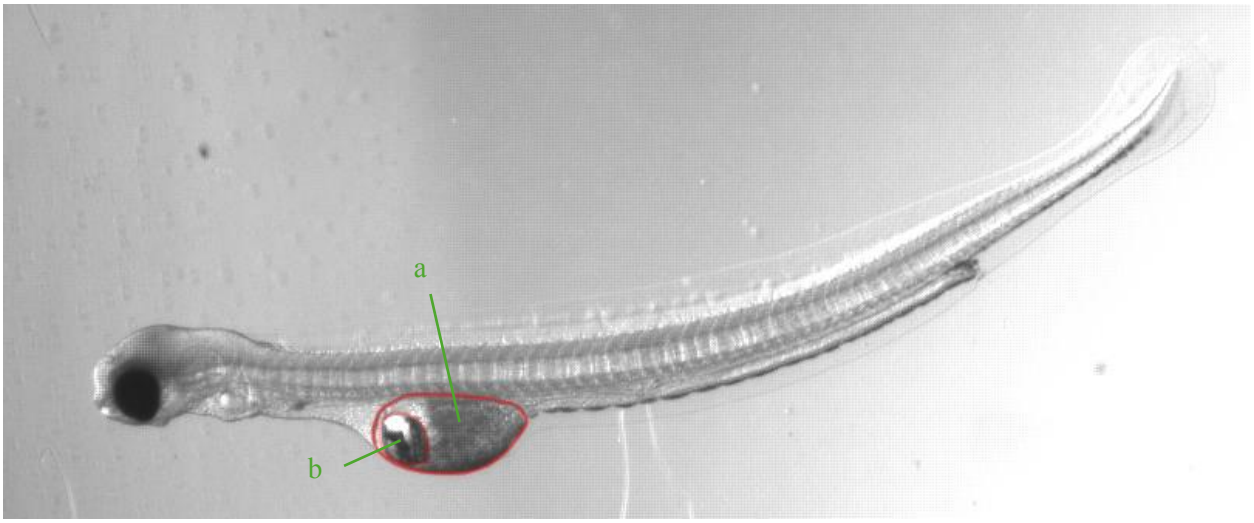

Figure 1. Surf smelt larvae just after hatch showing endogenous energy reserves: yolk (a) and oil globule (b).

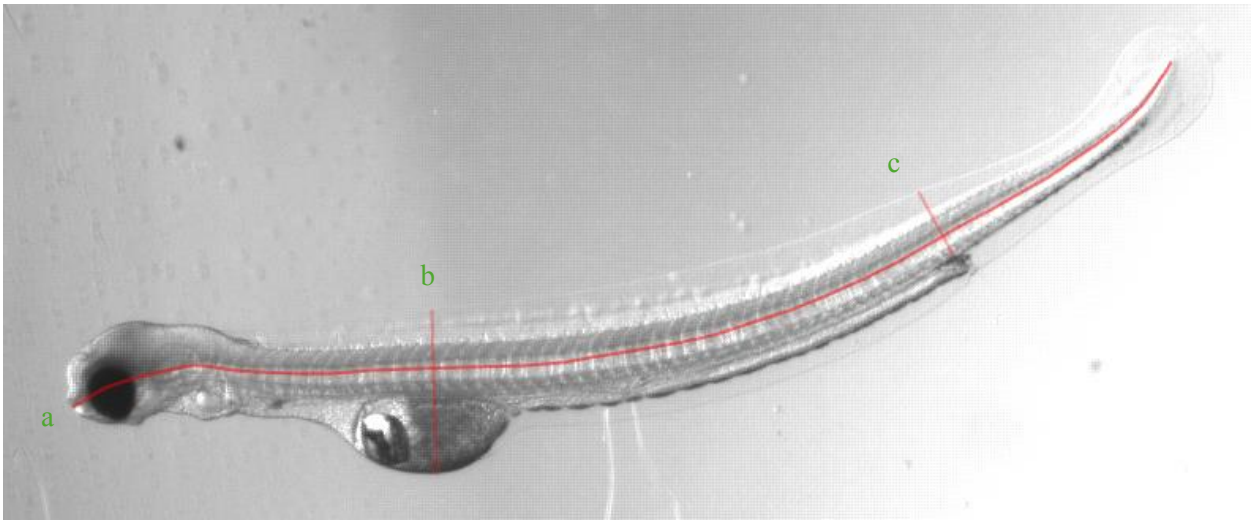

Figure 2. Morphological features measured during Experiments 1 & 2: standard length (a): SL, deepest body depth (b): BD1, and body depth at the anus (c): BD2.

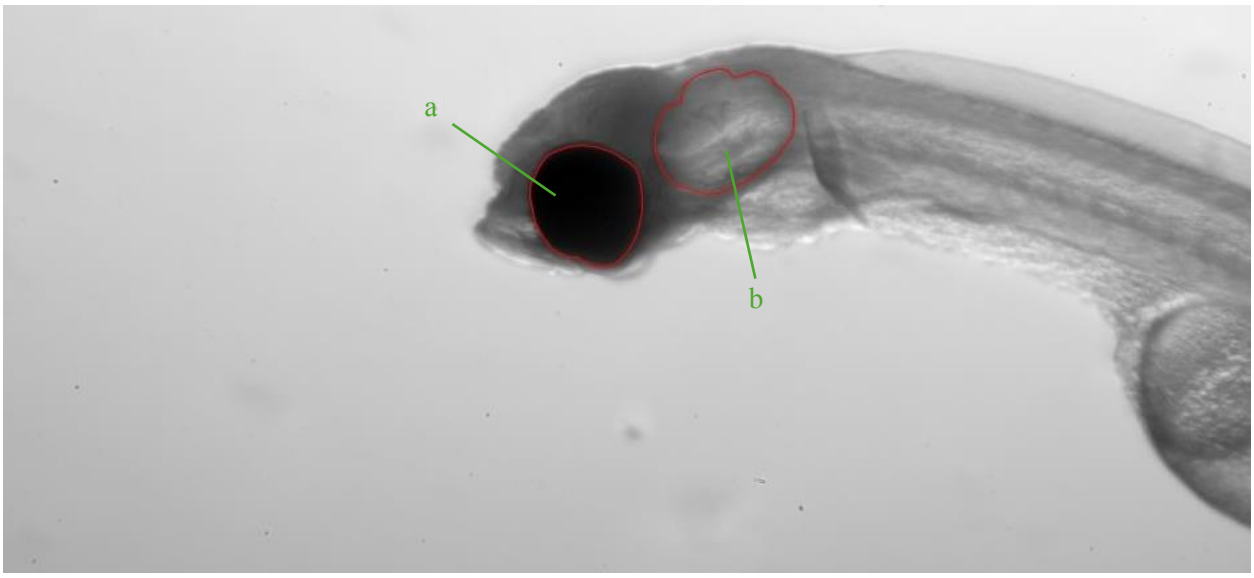

Figure 3. Sensory perception features measured in Experiment 2: eye (a) and otic capsule (b).

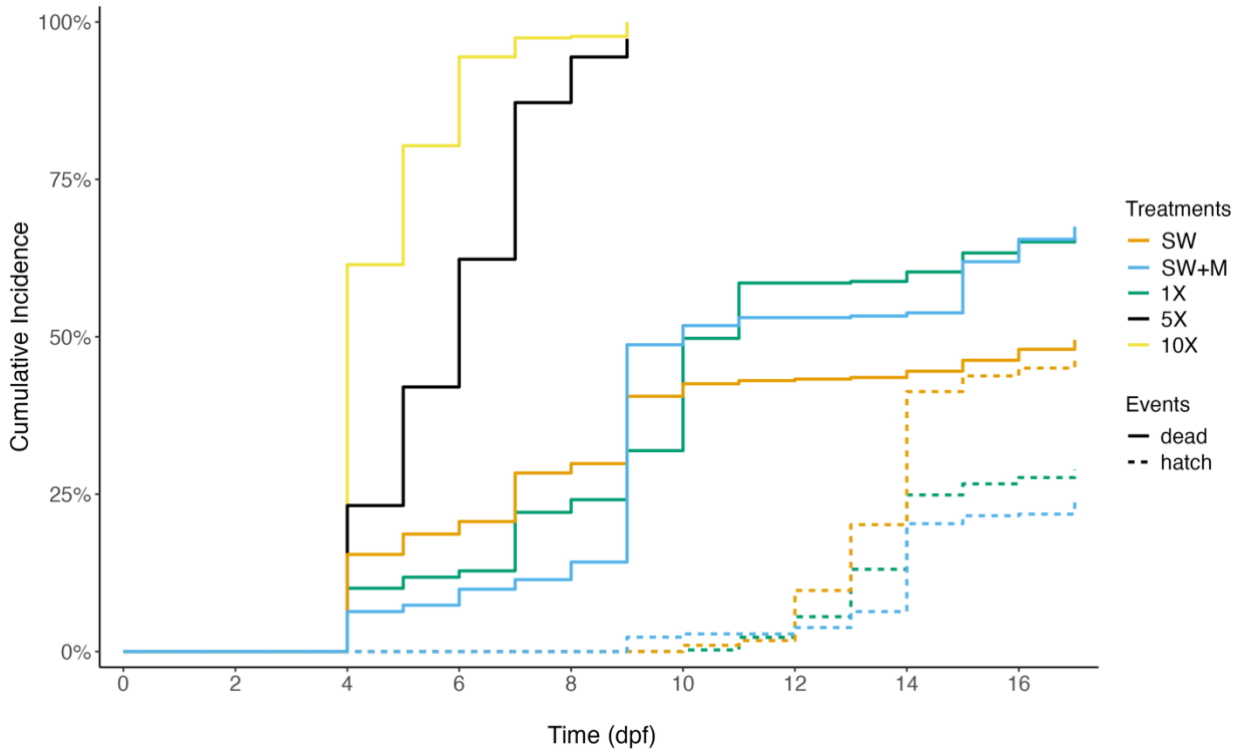

Figure 4. Cumulative incidence of competing events death and hatch of surf smelt embryos reared in SW and SW+M controls and 1X, 5X, and 10X PUA treatments (Experiment 1). Solid lines represent the cumulative incidence of death, and dashed lines represent the cumulative incidence of hatching events.

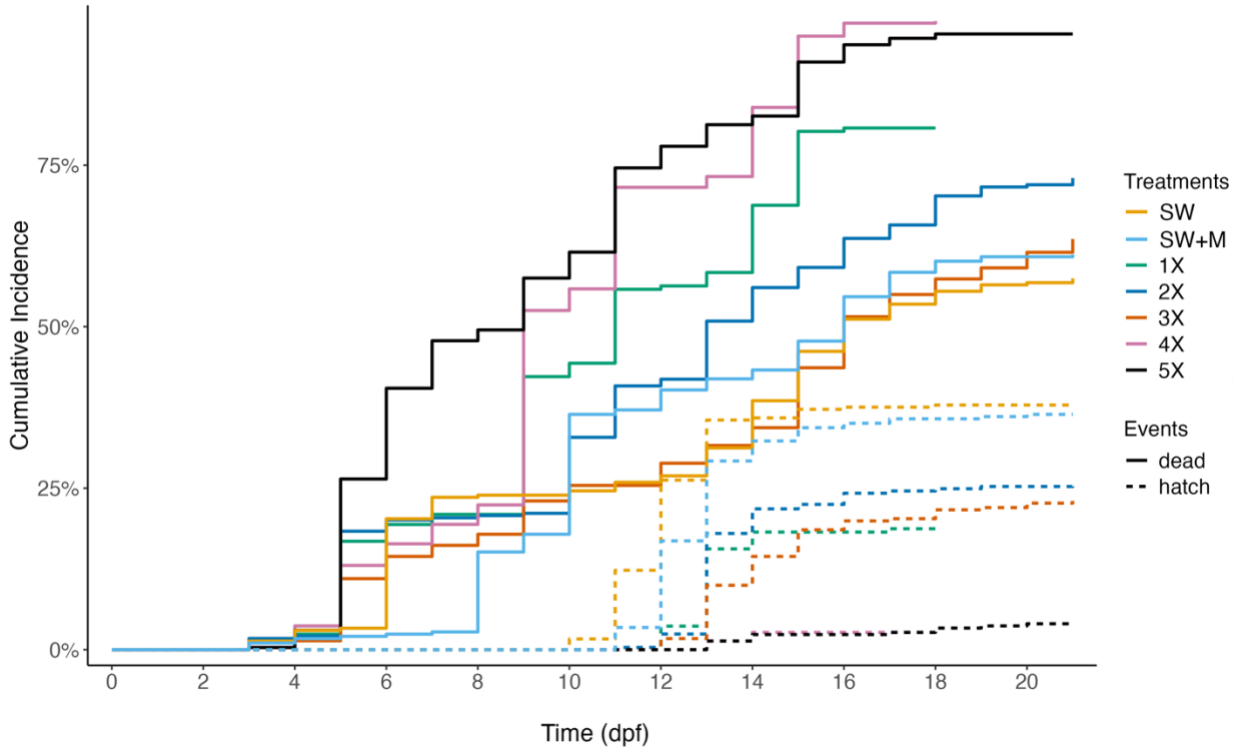

Figure 5. Cumulative incidence of competing events death and hatch of surf smelt embryos reared in SW and SW+M controls and 1X – 5X PUA treatments (Experiment 2). Solid lines represent the cumulative incidence of death, and dashed lines represent the cumulative incidence of hatching event.

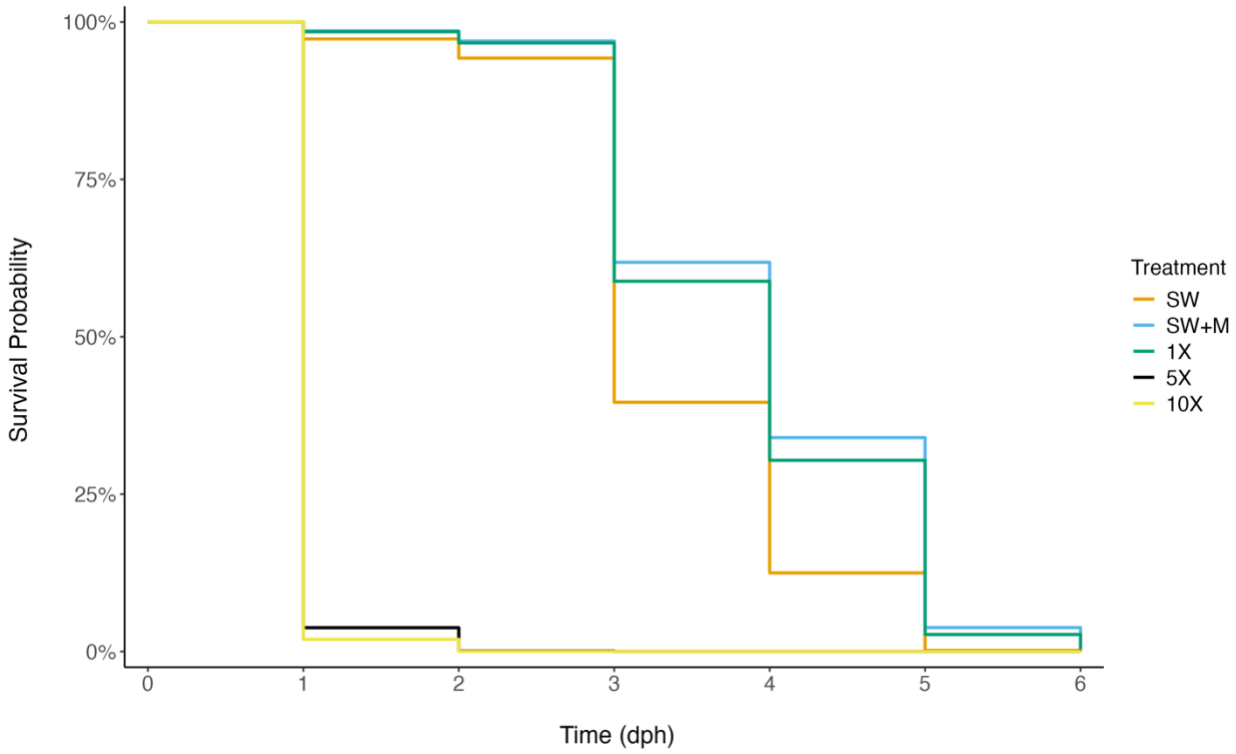

Figure 6. Survival probability of naïve larvae reared in autoclaved filtered seawater until hatch and exposed to 1X, 5X, and 10X PUA treatments one day post-hatch (Experiment 4).

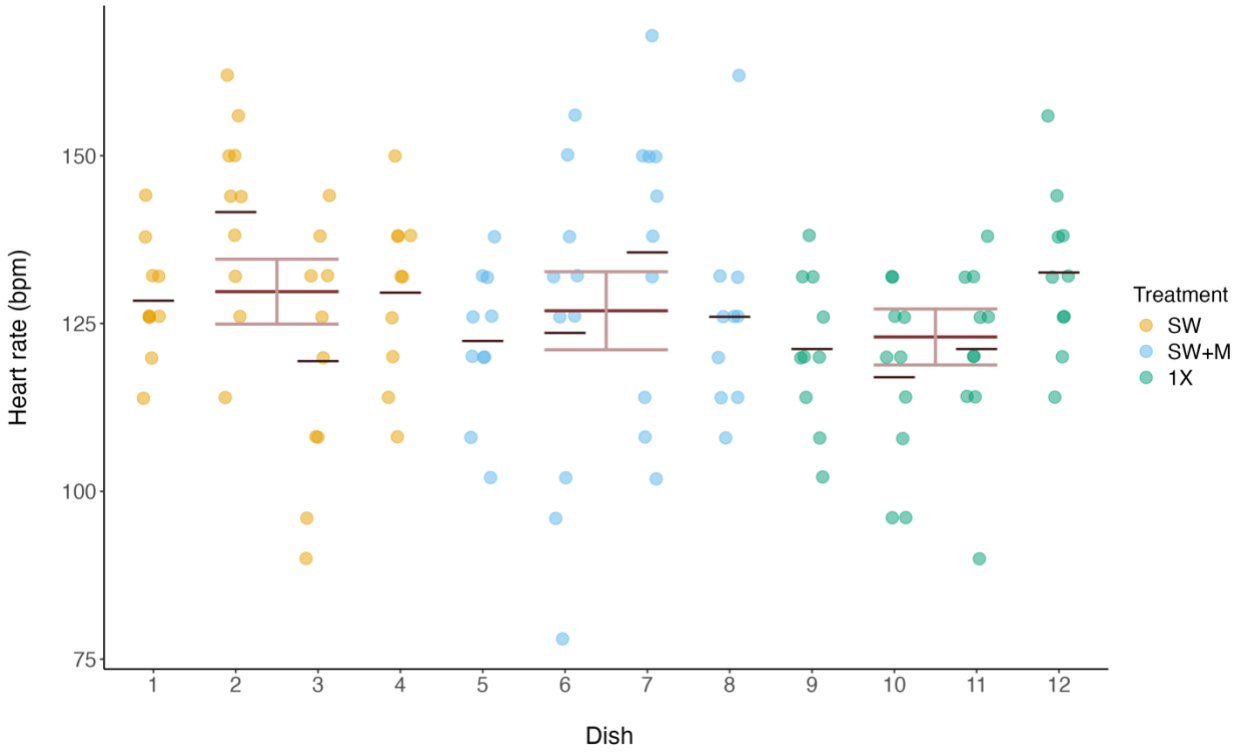

Figure 7. Heart rates of embryos reared in SW and SW+M controls and 1X, 5X, and 10X PUA treatments (Experiment 1). Heart rates of 5X and 10X embryos are absent due to 100% mortality prior to heart development. Data points are slightly offset so individual fish heart rates are discernible. Numbers on the x-axis represent individual Petri dishes. Short black horizontal bars represent the average heart rates of embryos in each Petri dish, dark brown horizontal bars represent the average heart rates of embryos in each treatment, and light brown bars represent the standard errors of the treatment average.

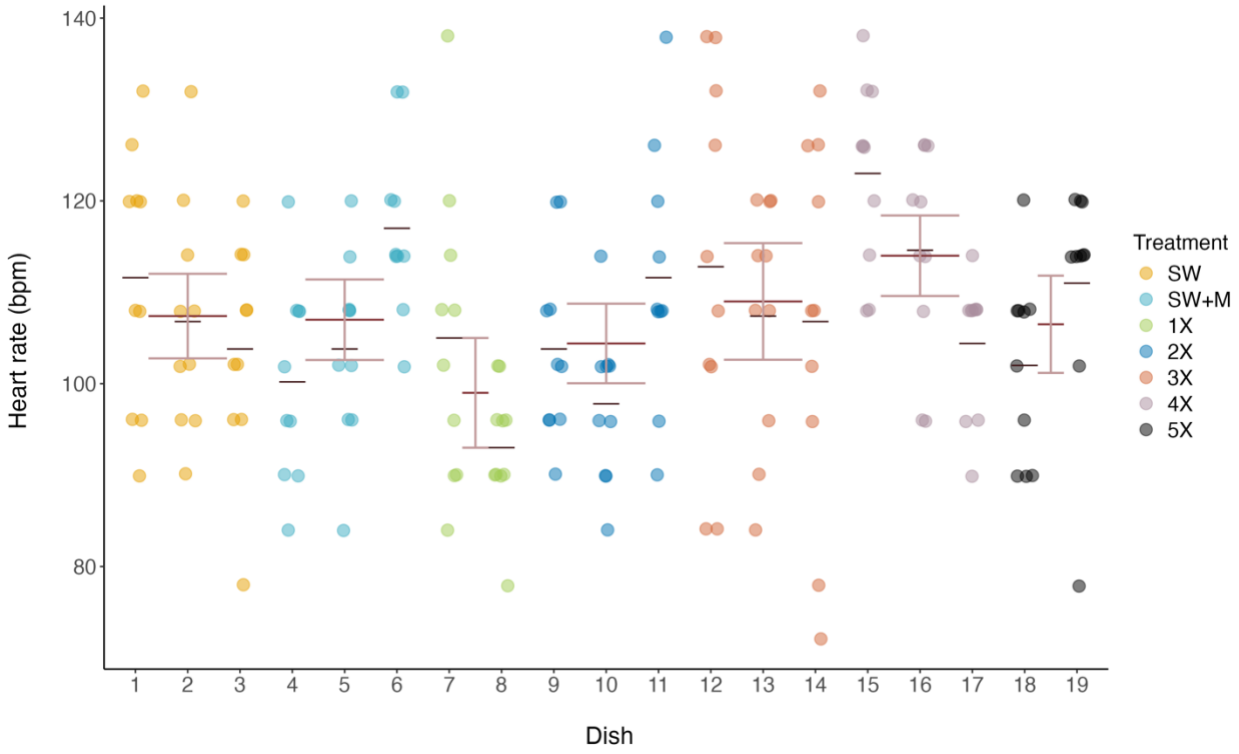

Figure 8. Heart rates of embryos reared in SW and SW+M controls and 1X – 5X PUA treatments (Experiment 2). 1X and 5X treatments are missing one replicate each due to experimenter error (1X) and expiration of embryos (5X). Data points are slightly offset so individual fish heart rates are discernible. Numbers on the x-axis represent individual Petri dishes. Short black horizontal bars represent the average heart rates of embryos in each Petri dish, dark brown horizontal bars represent the average heart rates of embryos in each treatment, and light brown bars represent the standard errors of the treatment average.

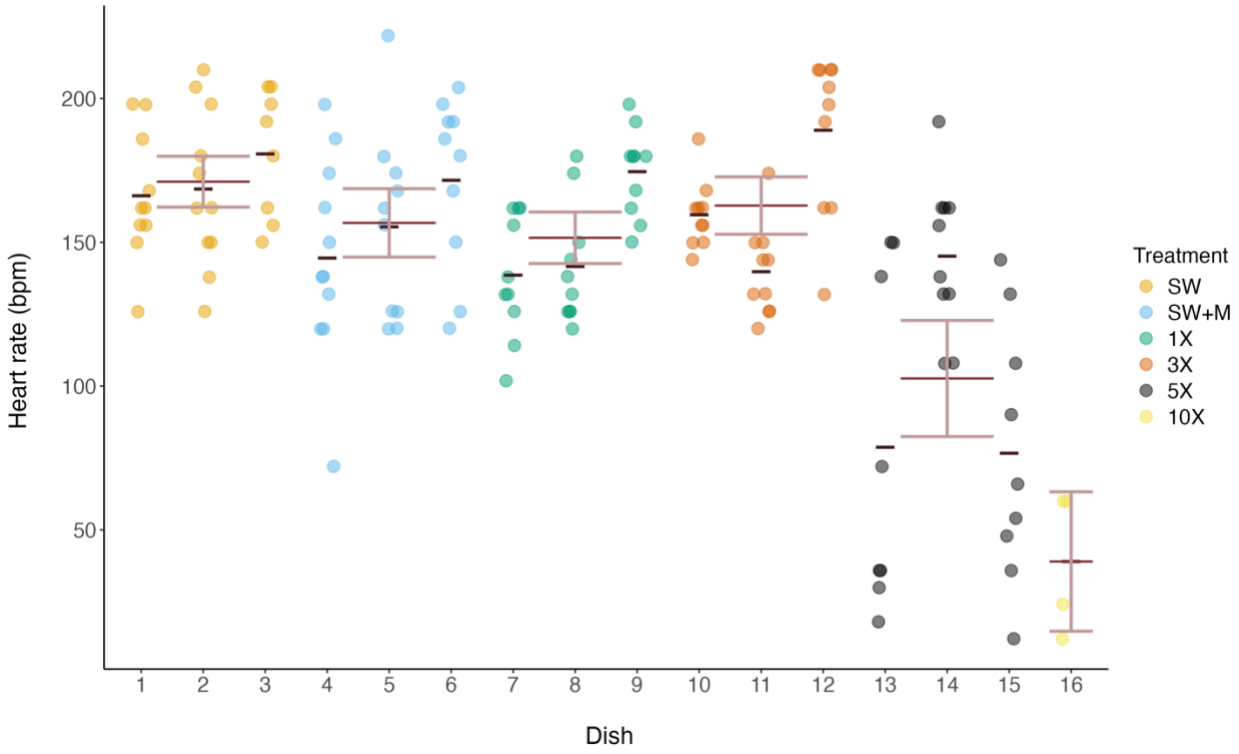

Figure 9. Heart rates of naïve embryos acutely exposed to 1X, 3X, 5X, and 10X PUA treatments, and the two controls: SW and SW+M (Experiment 3). Number of observations in 5X treatment varies and 10X treatment is missing two replicates due to expiration of embryos. Data points are slightly offset so individual fish heart rates are discernible. Numbers on the x-axis represent individual Petri dishes. Short black horizontal bars represent the average heart rates of embryos in each Petri dish, dark brown horizontal bars represent the average heart rates of embryos in each treatment, and light brown bars represent the standard errors of the treatment average.

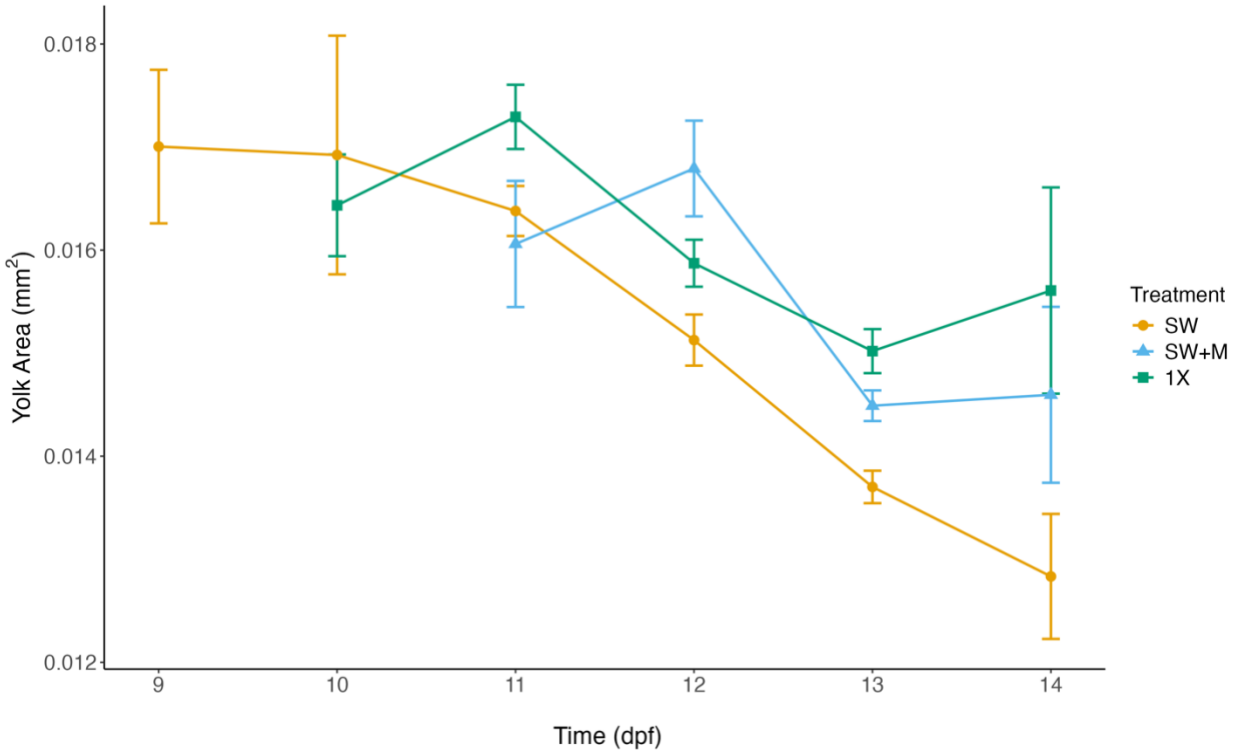

Figure 10. Yolk area measurements of surf smelt at hatch reared in SW and SW+M controls and 1X PUA treatment (Experiment 1). Each data point represents the average yolk area for all surf smelt larvae that hatched on the corresponding day post-fertilization. Horizontal bars show the standard errors for the mean.

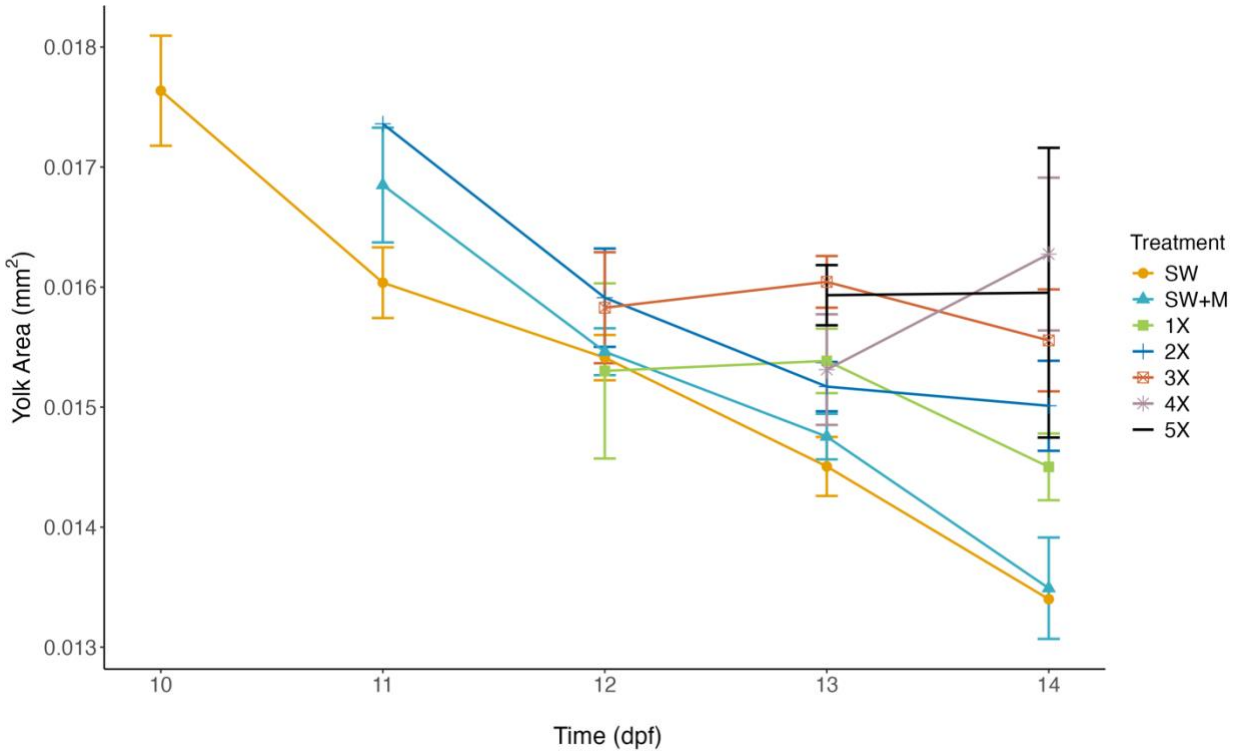

Figure 11. Yolk area measurements of surf smelt at hatch reared in SW and SW+M controls and 1X – 5X PUA treatments (Experiment 2). Each data point represents the average yolk area for all surf smelt larvae that hatched on the corresponding day post-fertilization. Horizontal bars show the standard errors for the mean.

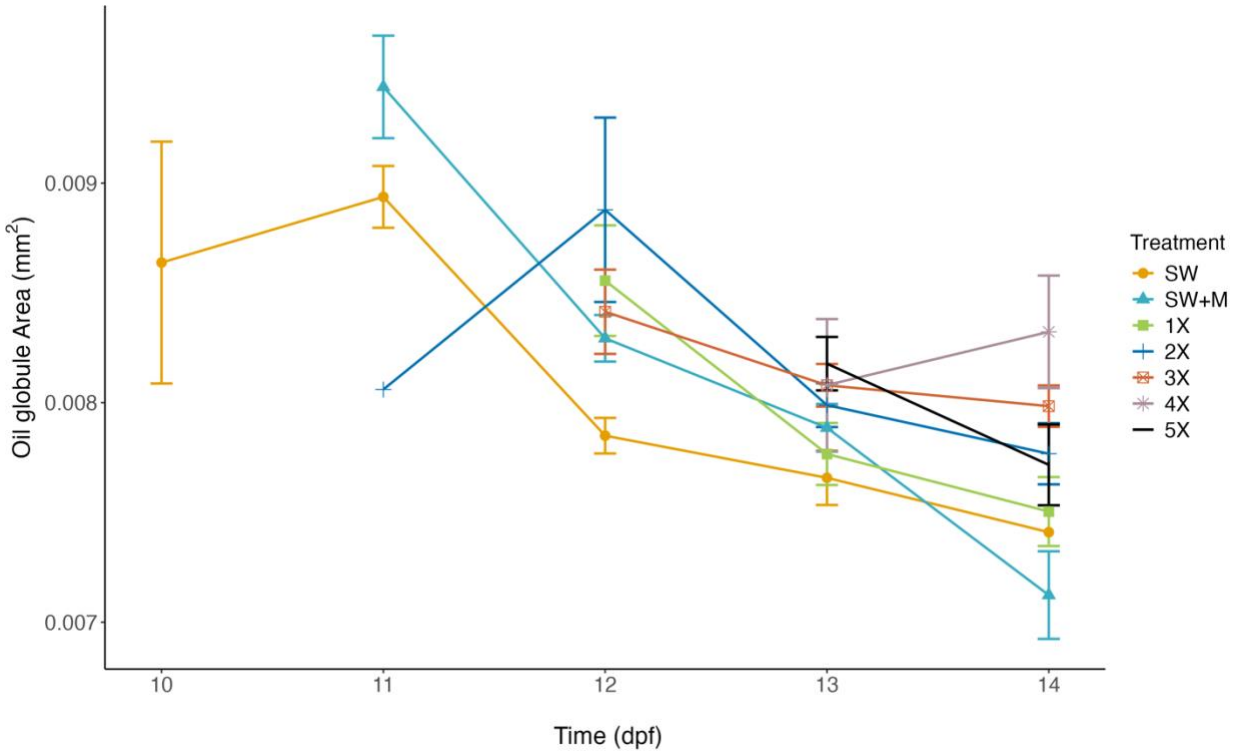

Figure 12. Oil globule area measurements of surf smelt at hatch reared in SW and SW+M controls and 1X – 5X PUA treatments (Experiment 2). Each data point represents the average oil globule for all surf smelt larvae that hatched on the corresponding day post-fertilization. Horizontal bars show the standard errors for the mean.

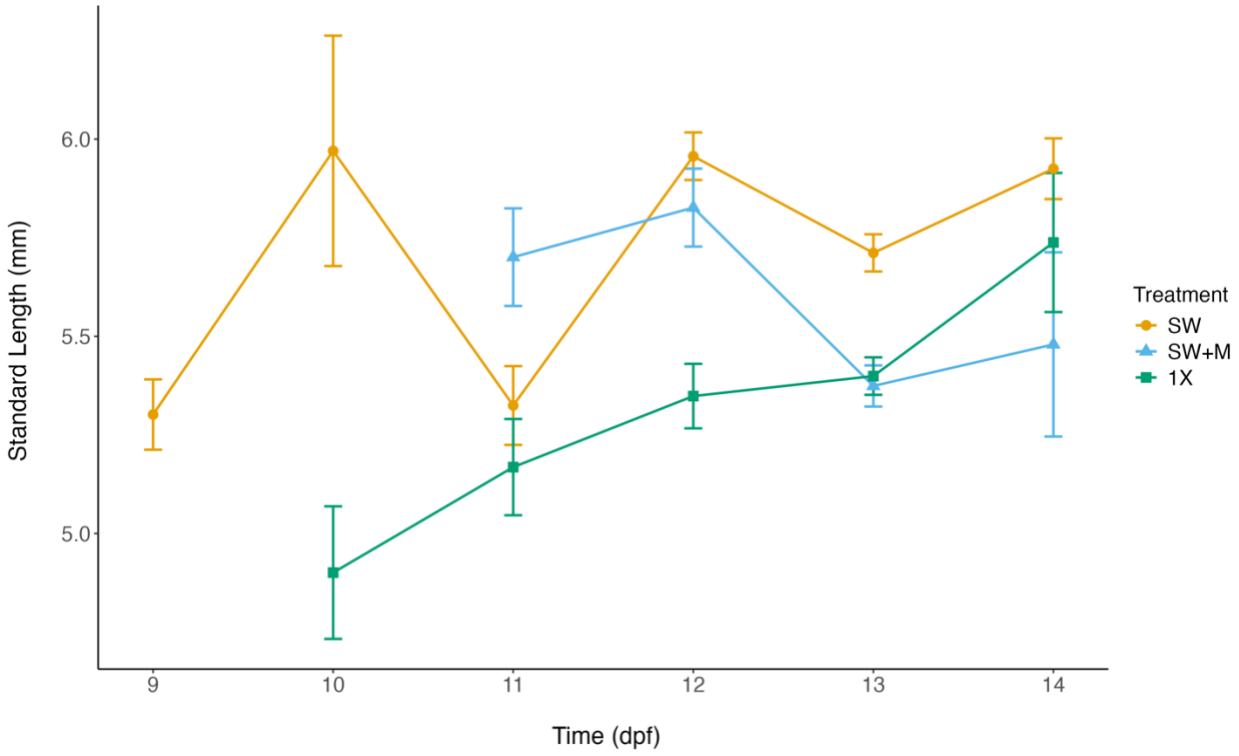

Figure 13. Standard length measurements of surf smelt at hatch reared in SW and SW+M controls and 1X PUA treatment (Experiment 1). Each data point represents the average standard length for all surf smelt larvae that hatched on the corresponding day post-fertilization. Horizontal bars show the standard errors for the mean.

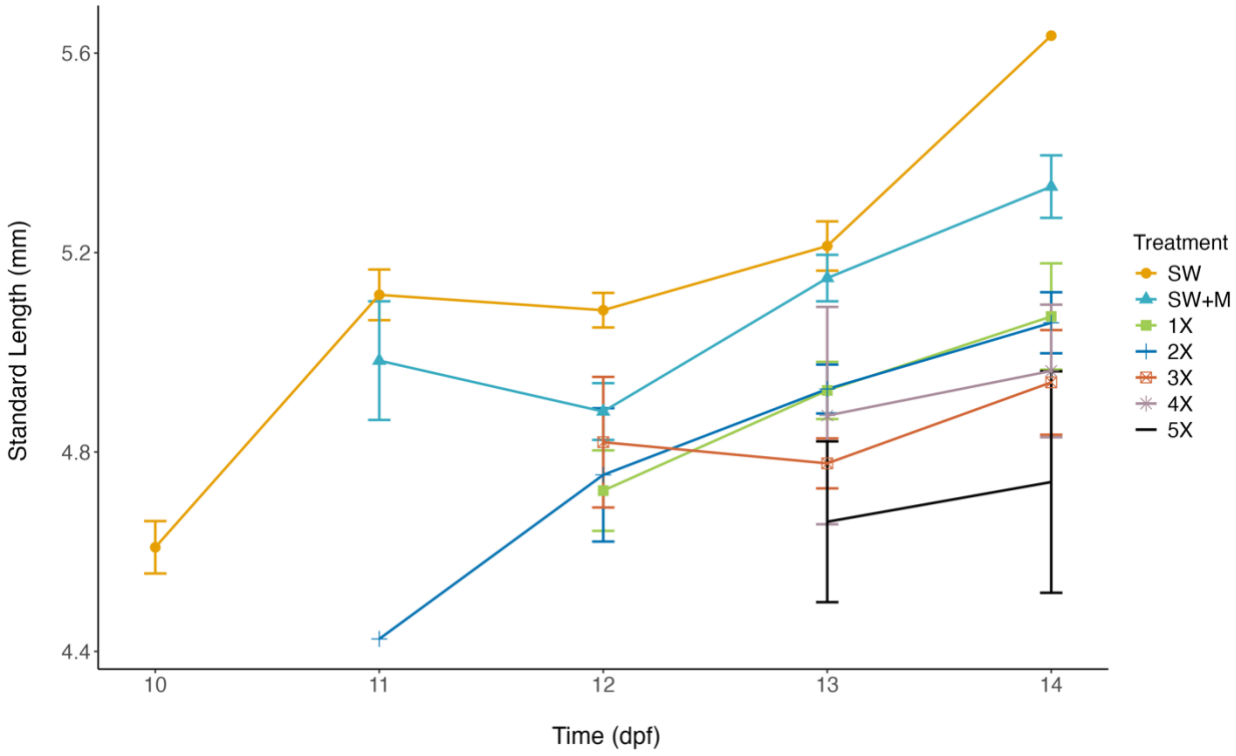

Figure 14. Standard length measurements of surf smelt at hatch reared in SW and SW+M controls and 1X – 5X PUA treatments (Experiment 2). Each data point represents the average standard length for all surf smelt larvae that hatched on the corresponding day post-fertilization. Horizontal bars show the standard errors for the mean.

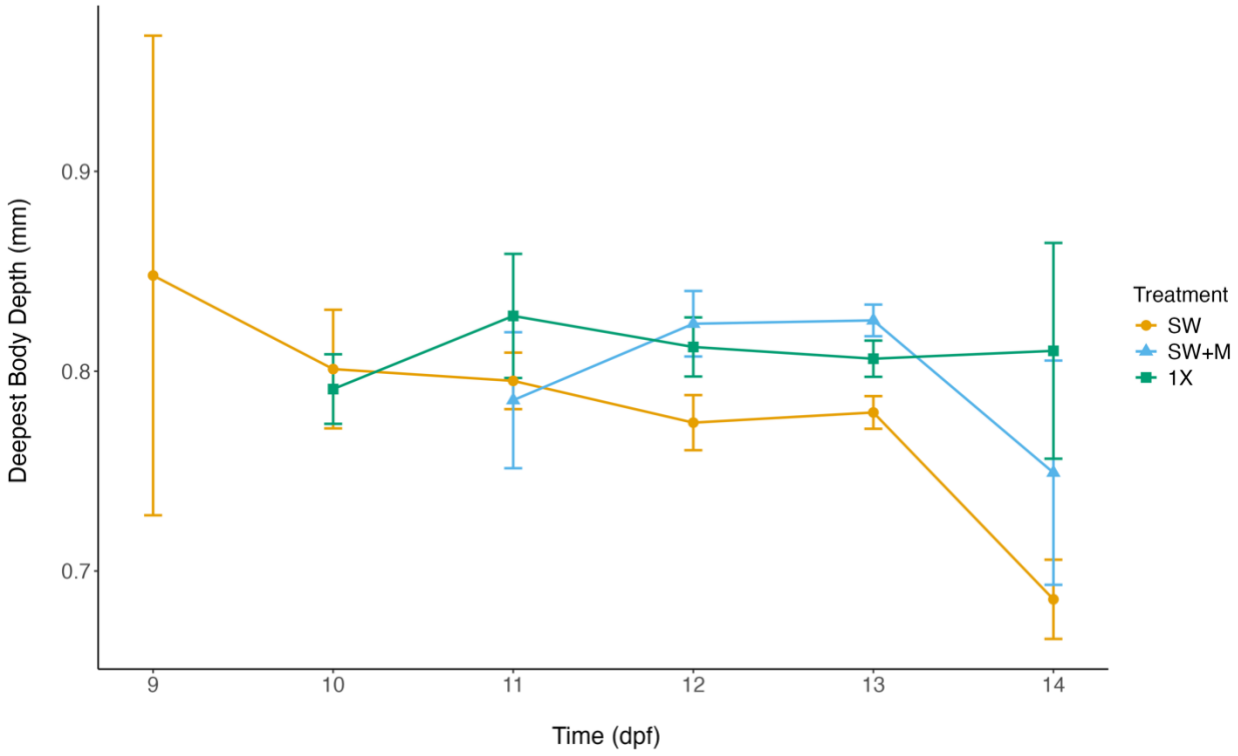

Figure 15. Deepest body depth measurements of surf smelt at hatch reared in SW and SW+M controls and 1X PUA treatment (Experiment 1). Each data point represents the average deepest body depth for all surf smelt larvae that hatched on the corresponding day post-fertilization. Horizontal bars show the standard errors for the mean.

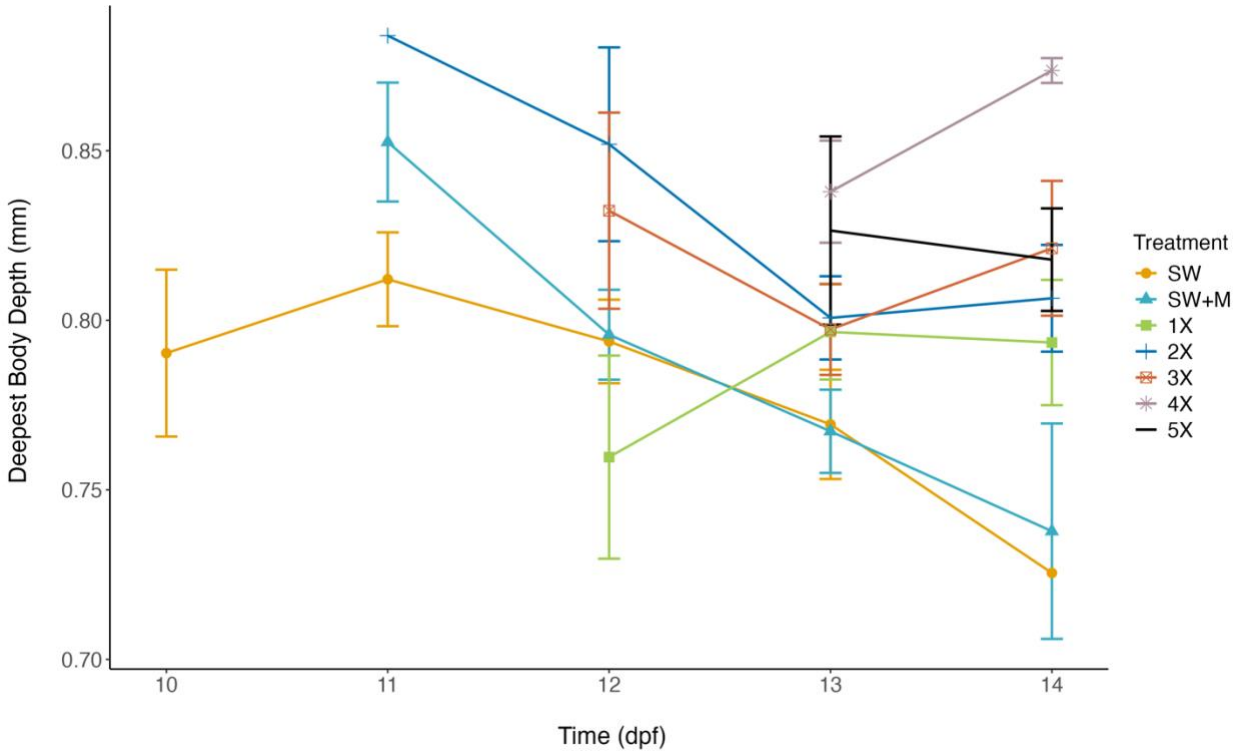

Figure 16. Deepest body depth measurements of surf smelt at hatch reared in SW and SW+M controls and 1X – 5X PUA treatments (Experiment 2). Each data point represents the average deepest body depth for all surf smelt larvae that hatched on the corresponding day post-fertilization. Horizontal bars show the standard errors for the mean.

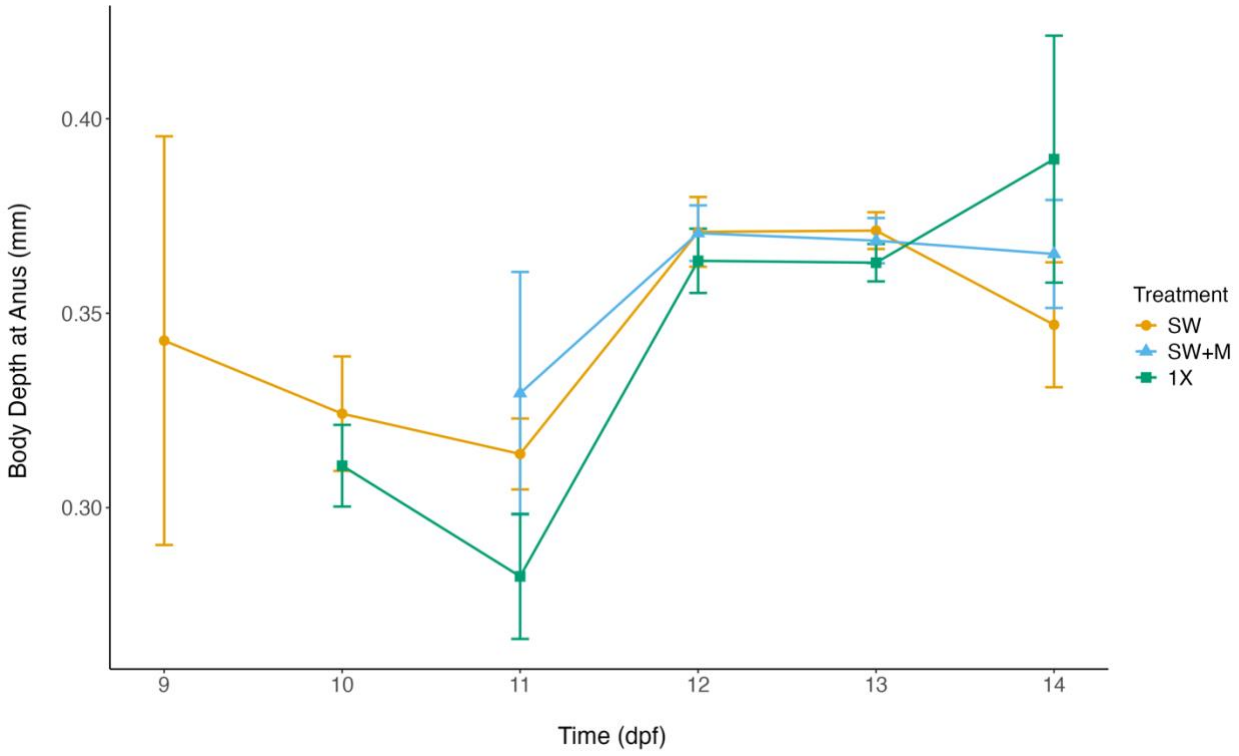

Figure 17. Body depth at anus measurements of surf smelt at hatch reared in SW and SW+M controls and 1X PUA treatment (Experiment 1). Each data point represents the average body depth at anus for all surf smelt larvae that hatched on the corresponding day post-fertilization. Horizontal bars show the standard errors for the mean.

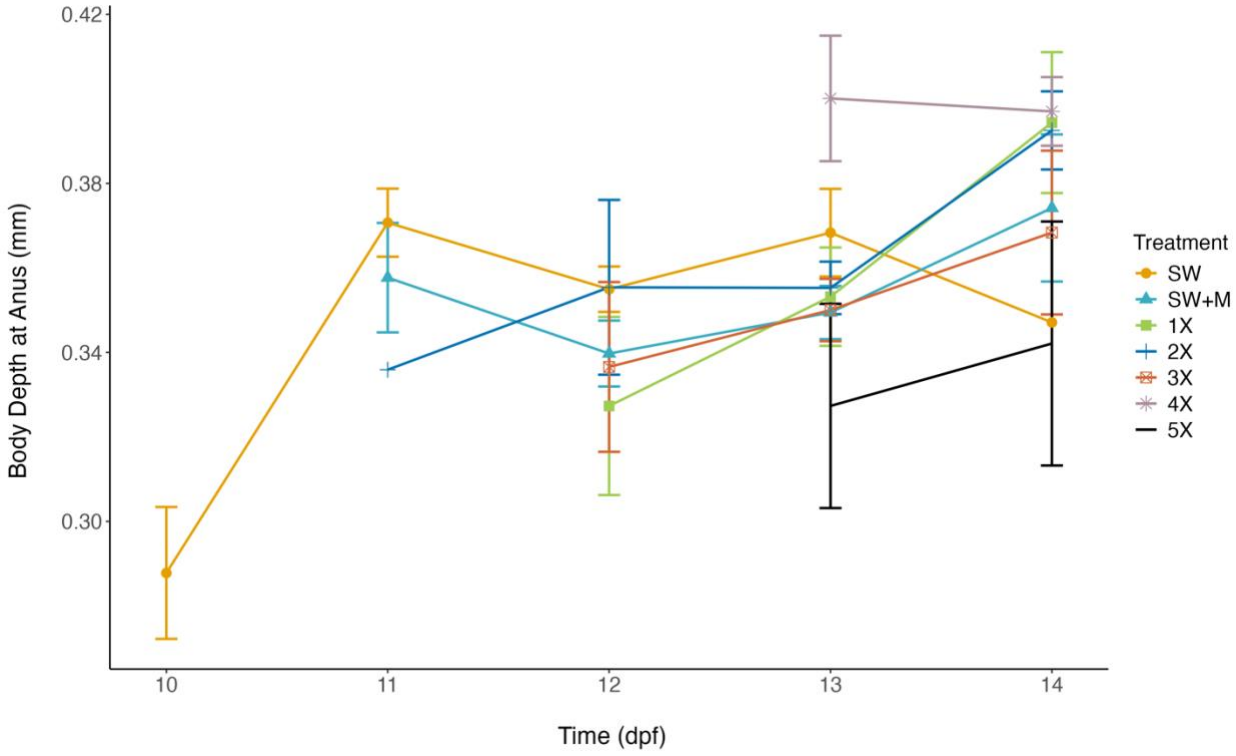

Figure 18. Body depth at anus measurements of surf smelt at hatch reared in SW and SW+M controls and 1X – 5X PUA treatments (Experiment 2). Each data point represents the average body depth at anus for all surf smelt larvae that hatched on the corresponding day post-fertilization. Horizontal bars show the standard errors for the mean.

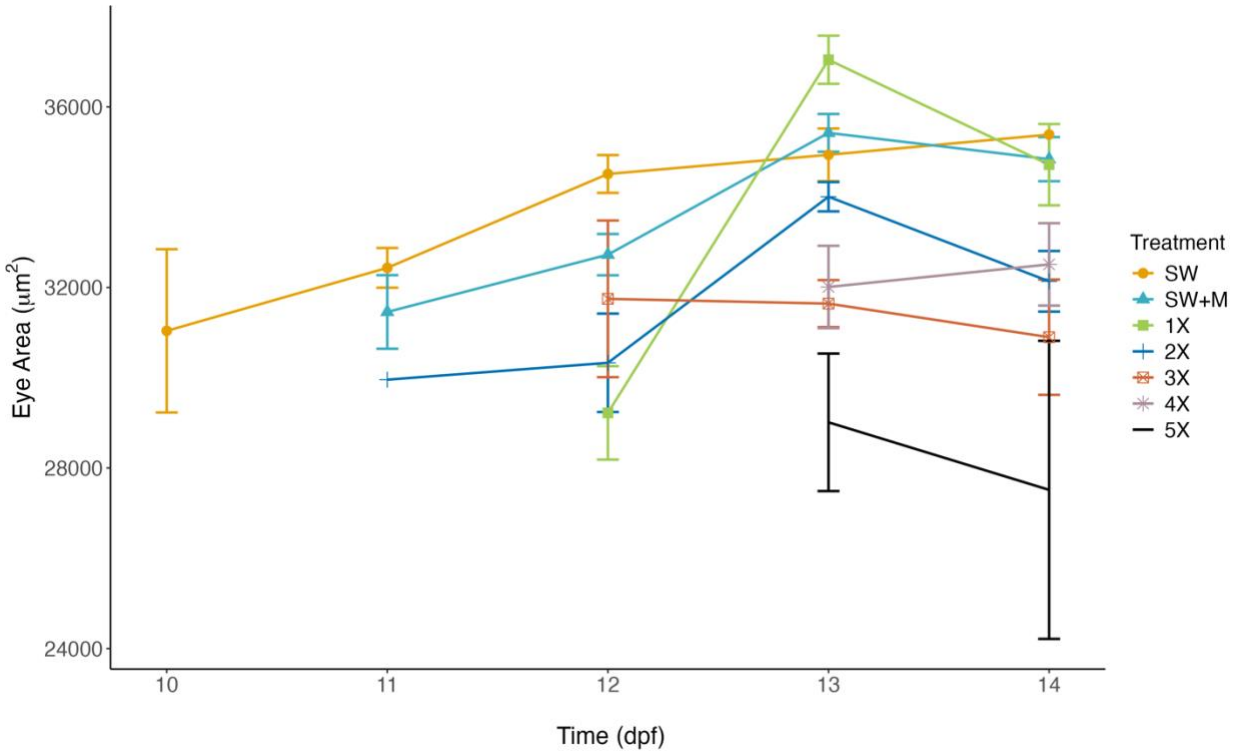

Figure 19. Eye area measurements of surf smelt at hatch reared in SW and SW+M controls and 1X – 5X PUA treatments (Experiment 2). Each data point represents the average eye area for all surf smelt larvae that hatched on the corresponding day post-fertilization. Horizontal bars show the standard errors for the mean.

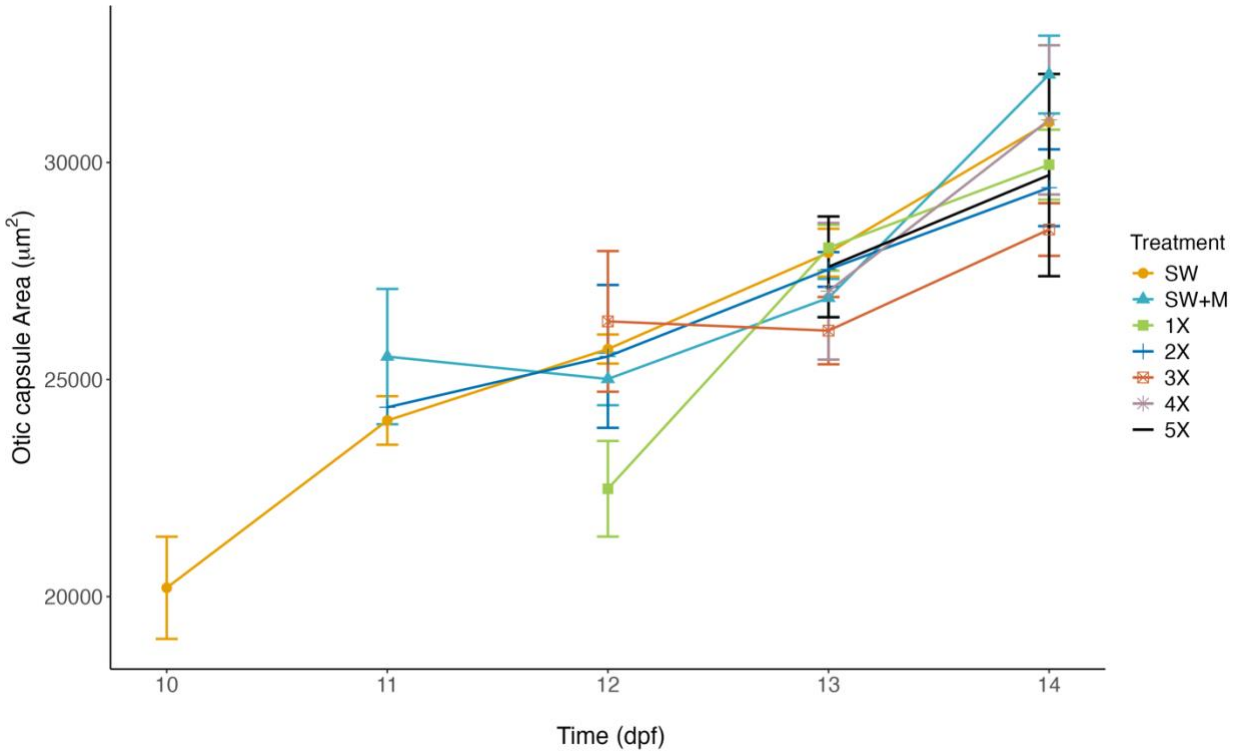

Figure 20. Otic capsule measurements of surf smelt at hatch reared in SW and SW+M controls and 1X – 5X PUA treatments (Experiment 2). Each data point represents the average otic capsule area for all surf smelt larvae that hatched on the corresponding day post-fertilization. Horizontal bars show the standard errors for the mean.

Table 1. Concentrations ($\mu g/mL$) of polyunsaturated aldehydes for different treatment levels.

Levels	**Heptadienal**	**Octadienal**	**Decadienal**
1X	0.66	1.12	0.38
2X	1.32	2.24	0.76
3X	1.98	3.36	1.14
4X	2.64	4.48	1.52
5X	3.3	5.6	1.9
10X	6.6	11.2	3.8

Table 2. Experiment number and design. Metrics are abbreviated as: S (survival), HR (heart rate), HSR (hatch success rate), E (endogenous energy usage), M (morphometrics), and SP (sensory perception).

Experiments	PUA Treatment Levels	Duration (hrs)	S	HR	HSR	E	M	SP
1	1X, 5X, 10X	384	✓	✓	✓	✓	✓	
2	1X, 2X, 3X, 4X, 5X	552	✓	✓	✓	✓	✓	✓
3	1X, 3X, 5X, 10X	5		✓				
4	1X, 5X, 10X	168	✓					

Table 3. Number of embryos that hatched each day during experiments up to 14 days post fertilization (dpf).

Experiment	Treatment	9 dpf	10 dpf	11 dpf	12 dpf	13 dpf	14 dpf	Total
	SW	2	3	33	40	82	9	169
1	SW+M	0	0	4	8	54	5	71
	1X	0	8	13	29	45	5	100
	Total	2	11	50	77	181	19	340
	SW	-	5	32	42	26	1	106
	SW+M	-	0	10	39	36	9	94
	1X	-	0	0	7	23	5	35*
2	2X	-	0	1	6	45	11	63
	3X	-	0	0	5	24	10	39
	4X	-	0	0	0	4	4	8
	5X	-	0	0	0	4	3	7
	Total	-	5	43	99	162	43	352

* Number of 1X treatment hatches is relatively low due to a loss of one replicate

Table 4. Competing risk analysis with subdistribution hazard model in Experiment 1 for embryonic survival relative to SW control. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value
SW+M	394	0.288	0.093	1.33	1.11 - 1.60	0.002
1X	398	0.288	0.0694	1.33	1.11 - 1.61	0.002
5X	414	1.95	0.100	7.03	5.78 - 8.55	< 0.001
10X	397	2.54	0.110	12.7	10.3 - 15.8	< 0.001

Table 5. Competing risk analysis with subdistribution hazard model during Experiment 2 for embryonic survival to SW control. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value
SW+M	291	0.098	0.105	1.10	0.90 - 1.36	0.35
1X	292	0.723	0.108	2.06	1.67 - 2.55	< 0.001
2X	289	0.399	0.101	1.49	1.22 - 1.81	< 0.001
3X	291	0.086	0.101	1.09	0.89 - 1.33	0.40
4X	299	1.15	0.088	3.16	2.66 - 3.76	< 0.001
5X	299	1.27	0.097	3.55	2.94 - 4.30	< 0.001

Table 6. Cox proportional hazard model for naïve larval survival during Experiment 4 relative to SW control. HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	HR	95% CI	p-value	Adj. p-value
SW+M	78	-0.6431	0.5256	0.27 - 1.04	0.0631	0.2526
1X	70	-0.3544	0.7016	0.35 - 1.39	0.3116	1.0000
5X	74	5.017	151.0	53.8 - 424	1.619e-21	6.477e-21
10X	82	5.191	179.7	62.7 - 515	4.368e-22	1.747e-21

Table 7. Competing risk analysis with subdistribution hazard model during Experiment 1 for hatch success rate relative to SW control. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value
SW+M	394	-0.790	0.118	0.45	0.36 - 0.57	< 0.001
1X	398	-0.554	0.112	0.57	0.46 - 0.72	< 0.001
5X	414	-11.6	0.084	0.00	0.00 - 0.00	< 0.001
10X	397	-11.6	0.084	0.00	0.00 - 0.00	< 0.001

Table 8. Competing risk analysis with subdistribution hazard model during Experiment 2 for hatch success rate relative to SW control. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value
SW+M	291	-0.136	0.135	0.87	0.67 - 1.14	0.31
1X	292	-0.998	0.187	0.37	0.26 - 0.53	< 0.001
2X	289	-0.590	0.144	0.55	0.42 - 0.73	< 0.001
3X	291	-0.724	0.147	0.48	0.36 - 0.6	< 0.001
4X	299	-2.92	0.363	0.05	0.03 - 0.11	< 0.001
5X	299	-2.51	0.299	0.08	0.05 - 0.15	< 0.001

Table 9. Summary of generalized linear mixed model for heart rates of embryos in Experiment 1.

	Value	**Std. Error**	**t-value**	**p-value**
Intercept	4.865	2.526e-02	192.6	< 2e-16
SW+M	-2.187e-02	3.552e-02	-0.62	0.538
1X	-5.319e-02	3.527e-02	-1.51	0.132

Table 10. Summary of generalized linear mixed model for heart rates of embryos in Experiment 2.

	Estimate	**Std. Error**	**z-value**	**p-value**
Intercept	4.676	2.999e-02	155.9	< 2e-16
SW+M	-4.285e-03	4.238e-02	-0.10	0.9195
1X	-8.190e-02	4.649e-02	-1.76	0.0781
2X	-2.865e-02	4.216e-02	-0.68	0.4968
3X	1.483e-02	4.255e-02	0.35	0.7274
4X	5.913e-02	4.297e-02	1.38	0.1688
5X	-8.552e-03	4.732e-02	-0.18	0.8566

Table 11. Summary of generalized linear mixed model for heart rates of embryos in Experiment 3.

	Estimate	**Std. Error**	**z-value**	**p-value**
Intercept	5.145	5.540e-02	92.87	< 2e-16
SW+M	-8.979e-02	8.343e-02	-1.08	0.2818
1X	-0.1286	7.752e-02	-1.66	9.727e-02
3X	-5.917e-02	7.689e-02	-0.77	0.4416
5X	-0.5286	0.1382	-3.82	1.310e-04
10X	-1.479	0.3240	-4.56	5e-06

Table 12. Summary of linear mixed model for yolk area of surf smelt at hatch in Experiment 1. DF: degrees of freedom.

	Value	**Std. Error**	**DF**	**t-value**	**p-value**
Intercept	1.450e-02	2.169e-04	329	66.84	0.0000
SW+M	7.318e-04	3.699e-04	8	1.979	0.0832
1X	9.723e-04	3.288e-04	8	2.957	0.0182
DPF	-9.961e-04	8.500e-05	329	-11.72	0.0000

Table 13. Summary of generalized linear square model for yolk area of surf smelt at hatch in Experiment 2.

	Value	**Std. Error**	**t-value**	**p-value**
Intercept	1.494e-02	1.419e-04	105.3	0.0000
SW+M	1.4134e-04	1.928e-04	0.7334	0.4638
1X	5.948e-04	2.730e-04	2.178	0.0301
2X	6.803e-04	2.348e-04	2.898	0.0040
3X	1.384e-03	2.709e-04	5.110	0.0000
4X	1.570e-03	5.501e-04	3.132	0.0019
5X	1.663e-03	5.286e-04	3.146	0.0018
DPF	-7.599e-04	9.383e-05	-8.098	0.0000

Table 14. Summary of generalized linear mixed model for oil globule area of surf smelt at hatch in Experiment 2.

	Estimate	**Std. Error**	**z-value**	**p-value**
Intercept	7.814e-03	7.161e-05	109.1	< 2e-16
SW+M	2.547e-04	9.959e-05	2.56	1.053e-02
1X	2.604e-04	1.295e-04	2.01	4.427e-02
2X	4.781e-04	1.118e-04	4.28	1.90e-05
3X	6.071e-04	1.264e-04	4.80	1.57e-06
4X	9.173e-04	2.139e-04	4.29	1.80e-05
5X	5.997e-04	2.273e-04	2.64	8.350e-03
DPF	-4.925e-04	4.844e-05	-10.17	< 2e-16

Table 15. Summary of generalized linear mixed model for standard length of surf smelt at hatch in Experiment 1.

	Estimate	**Std. Error**	**z-value**	**p-value**
Intercept	5.736	7.236e-02	79.28	< 2e-16
SW+M	-0.2239	0.1245	-1.80	7.206e-02
1X	-0.3566	0.1081	-3.30	9.780e-4
DPF	0.1069	2.739e-02	3.90	9.61e-05

Table 16. Summary of linear mixed model for standard length of surf smelt at hatch in Experiment 2. DF: degrees of freedom.

	Value	**Std. Error**	**DF**	**t-value**	**p-value**
Intercept	5.212	7.865e-02	289	66.27	0.0000
SW+M	-0.1663	0.1130	10	-1.471	0.1720
1X	-0.3679	0.1554	10	-2.367	0.0394
2X	-0.4460	0.1242	10	-3.592	0.0049
3X	-0.4655	0.1216	10	-3.829	0.0033
4X	-0.4722	0.1635	10	-2.889	0.0161
5X	-0.6889	0.1639	10	-4.204	0.0018
DPF	0.1483	2.180e-02	289	6.801	0.0000

Table 17. Summary of linear mixed model for deepest body depth of surf smelt at hatch in Experiment 1. DF: degrees of freedom.

	Value	**Std. Error**	**DF**	**t-value**	**p-value**
Intercept	0.7784	1.029e-02	328	75.62	0.0000
SW+M	3.509e-02	1.776e-02	8	1.975	0.0837
1X	2.639e-02	1.555e-02	8	1.697	0.1281
DPF	-3.940e-03	4.435e-03	328	-0.8885	0.3749

Table 18. Summary of generalized linear mixed model for deepest body depth of surf smelt at hatch in Experiment 2.

	Estimate	**Std. Error**	**z-value**	**p-value**
Intercept	0.7872	8.308e-03	94.76	< 2e-16
SW+M	2.398e-03	1.075e-02	0.22	0.8235
1X	1.263e-02	1.484e-02	0.85	0.3944
2X	3.398e-02	1.293e-02	2.63	8.575e-03
3X	3.485e-02	1.456e-02	2.39	1.673e-02
4X	8.517e-02	2.482e-02	3.43	5.99e-03
5X	5.061e-02	2.621e-02	1.93	5.352e-02
DPF	-1.729e-02	5.209e-03	-3.32	9.000e-04

Table 19. Summary of generalized linear mixed model for body depth at anus of surf smelt at hatch in Experiment 1.

	Estimate	**Std. Error**	**z-value**	**p-value**
Intercept	0.3591	3.642e-03	98.60	< 2e-16
SW+M	-6.493e-04	6.833e-03	-0.10	0.924
1X	-6.485e-03	5.963e-03	-1.09	0.277
DPF	1.845e-02	2.757e-03	6.69	2.18e-11

Table 20. Summary of generalized linear mixed model for body depth at anus of surf smelt at hatch in Experiment 2.

	Estimate	**Std. Error**	**z-value**	**p-value**
Intercept	0.3670	4.848e-03	75.70	< 2e-16
SW+M	-1.745e-02	6.589e-03	-2.65	8.092e-03
1X	-1.723e-02	9.331e-03	-1.85	6.476e-02
2X	-1.076e-02	8.023e-03	-1.34	0.1799
3X	-2.009e-02	9.259e-03	-2.17	3.001e-02
4X	2.154e-02	1.713e-02	1.26	0.2086
5X	-4.261e-02	1.807e-02	-2.36	1.833e-02
DPF	1.059e-02	3.207e-03	3.30	9.620e-04

Table 21. Summary of generalized linear mixed model for eye area of surf smelt at hatch in Experiment 2.

	Estimate	**Std. Error**	**z-value**	**p-value**
Intercept	10.46	1.613e-02	648.2	< 2e-16
SW+M	-2.929e-02	2.344e-02	-1.3	0.2113
1X	-1.507e-02	3.491e-02	-0.4	0.6659
2X	-7.373e-02	2.672e-02	-2.8	5.579e-03
3X	-0.1310	2.767e-02	-4.7	2.20e-06
4X	-0.1245	3.319e-02	-3.8	1.760e-04
5X	-0.2437	5.877e-02	-4.1	3.37e-05
DPF	4.296e-02	6.295e-03	6.8	8.83e-12

Table 22. Summary of generalized linear mixed model for otic capsule area of surf smelt at hatch in Experiment 2.

	Estimate	**Std. Error**	**z-value**	**p-value**
Intercept	10.20	1.320e-02	772.6	< 2e-16
SW+M	-1.541e-02	1.798e-02	-0.9	0.3915
1X	-2.501e-02	2.379e-02	-1.1	0.2932
2X	-2.173e-02	2.027e-02	-1.1	0.2837
3X	-6.021e-02	2.370e-02	-2.5	0.0111
4X	-6.212e-03	3.740e-02	-0.2	0.8681
5X	-2.168e-02	3.959e-02	-0.5	0.5839
DPF	8.507e-02	8.543e-03	-10.0	< 2e-16

Supplementary Materials

Table S1. Number of embryos hatched each day during Experiment 1.

Treatment	9	10	11	12	13	14	15	16	Total
SW	2	3	33	40	82	9	5	4	178
SW+M	0	0	4	8	54	5	1	7	79
1X	0	8	13	29	45	5	4	3	107
Total	2	11	50	77	181	19	10	14	364

Table S2. Number of embryos hatched each day during Experiment 2.

Treatment	10	11	12	13	14	15	16	17	18	19	20	21	Total
SW	5	32	42	26	1	4	1	0	1	0	0	1	106
SW+M	0	10	39	36	9	6	2	1	0	1	0	0	94
1X	0	0	7	23	5	0	0	0	1	0	0	0	35*
2X	0	1	6	45	11	2	5	0	1	1	0	0	63
3X	0	0	5	24	10	12	4	1	3	1	0	1	39
4X	0	0	0	4	4	0	0	0	0	0	0	0	8
5X	0	0	0	4	3	0	0	1	1	1	1	0	7
Total	5	43	99	162	43	24	12	3	7	4	1	2	405

*Number of 1X treatment hatches is relatively low due to a loss of one replicate

Table S3. Competing risk analysis with subdistribution hazard model in Experiment 1 for embryonic survival relative to SW+M control. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	394	-0.288	0.093	0.75	0.63 - 0.90	0.0019	0.0076
1X	398	0.000	0.076	1.00	0.86 - 1.16	> 0.99	1.000
5X	414	1.66	0.072	5.27	4.58 - 6.07	< 0.001	0.0000
10X	397	2.26	0.085	9.55	8.08 - 11.3	< 0.001	0.0000

Table S4. Competing risk analysis with subdistribution hazard model in Experiment 1 for embryonic survival relative to 1X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	394	-0.288	0.094	0.75	0.62 - 0.90	0.0023	0.0092
SW+M	398	0.000	0.076	1.00	0.86 - 1.16	1.0000	1.0000
5X	414	1.66	0.077	5.27	4.53 - 6.13	0.0000	0.0000
10X	397	2.26	0.089	9.54	8.01 - 11.4	0.0000	0.0000

Table S5. Competing risk analysis with subdistribution hazard model in Experiment 1 for embryonic survival relative to 5X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	394	-1.95	0.100	0.14	0.12 - 0.17	< 0.001	0.0000
SW+M	398	-1.66	0.072	0.19	0.16 - 0.22	< 0.001	0.0000
1X	414	-1.66	0.077	0.19	0.16 - 0.22	< 0.001	0.0000

10X	397	0.594	0.058	1.81	1.62 - 2.03	< 0.001	0.0000

Table S6. Competing risk analysis with subdistribution hazard model in Experiment 1 for embryonic survival relative to 10X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	**N**	**Coefficient**	**SE**	**HR**	**95% CI**	**p-value**	**Adj. p-value**
SW	394	-2.54	0.110	0.08	0.06 - 0.10	< 0.001	0.0000
SW+M	398	-2.26	0.085	0.10	0.09 - 0.12	< 0.001	0.0000
1X	414	-2.26	0.089	0.10	0.09 - 0.12	< 0.001	0.0000
5X	397	-0.594	0.058	0.55	0.49 - 0.62	< 0.001	0.0000

Table S7. Competing risk analysis with subdistribution hazard model in Experiment 2 for embryonic survival relative to SW+M control. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	**N**	**Coefficient**	**SE**	**HR**	**95% CI**	**p-value**	**Adj. p-value**
SW	301	-0.098	0.105	0.91	0.74 - 1.11	0.35	1.000
1X	292	0.625	0.106	1.87	1.52 - 2.60	< 0.001	2.52e-08
2X	289	0.301	0.099	1.35	1.11 - 1.64	0.002	0.0144
3X	291	-0.012	0.100	0.99	0.81 - 1.20	0.90	1.000
4X	299	1.05	0.086	2.86	2.42 - 3.39	< 0.001	0.0000
5X	299	1.17	0.094	3.22	2.68 - 3.88	< 0.001	0.0000

Table S8. Competing risk analysis with subdistribution hazard model in Experiment 2 for embryonic survival relative to 1X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	301	-0.723	0.108	0.49	0.39 - 0.60	< 0.001	1.44e-10
SW+M	291	-0.625	0.106	0.54	0.43 - 0.66	< 0.001	2.52e-08
2X	289	-0.324	0.102	0.72	0.59 - 0.88	0.002	0.009
3X	291	-0.637	0.104	0.53	0.43 - 0.65	< 0.001	4.50e-09
4X	299	0.427	0.087	1.53	1.29 - 1.82	< 0.001	5.70e-06
5X	299	0.545	0.096	1.72	1.43 - 2.08	< 0.001	7.80e-08

Table S9. Competing risk analysis with subdistribution hazard model in Experiment 2 for embryonic survival relative to 2X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	301	-0.399	0.101	0.67	0.55 - 0.82	< 0.001	4.32e-04
SW+M	291	-0.301	0.099	0.74	0.61 - 0.90	0.002	0.144
1X	292	0.324	0.102	1.38	1.13 - 1.69	0.002	0.009
3X	291	-0.313	0.095	0.73	0.61 - 0.88	0.001	0.006
4X	299	0.751	0.080	2.12	1.81 - 2.42	< 0.001	0.0000
5X	299	0.869	0.089	2.38	2.00 - 2.84	< 0.001	0.0000

Table S10. Competing risk analysis with subdistribution hazard model in Experiment 2 for embryonic survival relative to 3X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	301	-0.086	0.101	0.92	0.75 - 1.12	0.40	1.0000
SW+M	291	0.012	0.100	1.01	0.83 - 1.23	0.90	1.0000
1X	292	0.637	0.104	1.89	1.54 - 2.32	< 0.001	4.5e-09
2X	289	0.313	0.095	1.37	1.13 - 1.65	0.001	0.006
4X	299	1.06	0.082	2.90	2.47 - 3.40	< 0.001	0.0000
5X	299	1.18	0.091	3.26	2.73 - 3.90	< 0.001	0.0000

Table S11. Competing risk analysis with subdistribution hazard model in Experiment 2 for embryonic survival relative to 4X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	301	-1.15	0.088	0.32	0.27 - 0.38	0.0000	0.0000
SW+M	291	-1.05	0.089	0.35	0.30 - 0.41	0.0000	0.0000
1X	292	-0.427	0.087	0.65	0.55 - 0.77	9.5e-07	5.7e-06
2X	289	-0.751	0.080	0.47	0.40 - 0.55	0.0000	0.0000
3X	291	-1.06	0.082	0.35	0.29 - 0.41	0.0000	0.0000
5X	299	0.118	0.072	1.12	0.98 - 1.29	0.10	0.60

Table S12. Competing risk analysis with subdistribution hazard model in Experiment 2 for embryonic survival relative to 5X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	301	-1.27	0.097	0.28	0.23 - 0.34	0.0000	0.0000
SW+M	291	-1.17	0.094	0.31	0.26 - 0.37	0.0000	0.0000
1X	292	-0.545	0.096	0.58	0.48 - 0.70	1.3e-08	7.8e-08
2X	289	-0.869	0.089	0.42	0.35 - 0.50	0.0000	0.0000
3X	291	-1.18	0.091	0.31	0.26 - 0.37	0.0000	0.0000
4X	299	-0.118	0.072	0.89	0.77 - 1.02	0.10	0.60

Table S13. Cox proportional hazard model for naïve larval survival during Experiment 4 relative to SW+M control. P-values were Bonferroni corrected in adj. p-values. HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	HR	95% CI	p-value	Adj. p-value
SW	75	0.6431	1.903	0.97 - 3.75	0.0631	0.2526
1X	70	0.2888	1.335	0.67 - 265	0.409	1.0000
5X	74	5.660	287.2	101 - 814	1.708e-26	6.833e-26
10X	82	5.834	342.8	118 - 989	5.006e-27	2.002e-26

Table S14. Cox proportional hazard model for naïve larval survival during Experiment 4 relative to 1X PUA treatment. P-values were Bonferroni corrected in adj. p-values. HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	HR	95% CI	p-value	Adj. p-value
SW	75	0.3544	1.425	0.72 - 2.83	0.3116	1.0000
SW+M	78	-0.2888	0.7492	0.38 - 1.49	0.4091	1.0000
5X	74	5.371	215.2	76.2 - 608	3.801e-24	1.520e-23
10X	82	5.546	256.1	88.8 - 739	1.065e-24	4.261e-24

Table S15. Cox proportional hazard model for naïve larval survival during Experiment 4 relative to 5X PUA treatment. P-values were Bonferroni corrected in adj. p-values. HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	HR	95% CI	p-value	Adj. p-value
SW	75	-5.017	6.624e-03	0.00 - 0.02	1.619e-21	6.477e-21
SW+M	78	-5.660	3.482e-03	0.00 - 0.01	1.708e-26	6.833e-26
1X	70	-5.371	4.647e-03	0.00 - 0.01	3.801e-24	1.520e-23
10X	82	0.1740	1.190	0.60 - 2.35	0.615	1.0000

Table S16. Cox proportional hazard model for naïve larval survival during Experiment 4 relative to 10X PUA treatment. P-values were Bonferroni corrected in adj. p-values. HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	HR	95% CI	p-value	Adj. p-value
SW	75	-5.191	5.566e-03	0.00 - 0.02	4.368e-22	1.747e-21
SW+M	78	-5.834	2.926e-03	0.00 - 0.01	5.006e-27	2.002e-26
1X	70	-5.546	3.905e-03	0.00 - 0.01	1.065e-24	4.261e-24

5X	74	-0.174	0.8403	0.43 - 1.66	0.615	1.0000

Table S17. Competing risk analysis with subdistribution hazard model during Experiment 1 for hatch success rate relative to SW+M control. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	402	0.790	0.118	2.20	1.75 - 2.78	2.4e-11	9.60e-11
1X	398	0.236	0.132	1.27	0.98 - 1.64	0.074	0.296
5X	414	-10.8	0.110	0.00	0.00 - 0.00	0.0000	0.0000
10X	397	-10.8	0.110	0.00	0.00 - 0.00	0.0000	0.0000

Table S18. Competing risk analysis with subdistribution hazard model during Experiment 1 for hatch success rate relative to 1X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	402	0.554	0.112	1.74	1.40 - 2.17	7.7e-07	3.08e-06
SW+M	394	-0.236	0.132	0.79	0.61 - 1.02	0.074	0.296
5X	414	-11.0	0.103	0.00	0.00 - 0.00	0.0000	0.0000
10X	397	-11.0	0.103	0.00	0.00 - 0.00	0.0000	0.0000

Table S19. Competing risk analysis with subdistribution hazard model during Experiment 1 for hatch success rate relative to 5X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	402	11.6	0.084	108,095	91,679 - 127,450	0.0000	0.0000

SW+M	394	10.8	0.110	49,036	39,533 - 60,825	0.0000	0.0000
1X	398	11.0	0.103	62,117	50,803 - 75,950	0.0000	0.0000
10X	397	0.000	0.071	1.00	0.87 - 1.15	1.0000	1.0000

Table S20. Competing risk analysis with subdistribution hazard model during Experiment 1 for hatch success rate relative to 10X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	**N**	**Coefficient**	**SE**	**HR**	**95% CI**	**p-value**	**Adj. p-value**
SW	402	11.6	0.084	108,100	91,636 - 127,522	0.0000	0.0000
SW+M	394	10.8	0.110	49,039	39.523 - 60.845	0.0000	0.0000
1X	398	11.0	0.103	62,120	50,784 - 78,986	0.0000	0.0000
5X	414	0.000	0.071	1.00	0.87 - 1.15	1.0000	1.0000

Table S21. Competing risk analysis with subdistribution hazard model during Experiment 2 for hatch success rate relative to SW+M control. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	**N**	**Coefficient**	**SE**	**HR**	**95% CI**	**p-value**	**Adj. p-value**
SW	301	0.136	0.135	1.146	0.88 - 1.49	0.31	1.0000
1X	292	-0.862	0.186	0.4223	0.29 - 0.61	3.7e-06	2.22e-05
2X	289	-0.454	0.143	0.6349	0.48 - 0.84	1.4e-03	8.40e-03
3X	291	-0.588	0.146	0.5554	0.42 - 0.74	5.6e-05	3.36e-04
4X	299	-2.79	0.363	6.17e-02	0.03 - 0.13	1.6e-14	9.60e-14
5X	299	-2.37	0.299	9.31e-02	0.05 - 0.17	1.8e-15	1.08e-14

Table S22. Competing risk analysis with subdistribution hazard model during Experiment 2 for hatch success rate relative to 1X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	301	0.998	0.187	2.713	1.88 - 3.91	9.7e-08	5.82e-07
SW+M	291	0.862	0.186	2.368	1.64 - 3.41	3.7e-06	2.22e-05
2X	289	0.408	0.194	1.503	1.03 - 2.20	0.036	0.216
3X	291	0.274	0.197	1.315	0.89 - 1.93	0.16	0.960
4X	299	-1.92	0.386	0.1461	0.07 - 0.31	6.3e-07	3.78e-06
5X	299	-1.51	0.327	0.2204	0.12 - 0.42	3.6e-06	2.16e-05

Table S23. Competing risk analysis with subdistribution hazard model during Experiment 2 for hatch success rate relative to 2X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	301	0.590	0.144	1.805	1.36 - 2.39	4.1e-05	2.46e-04
SW+M	291	0.454	0.143	1.575	1.19 - 2.08	0.0014	8.40e-03
1X	292	-0.408	0.194	0.6652	0.45 - 0.97	0.036	0.216
3X	291	-0.134	0.145	0.8748	0.64 - 1.19	0.39	1.0000
4X	299	-2.33	0.367	0.0972	0.05 - 0.20	2.2e-10	1.32e-09
5X	299	-1.92	0.304	0.1466	0.08 - 0.24	2.6e-10	1.56e-09

Table S24. Competing risk analysis with subdistribution hazard model during Experiment 2 for hatch success rate relative to 3X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	301	0.724	0.147	2.063	1.55 - 2.75	8.8e-07	5.28e-06
SW+M	291	0.588	0.146	1.801	1.35 - 2.40	5.6e-05	3.36e-04
1X	292	-0.274	0.197	0.7604	0.52 - 1.12	0.16	0.96
2X	289	0.134	0.156	1.143	0.84 - 1.55	0.39	1.0000
4X	299	-2.20	0.369	0.1111	0.05 - 0.23	2.6e-09	1.56e-08
5X	299	-1.79	0.306	0.1676	0.09 - 0.31	5.2e-09	3.12e-08

Table S25. Competing risk analysis with subdistribution hazard model during Experiment 2 for hatch success rate relative to 4X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	301	2.92	0.363	18.56	9.11 - 97.8	8.9e-16	5.34e-15
SW+M	291	2.79	0.363	16.20	7.96 - 33.0	1.6e-14	9.60e-14
1X	292	1.92	0.386	6.843	3.21 - 14.6	6.3e-07	3.78e-06
2X	289	2.33	0.367	10.29	5.01 - 21.1	2.2e-10	1.32e-09
3X	291	2.20	0.369	8.999	4.37 - 18.5	2.6e-09	1.56e-08
5X	299	0.411	0.452	1.508	0.62 - 3.66	0.36	1.0000

Table S26. Competing risk analysis with subdistribution hazard model during Experiment 2 for hatch success rate relative to 5X PUA treatment. P-values were Bonferroni corrected in adj. p-values. SE: standard error; HR: hazard ratio; CI: confidence interval.

Treatment	N	Coefficient	SE	HR	95% CI	p-value	Adj. p-value
SW	301	2.51	0.299	12.31	6.85 - 22.1	0.0000	0.0000
SW+M	291	2.37	0.299	10.74	5.98 - 19.3	1.8e-15	1.08e-14
1X	292	1.51	0.327	4.537	2.39 - 8.60	3.6e-05	2.16e-05
2X	289	1.92	0.304	6.820	3.76 - 12.4	2.6e-10	1.56e-09
3X	291	1.79	0.306	5.966	3.28 - 10.9	5.2e-09	3.12e-08
4X	299	-0.411	0.452	0.6630	0.27 - 1.6.1	0.36	1.0000

Table S27. Pairwise comparison of marginal means of embryonic heart rates in Experiment 1. SE: standard error.

Contrast	ratio	SE	z ratio	p-value
SW / SW+M	1.02	3.63e-02	0.616	0.8116
SW / 1X	1.05	3.72e-02	1.508	0.2871
SW+M / 1X	1.03	3.62e-02	0.893	0.6448

Table S28. Summary of marginal means of embryonic heart rates in Experiment 1. SE: standard error; CL: confidence level.

Treatment	Response	SE	Lower CL	Upper CL
SW	130	3.27	123	136
SW+M	127	3.17	121	133
1X	123	3.03	117	129

Table S29. Pairwise comparison of marginal means of embryonic heart rates in Experiment 2. SE: standard error.

Contrast	Estimate	SE	z ratio	p-value
SW – (SW+M)	4.28e-03	4.24e-02	0.101	1.0000
SW – 1X	8.190e-02	4.65e-02	1.762	0.5741
SW – 2X	2.865e-02	4.22e-02	0.680	0.9937
SW – 3X	-1.483e-02	4.25e-02	-0.349	0.9999
SW – 4X	-5.913e-02	4.30e-02	-1.376	0.8149
SW – 5X	8.55e-03	4.73e-02	0.181	1.0000
(SW+M) – 1X	7.761e-02	4.65e-02	1.671	0.6358
(SW+M) – 2X	2.437e-02	4.21e-02	0.578	0.9974
(SW+M) – 3X	-1.912e-02	4.25e-02	-0.450	0.9994
(SW+M) – 4X	-6.341e-02	4.29e-02	-1.477	0.7589
(SW+M) – 5X	4.27e-03	4.73e-02	0.090	1.0000
1X – 2X	-5.325e-02	4.63e-02	-1.151	0.9121
1X – 3X	-9.673e-02	4.66e-02	-2.075	0.3674
1X – 4X	-0.1410	4.70e-02	-3.001	0.0429
1X – 5X	-7.334e-02	5.10e-02	-1.438	0.7811
2X – 3X	-4.349e-02	4.23e-02	-1.028	0.9477
2X – 4X	-8.778e-02	4.27e-02	-2.054	0.3802
2X – 5X	-2.010e-02	4.71e-02	-0.427	0.9995

3X – 4X	-4.429e-02	4.31e-02	-1.028	0.9478
3X – 5X	2.339e-02	4.74e-02	0.493	0.9990
4X – 5X	6.768e-02	4.78e-02	1.415	0.7938

Table S30. Summary of marginal means of embryonic heart rates in Experiment 2. SE: standard error; CL: confidence level.

Treatment	**Emmean**	**SE**	**Lower CL**	**Upper CL**
SW	107.4	3.22	101.2	114
SW+M	106.9	3.20	100.8	113
1X	98.9	3.51	92.3	106
2X	104.3	3.09	98.5	111
3X	109.0	3.29	102.7	116
4X	113.9	3.51	107.2	121
5X	106.5	3.90	99.1	114

Table S31. Pairwise comparison of marginal means of embryonic heart rates of naïve embryos in Experiment 3. SE: standard error.

Contrast	**Estimate**	**SE**	**z ratio**	**p-value**
SW – (SW+M)	0.0901	0.123	0.735	0.9776
SW – 1X	0.1280	0.123	1.041	0.9040
SW – 3X	0.0584	0.123	0.476	0.9970
SW – 5X	0.5665	0.125	4.528	0.0001

SW – 10X	1.481	0.215	6.873	< 0.0001
(SW+M) – 1X	0.0379	0.122	0.310	0.9996
(SW+M) – 3X	-0.0317	0.122	-0.259	0.9998
(SW+M) – 5X	0.4764	0.124	3.830	0.0018
(SW+M) – 10X	1.391	0.215	6.468	< 0.0001
1X – 3X	-0.0695	0.122	-0.568	0.9931
1X – 5X	0.4386	0.125	3.516	0.0058
1X – 10X	1.353	0.215	6.285	< 0.0001
3X – 5X	0.5081	0.125	4.076	0.0007
3X – 10X	1.423	0.215	6.609	< 0.0001
5X – 10X	0.9144	0.217	4.233	0.0003

Table S32. Summary of marginal means of embryonic heart rates of naïve embryos in Experiment 3. SE: standard error; CL: confidence level.

Treatment	**Emmean**	**SE**	**Lower CL**	**Upper CL**
SW	171.8	14.97	144.9	203.8
SW+M	157.0	13.53	132.6	185.9
1X	151.2	13.10	127.6	179.2
3X	162.1	14.03	136.8	192.1
5X	97.5	8.75	81.8	116.3
10X	39.1	7.70	26.6	57.5

Table S33. Pairwise comparison of marginal means of yolk area of surf smelt at hatch in Experiment 1. SE: standard error.

Contrast	Estimate	SE	t ratio	p-value
SW – SW+M	-7.32e-04	3.70e-04	-1.978	0.1796
SW – 1X	-9.72e-04	3.29e-04	-2.957	0.0432
SW+M – 1X	-2.40e-04	3.9e-04	-0.618	0.8144

Table S34. Summary of marginal means of yolk area of surf smelt at hatch in Experiment 1. SE: standard error; CL: confidence level.

Treatment	Emmean	SE	Lower CL	Upper CL
SW	1.45e-02	2.17e-04	1.40e-02	1.50e-02
SW+M	1.52e-02	2.99e-04	1.45e-02	1.59e-02
1X	1.55e-02	2.48e-04	1.49e-02	1.60e-02

Table S35. Pairwise comparison of marginal means of yolk area of surf smelt at hatch in Experiment 2. SE: standard error.

Contrast	Estimate	SE	t ratio	p-value
SW – (SW+M)	-1.41e-04	1.93e-04	-0.733	0.9904
SW – 1X	-5.95e-04	2.73e-04	-2.178	0.3100
SW – 2X	-6.80e-04	2.35e-04	-2.898	0.0604
SW – 3X	-1.38e-03	2.71e-04	-5.110	< 0.0001
SW – 4X	-1.57e-03	5.01e-04	-3.132	0.0308
SW – 5X	-1.66e-03	5.29e-04	-3.146	0.0295

(SW+M) – 1X	-4.53e-04	2.62e-04	-1.734	0.5939
(SW+M) – 2X	-5.39e-04	2.19e-04	-2.464	0.1761
(SW+M) – 3X	-1.24e-03	2.56e-04	-4.865	< 0.0001
(SW+M) – 4X	-1.43e-03	4.89e-04	-2.921	0.0567
(SW+M) – 5X	-1.52e-03	5.18e-04	-2.939	0.0540
1X – 2X	-8.56e-05	2.75e-04	-0.312	0.9999
1X – 3X	-7.90e-04	3.04e-04	-2.601	0.1287
1X – 4X	-9.75e-04	5.13e-04	-1.901	0.4807
1X – 5X	-1.07e-03	5.41e-04	-1.975	0.4323
2X – 3X	-7.04e-04	2.65e-04	-2.654	0.1135
2X – 4X	-8.89e-04	4.90e-04	-1.814	0.5397
2X – 5X	-9.83e-04	5.20e-04	-1.891	0.4879
3X – 4X	-1.85e-04	5.06e-04	-0.366	0.9998
3X – 5X	-2.79e-04	5.35e-04	-0.521	0.9985
4X – 5X	-9.34e-05	6.74e-04	-0.139	1.0000

Table S36. Summary of marginal means of yolk area of surf smelt at hatch in Experiment 2. SE: standard error; CL: confidence level.

Treatment	**Emmean**	**SE**	**Lower CL**	**Upper CL**
SW	0.0149	1.42e-04	0.0147	0.0152
SW+M	0.0151	1.34e-04	0.0148	0.0153

1X	0.0155	2.23e-04	0.0151	0.0160
2X	0.0156	1.70e-04	0.0153	0.0160
3X	0.0163	2.15e-04	0.0159	0.0168
4X	0.0165	4.69e-04	0.0156	0.0174
5X	0.0166	4.99e-04	0.0156	0.0176

Table S37. Pairwise comparison of marginal means of oil globule area of surf smelt at hatch in Experiment 2. SE: standard error.

Contrast	**Estimate**	**SE**	**t ratio**	**p-value**
SW – (SW+M)	-2.55e-04	9.96e-05	-2.558	0.1425
SW – 1X	-2.60e-04	1.29e-04	-2.012	0.4092
SW – 2X	-4.78e-04	1.12e-04	-4.276	0.0005
SW – 3X	-6.07e-04	1.26e-04	-4.802	< 0.0001
SW – 4X	-9.17e-04	2.14e-04	-4.289	0.0005
SW – 5X	-6.00e-04	2.27e-04	-2.638	0.1181
(SW+M) – 1X	-5.70e-06	1.21e-04	-0.047	1.0000
(SW+M) – 2X	-2.23e-04	1.01e-04	-2.209	0.2934
(SW+M) – 3X	-3.52e-04	1.17e-04	-3.022	0.0426
(SW+M) – 4X	-6.63e-04	2.06e-04	-3.212	0.0241
(SW+M) – 5X	-3.45e-04	2.20e-04	-1.569	0.7024
1X – 2X	-2.18e-04	1.24e-04	-1.752	0.5815

1X – 3X	-3.47e-04	1.36e-04	-2.542	0.1479
1X – 4X	-6.57e-04	2.17e-04	-3.030	0.0417
1X – 5X	-3.39e-04	2.30e-04	-1.473	0.7606
2X – 3X	-1.29e-04	1.18e-04	-1.096	0.9291
2X – 4X	-4.39e-04	2.05e-04	-2.140	0.3315
2X – 5X	-1.22e-04	2.20e-04	-0.554	0.9980
3X – 4X	-3.10e-04	2.12e-04	-1.463	0.7663
3X – 5X	7.42e-06	2.26e-04	0.033	1.0000
4X – 5X	3.18e-04	2.81e-04	1.130	0.9184

Table S38. Summary of marginal means of oil globule area of surf smelt at hatch in Experiment 2. SE: standard error; CL: confidence level.

Treatment	Emmean	SE	Lower CL	Upper CL
SW	7.81e-03	7.16e-05	7.67e-03	7.95e-03
SW+M	8.07e-03	6.68e-05	7.94e-03	8.20e-03
1X	8.07e-03	1.03e-04	7.87e-03	8.28e-03
2X	8.29e-03	7.88e-05	8.14e-03	8.45e-03
3X	8.42e-03	9.76e-05	8.23e-03	8.61e-03
4X	8.73e-03	1.97e-04	8.34e-03	9.12e-03
5X	8.41e-03	2.11e-04	8.00e-03	8.83e-03

Table S39. Pairwise comparison of marginal means of standard length of surf smelt at hatch in Experiment 1. SE: standard error.

Contrast	Estimate	SE	t ratio	p-value
SW – SW+M	0.224	0.124	1799	0.1716
SW - 1X	0.357	0.108	3.298	0.0031
SW+M – 1X	0.133	0.129	1.029	0.5591

Table S40. Summary of marginal means of standard length of surf smelt at hatch in Experiment 1. SE: standard error; CL: confidence level.

Treatment	Emmean	SE	Lower CL	Upper CL
SW	5.74	0.0724	5.59	5.88
SW+M	5.51	0.1019	5.31	5.71
1X	5.38	0.0810	5.22	5.54

Table S41. Pairwise comparison of marginal means of standard length of surf smelt at hatch in Experiment 2. SE: standard error.

Contrast	Estimate	SE	t ratio	p-value
SW – (SW+M)	0.1663	0.113	1.471	0.7549
SW – 1X	0.3679	0.155	2.367	0.3017
SW – 2X	0.4460	0.124	3.592	0.0524
SW – 3X	0.4655	0.122	3.829	0.0368
SW – 4X	0.4722	0.163	2.889	0.1478
SW – 5X	0.6889	0.164	4.204	0.0211

(SW+M) – 1X	0.2016	0.156	1.295	0.8402
(SW+M) – 2X	0.2797	0.124	2.252	0.3489
(SW+M) – 3X	0.2992	0.121	2.469	0.2644
(SW+M) – 4X	0.3059	0.162	1.884	0.5295
(SW+M) – 5X	0.5226	0.163	3.207	0.0929
1X – 2X	0.0781	0.162	0.481	0.9986
1X – 3X	0.0976	0.160	0.610	0.9949
1X – 4X	0.1043	0.193	0.541	0.9973
1X – 5X	0.3210	0.193	1.660	0.6526
2X – 3X	0.0195	0.129	0.151	1.0000
2X – 4X	0.0262	0.168	0.156	1.0000
2X – 5X	0.2429	0.169	1.440	0.7710
3X – 4X	0.0067	0.165	0.040	1.0000
3X – 5X	0.2234	0.166	1.345	0.8174
4X – 5X	0.2167	0.197	1.098	0.9152

Table S42. Summary of marginal means of standard length of surf smelt at hatch in Experiment 2. SE: standard error; CL: confidence level.

Treatment	**Emmean**	**SE**	**Lower CL**	**Upper CL**
SW	5.21	0.0786	5.05	5.38
SW+M	5.05	0.0810	4.87	5.23

1X	4.84	0.1330	4.55	5.14
2X	4.77	0.0943	4.56	4.98
3X	4.75	0.0903	4.55	4.95
4X	4.74	0.1409	4.43	505
5X	4.52	0.1415	4.21	4.84

Table S43. Pairwise comparison of marginal means of deepest body depth of surf smelt at hatch in Experiment 1. SE: standard error.

Contrast	**Estimate**	**SE**	**t ratio**	**p-value**
SW – SW+M	-0.0351	0.0178	-1.975	0.1805
SW - 1X	-0.0264	0.0155	-1.697	0.2641
SW+M – 1X	0.0087	0.0187	0.466	0.8888

Table S44. Summary of marginal means of deepest body depth of surf smelt at hatch in Experiment 1. SE: standard error; CL: confidence level.

Treatment	**Emmean**	**SE**	**Lower CL**	**Upper CL**
SW	0.778	0.0103	0.755	0.801
SW+M	0.813	0.145	0.780	0.847
1X	0.805	0.117	0.778	0.832

Table S45. Pairwise comparison of marginal means of deepest body depth of surf smelt at hatch in Experiment 2. SE: standard error.

Contrast	Estimate	SE	t ratio	p-value
SW – (SW+M)	-2.398e-03	0.0107	-0.223	1.0000
SW – 1X	-1.263e-02	0.0148	-0.852	0.9791
SW – 2X	-3.398e-02	0.0129	-2.629	0.1207
SW – 3X	-3.485e-02	0.0146	-2.393	0.2048
SW – 4X	-8.517e-02	0.0248	-3.432	0.0119
SW – 5X	-5.06`e-02	0.0262	-1.931	0.4614
(SW+M) – 1X	-1.024e-02	0.0141	-0.723	0.9911
(SW+M) – 2X	-3.158e-02	0.0120	-2.642	0.1170
(SW+M) – 3X	-3.245e-02	0.0137	-2.360	0.2189
(SW+M) – 4X	-8.278e-02	0.0241	-3.441	0.0115
(SW+M) – 5X	-4.821e-02	0.0255	-1.888	0.4895
1X – 2X	-2.134e-02	0.0143	-1.497	0.7466
1X – 3X	-2.221e-02	0.0158	-1.402	0.8008
1X – 4X	-7.254e-02	0.0250	-2.901	0.0598
1X – 5X	-3.797e-02	0.0265	-1.435	0.7825
2X – 3X	-8.69e-04	0.0137	-0.063	1.0000
2X – 4X	-5.120e-02	0.0236	-2.169	0.3154
2X – 5X	-1.663e-02	0.0252	-0.661	0.9945

3X – 4X	-5.033e-02	0.0247	-2.041	0.3905
3X – 5X	-1.576e-02	0.0261	-0.603	0.9967
4X – 5X	3.457e-02	0.0323	1.070	0.9365

Table S46. Summary of marginal means of deepest body depth of surf smelt at hatch in Experiment 2. SE: standard error; CL: confidence level.

Treatment	**Emmean**	**SE**	**Lower CL**	**Upper CL**
SW	0.787	8.31e-03	0.771	0.804
SW+M	0.790	7.80e-03	0.774	0.805
1X	0.800	1.176e-02	0.777	0.823
2X	0.821	8.73e-03	0.804	0.838
3X	0.822	1.154e-02	0.800	0.844
4X	0.872	2.254e-02	0.828	0.917
5X	0.838	2.412e-02	0.790	0.885

Table S47. Pairwise comparison of marginal means of body depth at anus of surf smelt at hatch in Experiment 1. SE: standard error.

Contrast	**Estimate**	**SE**	**t ratio**	**p-value**
SW – SW+M	0.000649	0.00683	0.095	0.9950
SW - 1X	0.006485	0.00596	1.088	0.5224
SW+M – 1X	0.005836	0.00751	0.777	0.7172

Table S48. Summary of marginal means of body depth at anus of surf smelt at hatch in Experiment 1. SE: standard error; CL: confidence level.

Treatment	Emmean	SE	Lower CL	Upper CL
SW	0.359	3.64e-03	0.352	0.366
SW+M	0.358	5.73e-03	0.347	0.370
1X	0.353	4.74e-03	0.343	0.362

Table S49. Pairwise comparison of marginal means of body depth at anus of surf smelt at hatch in Experiment 2. SE: standard error.

Contrast	Estimate	SE	t ratio	p-value
SW – (SW+M)	1.745e-02	6.59e-03	2.648	0.1151
SW – 1X	1.723e-02	9.33e-03	1.847	0.5172
SW – 2X	1.076e-02	8.02e-03	1.341	0.8320
SW – 3X	2.009e-02	9.26e-03	2.170	0.3147
SW – 4X	-2.154e-02	1.713e-02	-1.257	0.8707
SW – 5X	4.261e-02	1.806e-02	2.359	0.2195
(SW+M) – 1X	-2.15e-04	8.94e-03	-0.024	1.0000
(SW+M) – 2X	-6.688e-03	7.48e-03	-0.894	0.9732
(SW+M) – 3X	2.642e-03	8.73e-03	0.303	0.9999
(SW+M) – 4X	-3.899e-02	1.671e-02	-2.333	0.2315
(SW+M) – 5X	2.516e-02	1.770e-02	1.422	0.7897
1X – 2X	-6.473e-03	9.38e-03	-0.690	0.9931

1X – 3X	2.857e-03	1.037e-02	0.275	1.0000
1X – 4X	-3.877e-02	1.752e-02	-2.213	0.2914
1X – 5X	2.538e-02	1.848e-02	1.373	0.8158
2X – 3X	9.33e-03	9.07e-03	1.029	0.9470
2X – 4X	-3.230e-02	1.676e-02	-1.927	0.4636
2X – 5X	3.185e-02	1.776e-02	1.793	0.5536
3X – 4X	-4.163e-02	1.730e-02	-2.406	0.1993
3X – 5X	2.252e-02	1.828e-02	1.232	0.8813
4X – 5X	6.415e-02	2.302e-02	2.787	0.0813

Table S50. Summary of marginal means of body depth at anus of surf smelt at hatch in Experiment 2. SE: standard error; CL: confidence level.

Treatment	**Emmean**	**SE**	**Lower CL**	**Upper CL**
SW	0.367	4.85e-03	0.357	0.377
SW+M	0.350	4.60e-03	0.341	0.359
1X	0.350	7.62e-03	0.335	0.365
2X	0.356	5.82e-03	0.345	0.368
3X	0.347	7.36e-03	0.332	0.361
4X	0.389	1.602e-02	0.357	0.420
5X	0.324	1.704e-02	0.291	0.358

Table S51. Pairwise comparison of marginal means of eye area of surf smelt at hatch in Experiment 2. SE: standard error.

Contrast	Estimate	SE	z ratio	p-value
SW – (SW+M)	0.0293	0.0234	1.250	0.8744
SW – 1X	0.0151	0.0349	0.432	0.9995
SW – 2X	0.0737	0.0267	2.760	0.0841
SW – 3X	0.1310	0.0277	4.735	< 0.0001
SW – 4X	0.1245	0.0332	3.752	0.0033
SW – 5X	0.2437	0.0588	4.147	0.0007
(SW+M) – 1X	-0.0142	0.0350	-0.407	0.9997
(SW+M) – 2X	0.0444	0.0266	1.673	0.6343
(SW+M) – 3X	0.1017	0.0273	3.722	0.0037
(SW+M) – 4X	0.0952	0.0326	2.925	0.0534
(SW+M) – 5X	0.2144	0.0585	3.663	0.0047
1X – 2X	0.0587	0.0366	1.601	0.6820
1X – 3X	0.1159	0.0372	3.116	0.0303
1X – 4X	0.1094	0.0410	2.667	0.1068
1X – 5X	0.2287	0.0637	3.592	0.0061
2X – 3X	0.0573	0.0292	1.963	0.4384
2X – 4X	0.0508	0.0339	1.499	0.7456
2X – 5X	0.1700	0.0594	2.863	0.0637

3X – 4X	-0.0065	0.0342	-0.190	1.0000
3X – 5X	0.1127	0.0597	1.889	0.4873
4X – 5X	0.1192	0.0620	1.923	0.4649

Table S52. Summary of marginal means of eye area of surf smelt at hatch in Experiment 2. SE: standard error; CL: confidence level.

Treatment	**Emmean**	**SE**	**Lower CL**	**Upper CL**
SW	34793	561	33710	35911
SW+M	33789	572	32686	34930
1X	34273	1049	32278	36391
2X	32321	666	31041	33653
3X	30521	662	29252	31846
4X	30720	860	29080	32453
5X	27268	1529	24429	30436

Table S53. Pairwise comparison of marginal means of otic capsule area of surf smelt at hatch in Experiment 2. SE: standard error.

Contrast	**ratio**	**SE**	**z ratio**	**p-value**
SW / (SW+M)	1.016	0.0183	0.857	0.9787
SW / 1X	1.025	0.0244	1.051	0.9420
SW / 2X	1.022	0.0207	1.072	0.9364
SW / 3X	1.062	0.0252	2.541	0.1449

SW / 4X	1.006	0.0376	0.166	1.0000
SW / 5X	1.022	0.0405	0.548	0.9981
(SW+M) / 1X	1.010	0.0222	0.436	0.9995
(SW+M) / 2X	1.006	0.0180	0.354	0.9998
(SW+M) / 3X	1.046	0.0226	2.070	0.3706
(SW+M) / 4X	0.991	0.0353	-0.258	1.0000
(SW+M) / 5X	1.006	0.0383	0.165	1.0000
1X / 2X	0.997	0.0223	-0.147	1.0000
1X / 3X	1.036	0.0264	1.380	0.8128
1X / 4X	0.981	0.0370	-0.498	0.9989
1X / 5X	0.997	0.0400	-0.083	1.0000
2X / 3X	1.039	0.0226	1.771	0.5678
2X / 4X	0.985	0.0348	-0.439	0.9995
2X / 5X	1.000	0.0378	-0.001	1.0000
3X / 4X	0.947	0.0354	-1.445	0.7773
3X / 5X	0.962	0.0382	-0.971	0.9602
4X / 5X	1.016	0.0490	0.321	0.9999

Table S54. Summary of marginal means of otic capsule area of surf smelt at hatch in Experiment 2. SE: standard error; CL: confidence level.

Treatment	Emmean	SE	Lower CL	Upper CL
SW	26957	356	26268	27664
SW+M	26545	313	26938	27166
1X	26291	496	25337	27281
2X	26378	366	25670	27104
3X	25382	469	24479	26318
4X	26790	909	25065	28633
5X	26379	960	24562	28330