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RESEARCH ARTICLE

Salt sensitivity of the morphometry of *Artemia franciscana* during development: a demonstration of 3D critical windows

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ABSTRACT

A 3D conceptual framework of 'critical windows' was used to examine whether the morphometry of *Artemia franciscana* is altered by salinity exposure during certain key periods of development. *Artemia franciscana* were hatched at 20 ppt (designated control salinity) and were then exposed to 10, 30, 40 or 50 ppt either chronically (days 1–15) or only on days 1–6, 7–9, 10–12 or 13–15. On day 15, maturity was assessed and morphometric characteristics, including mass, total body length, tail length and width, length of the third swimming appendage and eye diameter, were measured. Maturation and morphometry on day 15 were influenced by the exposure window and salinity dose. *Artemia franciscana* were generally larger following exposure to 10 and 40 ppt during days 1–6 and 7–9 when compared with days 10–12 and 13–15, in part due to a higher percentage of mature individuals. Exposure to different salinities on days 1–6 produced the greatest differences in morphometry, and thus this appears to be a period in development when *A. franciscana* is particularly sensitive to salinity. Viewing the developmental window as three-dimensional allowed more effective visualization of the complex interactions between exposure window, stressor dose and the magnitude of morphometric changes in *A. franciscana*.

KEY WORDS: Brine shrimp, Growth, Phenotype, Salinity, Maturation, Plasticity

INTRODUCTION

Developmental phenotypic plasticity involves the ability of a developing animal to alter its phenotype in response to intrinsic (genetic) or extrinsic (environmental) variables (Pigliucci et al., 2006; Burggren and Reyna, 2011; Hutchings, 2011). For example, during development, periods may exist when the emerging phenotype of an animal is particularly plastic or susceptible to an environmental stressor – these periods are often termed 'critical windows' or 'sensitive periods' (Pinkerton and Joad, 2000; Rice and Barone, 2000; van Aerle et al., 2002; Chan and Burggren, 2005; Hogan et al., 2008; Burggren and Reyna, 2011; Burggren et al., 2014; Burggren and Mueller, 2015). Across a range of animal taxa, from invertebrates to mammals, exposure to environmental, chemical or pharmacological stressors at certain time points of development is utilized to detect and understand critical windows during development (Green et al., 1986; Dzialowski et al., 2002; Maack and Segner, 2004; Chan and Burggren, 2005; Liu and Wong-Riley, 2010; Yuan et al., 2011; Tate et al., 2015). Phenotypic changes during critical windows have potential long-term implications for the animal's biology, ranging from molecular to morphological and physiological levels. Thus, study of how a phenotype is altered during a critical window is important not just for understanding developmental processes but also for revealing links between conditions during development and an animal's phenotype later in life (Burggren and Warburton, 2005).

Recently, the importance of critical windows and the experimental approaches used to investigate them have come into sharper focus (Burggren and Mueller, 2015; Mueller et al., 2015). The search for critical windows typically involves exposure to a particular stressor during distinct, separate periods of development. When phenotypic modification is detected, a critical window is then defined by the length of the preceding exposure period. Typical of studies that use this approach is the employment of a single dose of a stressor, and this approach contributes to how accurately a critical window is defined. A low dose of a stressor may detect that a critical window is present but a higher dose may reveal that the window is larger. This can occur because a higher dose induces an effect earlier in development that may also persist for longer (Burggren and Mueller, 2015; Mueller et al., 2015). While the presence or absence of phenotypic changes are reported, demonstration of the magnitude of the response in relation to exposure window and stressor dose is limited to a mere handful of studies (e.g. Degitz et al., 2000; Lavolpe et al., 2004; Hu et al., 2009; Yuan et al., 2011). Therefore, while typical studies of critical windows are useful for detecting periods of plasticity or susceptibility, they are limited in their ability to fully appreciate the phenotypic changes displayed by a developing animal.

In contrast to conventional approaches in critical window identification, a three-dimensional (3D) approach that incorporates different exposure windows and stressor doses, and examines the magnitude of the phenotype change, provides an opportunity to advance how critical windows are assessed (Burggren and Mueller, 2015). The 3D approach not only examines when a critical window is present, or how large it is, but also visualizes the interaction between the three important variables of time, dose and effect size.

Testing the 3D concept of critical windows is theoretically straightforward but demands large amounts of data. Such testing thus benefits from the use of animals that can be obtained and housed in large numbers and that have relatively rapid development times. Brine shrimp (*Artemia* spp.) can be hatched in large numbers as necessary using stored dormant cysts, hatched animals are at the same developmental stage (1st instar), they have rapid development times, and care and space requirements during development are relatively simple (Ward-Booth and Reiss, 1988; Dockery and Tomkins, 2000). *Artemia* is the only genus of saline invertebrate that is commercially harvested as embryos for use in aquaculture, and thus it is an excellent model for both basic and applied developmental research (Neumeyer et al., 2015). Furthermore, *Artemia* are remarkable osmoregulators, inhabiting salinities that range from as low as 10 ppt up to extreme levels of approximately

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250 ppt (Persoone and Sorgeloos, 1980). Previous research has indicated that phenotypic modification of *Artemia* occurs with altered environmental conditions (salinity, temperature, food availability) during development (Vanhaecke et al., 1984; Abatzopoulos et al., 2003; El-Bermawi et al., 2004; Pinto et al., 2013). We have previously examined the time-dependent effects of different levels of salinity on survival during development in *Artemia franciscana* (Burggren and Mueller, 2015; Table 1). We interpreted survival in the context of a 3D critical window, revealing that phenotypic modification is not necessarily constant across all of development, a finding that would not have been revealed with a chronic exposure study. Therefore, *A. franciscana* is an excellent model organism to test in detail the 3D critical window construct by examining the interaction between the dose of an environmental stressor and the exposure window on the magnitude of a phenotypic modification.

Several studies have indicated that survival, reproductive characteristics, life span, respiration, growth and morphology of *Artemia* all change following chronic exposure to different salinities (Conte, 1984; Triantaphyllidis et al., 1995; Browne and Wanigasekera, 2000; El-Bermawi et al., 2004; Neumeyer et al., 2015). However, it remains to be tested whether the morphometric phenotype of adult *Artemia* is altered following exposure to different salinities during specific windows of development. Thus, the aim of this study was to apply the 3D critical window construct to morphometric characteristics of *A. franciscana*, hatched from commercially available San Francisco Bay cysts, following a critical window experimental design that involved exposure to salinities of 10, 20, 30, 40 and 50 ppt during one of four windows over a 15 day development period at 25°C. Previously, we showed that survival was salinity dependent with exposures on days 1–6 and 7–9 of development, but less so with exposures on days 10–12 and 13–15 (Burggren and Mueller, 2015). High salinity exposure early in development (days 1–9) reduces survival and may also result in small, slowly growing individuals; alternatively, a selective effect may occur, in which the best individuals survive and grow large once returned to control conditions later in development. We hypothesized that high salinity exposure early in development (days 1–9) would reduce survival and produce a smaller morphometric phenotype on day 15, while low salinity exposure early in development would increase survival and produce larger individuals. Thus, we predicted a correlation between survival and the morphometric phenotype. We also hypothesized that the effect of salinity on morphometry would be less pronounced with exposure later in development (days 10–15).

Table 1. Critical window experimental design consisting of 21 different populations of *Artemia franciscana* showing the onset, termination, timing and dose of salinity exposure during development

	Exposure window (days)				
Population	1–6	7–9	10–12	13–15	Survival (%)
1	10 ppt	10 ppt	10 ppt	10 ppt	51.8±4.4
2	20 ppt	20 ppt	20 ppt	20 ppt	61.2±4.0
3	30 ppt	30 ppt	30 ppt	30 ppt	37.7±7.1
4	40 ppt	40 ppt	40 ppt	40 ppt	8.34±3.0
5	50 ppt	50 ppt	50 ppt	50 ppt	0
6	10 ppt	20 ppt	20 ppt	20 ppt	62.3±7.4
7	30 ppt	20 ppt	20 ppt	20 ppt	31.4±14.8
8	40 ppt	20 ppt	20 ppt	20 ppt	15.5±7.6
9	50 ppt	20 ppt	20 ppt	20 ppt	7.6±6.2
10	20 ppt	10 ppt	20 ppt	20 ppt	68.1±5.3
11	20 ppt	30 ppt	20 ppt	20 ppt	50.0±3.8
12	20 ppt	40 ppt	20 ppt	20 ppt	14.4±6.8
13	20 ppt	50 ppt	20 ppt	20 ppt	18.8±6.7
14	20 ppt	20 ppt	10 ppt	20 ppt	31.0±12.1
15	20 ppt	20 ppt	30 ppt	20 ppt	35.6±13.0
16	20 ppt	20 ppt	40 ppt	20 ppt	24.0±6.0
17	20 ppt	20 ppt	50 ppt	20 ppt	16.9±6.2
18	20 ppt	20 ppt	20 ppt	10 ppt	38.7±12.4
19	20 ppt	20 ppt	20 ppt	30 ppt	37.7±7.1
20	20 ppt	20 ppt	20 ppt	40 ppt	28.9±5.8
21	20 ppt	20 ppt	20 ppt	50 ppt	33.5±6.4

Light and dark shading show salinities below and above control values of 20 ppt, respectively. Day 1, *A. franciscana* at 1st instar (nauplius) stage (Heath, 1924). Survival (means±s.e.m.) at the end of each exposure is as previously published (fig. 7 in Burggren and Mueller, 2015).

MATERIALS AND METHODS

Artemia hatchery

A hatchery was created using the top half of a 2 l plastic bottle, inverted and held in place in a sand-filled jar. The bottle was filled with 800 ml of 20 ppt saline water, prepared using sea salt (Instant Ocean®, Blacksbury, VA, USA) and reverse osmosis water, buffered to pH 7 with sodium bicarbonate. A salinity of 20 ppt was chosen as the hatching medium because previous studies have shown that hatching of cysts occurs within 24 h at salinities of ~10–35 ppt (Jennings and Whitaker, 1941; Neumeyer et al., 2015), and we had the highest hatching success at 20 ppt in preliminary experiments. Salinity was checked using a hydrometer (Marineland, Blacksbury, VA, USA) and salinity probe (YSI Pro Plus, YSI Incorporated, Yellow Springs, OH, USA) and pH was checked using pH paper (pHydrion®, Micro Essential Laboratory, Brooklyn, NY, USA). After establishment of the appropriate water mix, approximately 700 mg to 1 g of commercially available *Artemia franciscana* Kellogg 1906 cysts from San Francisco Bay, CA, USA (San Francisco Bay Brand, Newark, CA, USA), were added to the hatchery. The hatchery was housed in a 2-door constant temperature chamber with an electronic temperature control (Ranco, Plain City, OH, USA) set to 25±1°C. Tubing attached to a small aquarium pump was placed into the bottom of the hatchery to create a gentle rolling boil of the water and cysts, and the top of the hatchery was covered with plastic wrap to reduce evaporation.

After approximately 24 h of incubation in 20 ppt water, the air line was removed, allowing the unhatched cysts to settle on the bottom of the hatchery and the cyst casings from hatched nauplii to float on the surface. Newly hatched nauplii were then siphoned out of the middle of the hatchery into a beaker and held at 25±1°C.

Salinity exposure during development

Developing *A. franciscana* were exposed to one of five salinities during four developmental exposure windows, as described in Table 1. The entirety of growth in 20 ppt water was designated as the overall control, because this salinity produced the highest survival in preliminary experiments and was consistent with the hatching salinity we used. Additionally, *A. franciscana* were exposed to 10, 30, 40 or 50 ppt either chronically from days 1 to 15 (treatments 1–5, Table 1), or during days 1–6, 7–9, 10–12 or 13–15 (treatments 5–21, Table 1). The salinity range of 10–50 ppt was chosen after preliminary experiments indicated that salinities above 50 ppt resulted in very high mortality of our newly hatched nauplii. Salinity stocks were created 24 h before use using reverse osmosis water, Instant Ocean® sea salt and sodium bicarbonate. Salinity and pH were checked as described above for the hatchery. The exposure periods used corresponded approximately to the following stages of Heath (1924): days 1–6: 1st to 6th instar; days 7–9: 7th to 8th instar; days 10–12: 9th to 10th instar; days 13–15: 11th to 12th instar

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(reproductive adults). However, these stages are only a guide because different environmental conditions, including salinity, can alter the developmental progression and molting of *Artemia* (Weisz, 1946).

Approximately 1 ml of densely congregated hatched nauplii (~600–800) was added to one of five, 15 l tanks housed within the thermostated environmental chamber. Large tanks were used during the initial window to ensure the highest survival at the beginning of the experiment. Each tank was filled with 2 l of stock (10, 20, 30, 40 or 50 ppt salinity) and 15 ml food mix consisting of 10 mg ml^{-1} *Spirulina* powder (Ta Aquaculture, Malta) and 2–3 drops of microalgae paste (Nutraplus Micro, Nutra-Kol, Mullaloo, WA, Australia). The food was pre-mixed and stored in the fridge and used for 3–4 days, after which fresh stock was made. An air line, with a rubber stopper acting as a weight, was added to each tank. Each air line was connected to a gang valve that was supplied with air from a linear air pump (Whitewater™ LT-11) at a rate of ~3 bubbles per second. The tanks were covered with plastic wrap to prevent evaporation. A mini compact fluorescent bulb (Coralife, Franklin, WI, USA) provided a mix of bright white and blue light on a 12 h:12 h cycle. *Artemia franciscana* were fed 3–6 ml of the food mix and dead individuals were removed daily.

At the end of day 6, *A. franciscana* were removed from the tanks and placed in beakers with water from the tank and maintained at 25±1°C. The tanks were replaced with 25, 236 ml labeled Ziploc® plastic containers. Each container was filled with 150 ml of the desired salinity stock (Table 1), 1 ml of food mix and 0.5 ml of live *Dunaliella salina* algae, cultured as per the supplier's instructions (no. 152160, Carolina Biological Supply Company, Burlington, NC, USA). An air line connected to the linear air pump via a 5-way gang valve provided air to each container at a rate of two bubbles per second. Forty *A. franciscana* were added to each treatment, taken from the tank with the appropriate salinity as per Table 1. *Artemia franciscana* were fed twice daily, with 0.25–0.3 ml of food mix and 0.25–0.3 ml live *D. salina* in the morning and food mix alone in the evening. Dead *A. franciscana* and waste were removed twice daily. At the end of days 9 and 12, *A. franciscana* were transferred to new containers containing fresh water at the relevant salinity as per Table 1. At the end of day 15, *A. franciscana* were preserved in 70% ethanol. Rearing success to day 15 ranged between 0% in *A. franciscana* exposed chronically (days 1–15) to 50 ppt up to 50% following exposure to 20 ppt during all exposure windows. Survival immediately after each exposure window is presented in Table 1 and in fig. 7 in Burggren and Mueller (2015).

The experiment described above was replicated four times, yielding *N* values that ranged from 5 to 20 per treatment.

Maturation and morphometry on day 15

For each individual *A. franciscana*, an image of the dorsal view of the body was taken at 10× magnification with a dissecting microscope (Wild M3Z, Leica Microsystems, Waukegan, IL, USA) and camera (DFC450, Leica Microsystems). Each individual was staged according to Heath (1924) to determine the percentage of mature individuals. Total body length (mm) was measured in duplicate using the segmented line tool in ImageJ (1.47t, National Institutes of Health, Bethesda, MD, USA) and the duplicates were averaged. Another 4–5 images, including images of the head, tail and appendages, were taken per individual using a light microscope (Eclipse E200, Nikon Instruments Inc., Melville, NY, USA) at 40× magnification. The length of the tail (mm) and third swimming appendage (mm) were measured in duplicate using the segmented line tool in ImageJ, and tail width (mm) and compound eye diameter (mm) were measured in duplicate using the straight line tool in ImageJ and the two measurements for each variable were averaged. The length of the third swimming appendage was measured because it was always clearly visible in images. To determine body mass, *A. franciscana* were blotted with Kimwipes to remove excess ethanol and weighed to ±0.01 mg on an electronic balance (XA 105DU, Mettler-Toledo, Columbus, OH, USA).

Data analysis

All statistical analyses were performed in SAS (version 9.2, SAS Institute Inc., Cary, NC, USA). The data were tested for normality and homogeneity of variances using Shapiro–Wilk's and O'Brien's test, respectively. All data met the assumption of homogeneity of variances. Data that did not meet the assumption of normality were log transformed. Maturation was presented as a percentage, so these values were arcsine square root transformed before analysis. For each variable, a two-way ANOVA was run with salinity, exposure window and the interaction between salinity and exposure window as the effects. A Student–Newman–Keuls (SNK) *post hoc* multiple pairwise comparison test was run when any of these variables was significant. When the interaction between salinity and exposure window was significant, a SNK test was run on the interaction term only. Data are presented as means±s.e.m. and differences were accepted as statistically significant at α=0.05.

Mean values for maturation and each morphometric variable for each treatment were plotted against exposure window and salinity dose on a 3D mesh plot in SigmaPlot (v. 12 Systat Software Inc., Chicago, IL, USA) to create a 3D representation of the relationship between exposure window, salinity and each variable.

RESULTS

The results presented for different morphometric characteristics in relation to salinity and exposure window, particularly in the 3D graphs, are best interpreted in the context of survival. Survival was previously shown to be significantly reduced following exposure to 40 and 50 ppt during days 1–6 and 7–9 (see fig. 7 in Burggren and Mueller, 2015), and should be considered when examining the salinity effects on morphometry during these exposure windows.

Maturation

On day 15, all *A. franciscana* were at the 8th instar stage or later, as distinguished by 11 fully functioning swimming appendages and the transformation of the second antennae (Heath, 1924). The percentage of *A. franciscana* on day 15 that reached sexual maturity (12th instar), as indicated by second antennae in males and the presence of oocytes in females, ranged from 6% to 50% across treatments (Fig. 1A). Maturation was significantly affected by salinity (P<0.001), exposure window (P=0.028) and the interaction between salinity and exposure window (P<0.001). Maturation following exposure to 40 ppt during days 1–6 (39%) and 7–9 (50%) was significantly greater than maturation following chronic exposure (days 1–15) to 40 and 50 ppt (0%, Fig. 1B). Maturation following 40 ppt on days 1–6 and 7–9 was also significantly greater than that following exposure to 50 ppt on days 7–9 and 13–15 and 30 ppt on days 1–6 and 10–12. More individuals reached adult stages following exposure to 10 ppt during days 10–12 compared with exposure to 30 ppt during the same window.

Body mass

Body mass (mg) of day 15 *A. franciscana* was significantly affected by salinity (P<0.001), exposure window (P<0.001) and the

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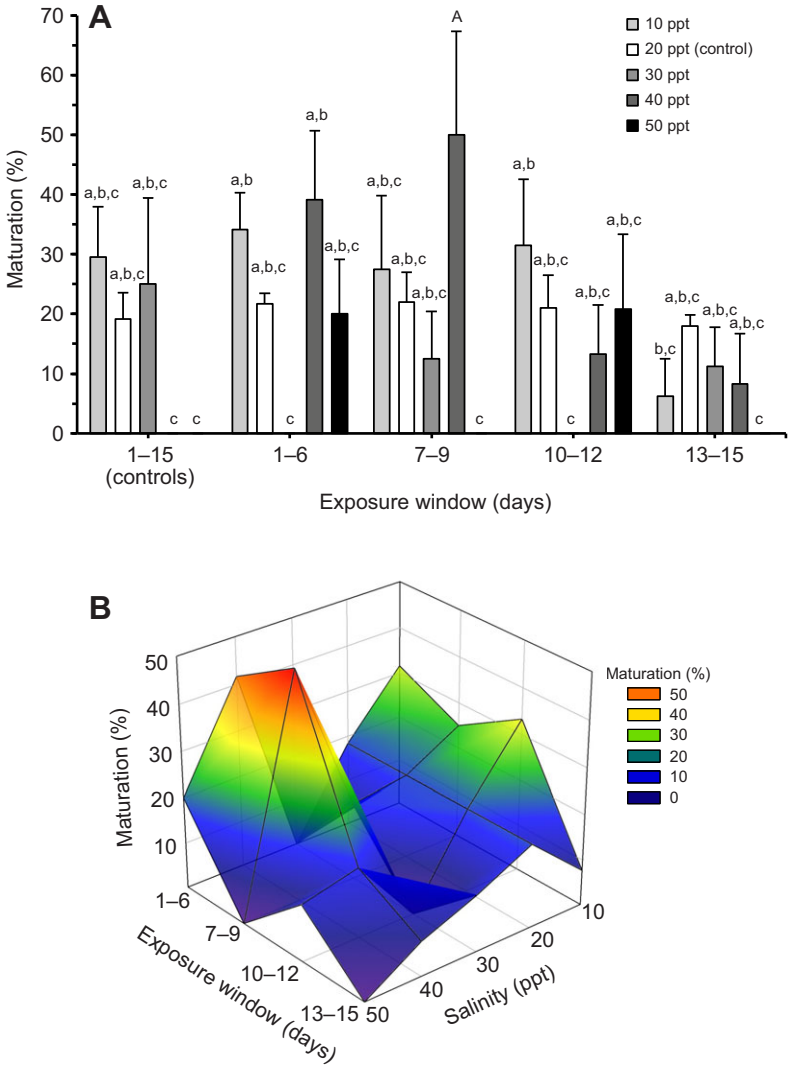

Fig. 1. Influence of salinity and exposure window on maturation. (A) Maturation of *Artemia franciscana*, expressed as a percentage of the total population, on day 15 following exposure to different salinities during different exposure windows in development. Maturation was determined by calculating the percentage of *Artemia* in each treatment that clearly displayed adult characteristics, as indicated by second antennae in males and the presence of oocytes in females (Heath, 1924). Different letters indicate significant differences between treatments (SNK multiple comparisons, $P<0.05$). Data are presented as means+s.e.m. ($N=4$). (B) Maturation plotted against exposure window and salinity.

interaction between salinity and exposure window ($P=0.005$). Body mass on day 15 following exposure to 10 and 40 ppt during days 1–6 was significantly greater than mass on day 15 after exposure to 10 and 40 ppt during days 13–15 (Fig. 2A). This is clearly visualized when the mean mass values from Fig. 2A are plotted on a 3D diagram (Fig. 2B).

Morphometric characteristics

Total body length, tail length and width, length of the third appendage and eye diameter were all significantly affected by salinity ($P<0.001$ for all) and exposure window ($P<0.001$ for all). However, the interaction between salinity and exposure window was not significant ($P>0.05$).

Total body length (mm) of *A. franciscana* was significantly greater when exposed to 10, 20 or 40 ppt compared with 30 and 50 ppt (Fig. 3A). *Artemia franciscana* were also longer on day 15 if they were exposed to a different salinity during days 1–6 and 7–9 compared with days 10–12 and 13–15 of development (Fig. 3B,C).

Tail length (mm) was significantly longer following exposure to 10 and 20 ppt compared with 30 and 50 ppt (Fig. 4A). Tail length following exposure to 40 ppt was similar to that for all other all treatments, excluding 50 ppt. Tail length was significantly greater in *A. franciscana* following exposure to a different salinity during days 1–6 and 7–9 compared with exposure during days 10–12 and 13–15 (Fig. 4B,C). Tail length under chronic salinity exposure (days 1–15) was only significantly different from tail length following exposure during days 1–6. Tail width (mm) was significantly greater following exposure to 10 ppt compared with all other salinities (Fig. 4D). Tail width following exposure to 40 ppt was also significantly greater compared with exposure to 50 ppt. Tail width of chronically exposed *A. franciscana* and those that experienced a different salinity during days 1–6 and 7–9 was significantly greater than for those that experienced a change in salinity during days 10–12 and 13–15 (Fig. 4E,F).

The length of the third swimming appendage (mm) at day 15 was significantly greater after exposure to 10 ppt compared with exposure to 30 and 50 ppt (Fig. 5A). Third appendage length was also significantly greater following exposure to 20 and 40 ppt compared with 50 ppt and following exposure to a different salinity during days 1–6 and 7–9 compared with exposure during days 10–12 and 13–15 (Fig. 5B). Chronic salinity exposure (days 1–15) resulted in a third appendage length that was only significantly smaller compared with early exposure during days 1–6.

Eye diameter (mm) of *A. franciscana* following exposure to 10 ppt was significantly greater than that following exposure to 30 and 50 ppt, with 20 and 40 ppt results being intermediate (Fig. 5D). Eye diameter following salinity exposure during days

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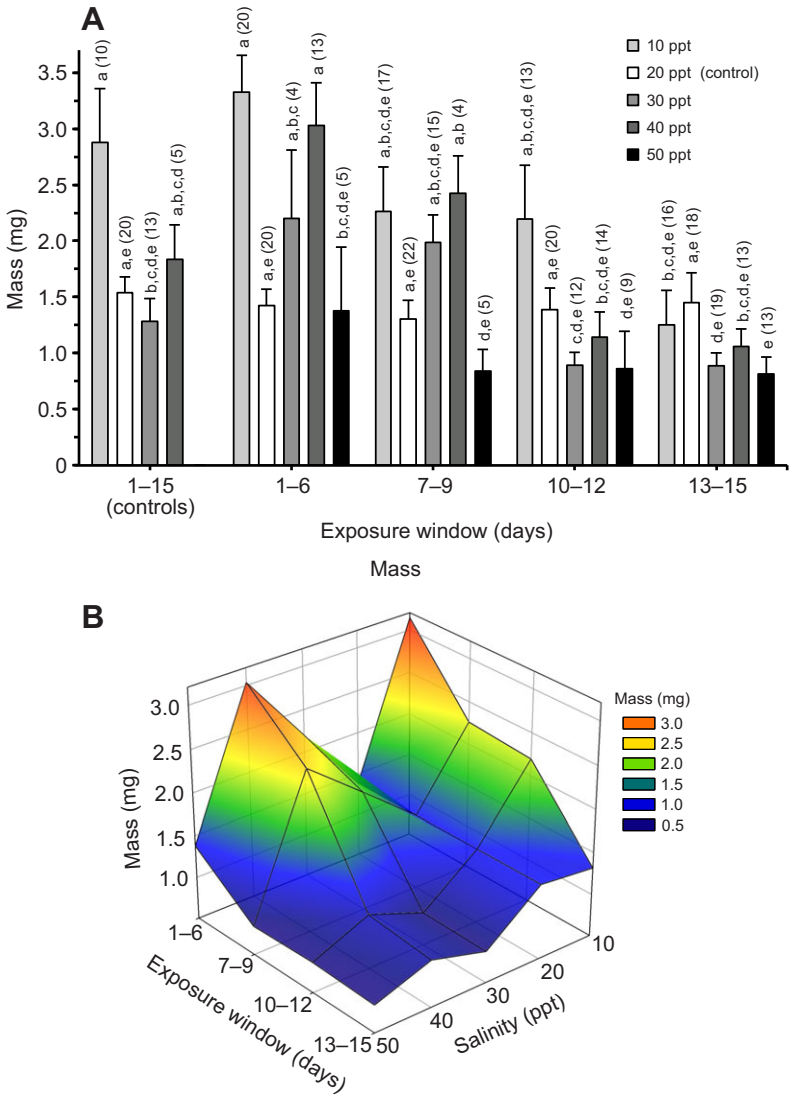

Fig. 2. Influence of salinity and exposure window on mass. (A) Mass of *A. franciscana* on day 15 following exposure to different salinities during different exposure windows in development. Different letters indicate significant differences between treatments (SNK multiple comparisons, $P<0.05$). *N* per treatment is indicated in parentheses. Data are presented as means+s.e.m. (B) Mean mass of *A. franciscana* plotted against exposure window and salinity.

1–6 was significantly greater than for all other exposure times, including chronic exposure from day 1 to 15 (Fig. 5E). Eye diameter following exposure on days 7–9 was also significantly greater than eye diameter following exposure late in development on days 13–15. The effects of salinity and exposure window on third appendage length and eye diameter are apparent in the 3D plots (Fig. 5C,F).

Overall, salinity had the strongest effect on maturation and morphometry during days 1–6 and 7–9, with development and growth particularly stimulated at 10 and 40 ppt. However, the effect of salinity on maturation and growth was reduced with exposure later in development (days 10–12 and 13–15).

DISCUSSION

Critique of methodologies

Most studies expose an animal to a single level (dose) of stressor during different exposure windows throughout development. For example, Ali et al. (2011), studied the effects of 10% ethanol exposure during certain developmental windows in zebrafish (*Danio rerio*) embryos, and found that mortality was highest during gastrulation, while at later stages mortality declined but severe morphological malformations were observed. Likewise, a single low oxygen treatment, either 10% or 15% hypoxia, has been used to examine critical windows of morphological, respiratory and cardiovascular development in bird and reptile embryos (e.g. Dzialowski et al., 2002; Chan and Burggren, 2005; Tate et al., 2015). That traditional approach serves as a useful first approximation for determining periods of development that may fall within critical windows. However, the experimental design used in this study, in which multiple levels of a stressor were used as well as multiple lengths of exposure (i.e. different widths of windows), provided useful insights for studying critical windows. By using exposure to five different salinities during four exposure windows throughout the development of *A. franciscana*, we were able to demonstrate how a 3D approach can provide more nuanced information on the magnitude of phenotypic change, and how it is related to the timing of exposure and level of stressor. Of course, the more data available to construct a time–exposure–response 3D profile, the more ‘smooth’ are the contours that represent the phenotypic modifications. Burggren and Mueller (2015), in a largely theoretical treatment of the concept of the critical window as a 3D construct, provided smooth contours relating the time–exposure–magnitude responses of developing animals. The 3D contours formed in the present study are somewhat ‘angular’, even with 21 different treatments involving five salinities and four different potential critical windows.

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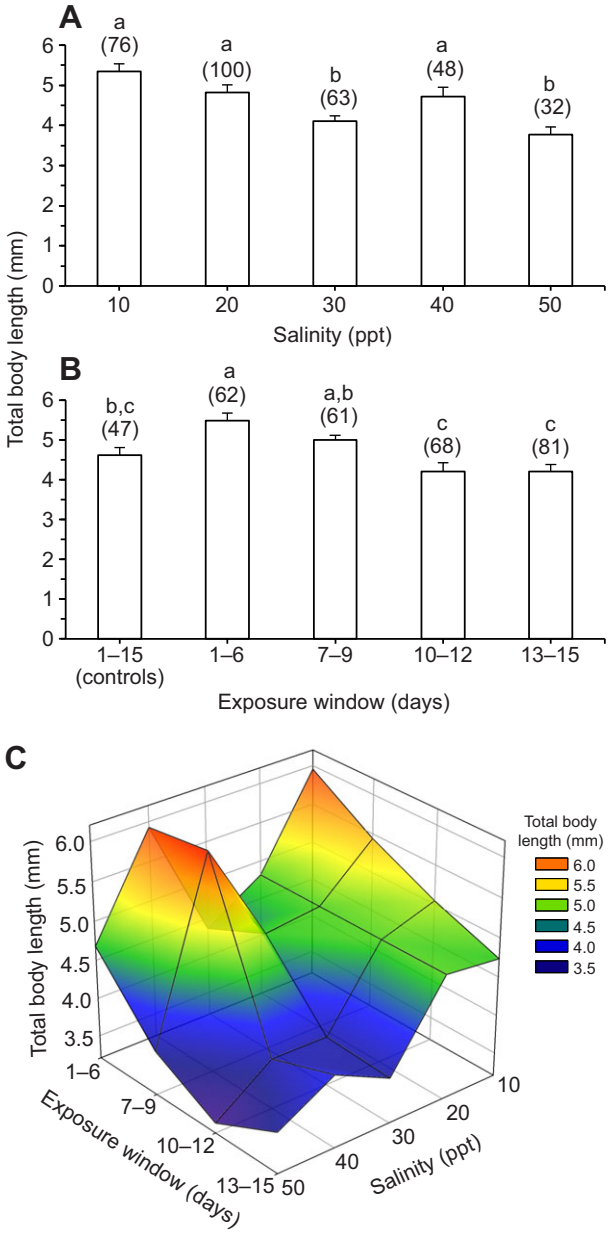

Fig. 3. Influence of salinity and exposure window on total body length. (A) Total body length of *A. franciscana* on day 15 following exposure to different salinities. Data represent means+s.e.m. of exposure to each salinity during all exposure windows. (B) Total body length following exposure to a different salinity during each exposure window. Different letters in A and B indicate significant differences between treatments (SNK multiple comparisons, $P<0.05$). *N* is indicated in parentheses. (C) Mean total body length plotted against exposure window and salinity.

While the 3D graphs (Figs 1B, 2B, 3C, 4C,F and 5C,F) are advantageous for visualizing the interaction between exposure window and stressor dose, they do not include the error terms for the treatment means that are represented in the conventional bar graphs. However, using the example of mass, we demonstrate how it is possible to overlay another layer on these graphs that is constructed from the standard error of the mean for each treatment (Fig. 6). These 3D graphs can be presented in either fashion but, without the error overlay, the interaction between exposure window, stressor dose and effect size is visually easier to examine. We have chosen to present the data in this study using both bar graphs with error terms and the 3D graph, so that a complete picture of the phenotypic modification that occurs following different salinity exposures can be readily visualized.

Despite the 3D graphs illustrating similar patterns of change with salinity and exposure window in all variables, maturation and mass were the only variables with a significant statistical interaction between salinity and exposure window. This finding most likely reflects the experimental design with a large number of treatments, and subsequent statistical analysis. Critical window studies often have a relatively high number of treatments, particularly compared with traditional chronic exposure studies. For example, this study had 21 treatments, but a study with chronic exposures of the same salinities (10, 20, 30, 40 and 50 ppt) would have just five treatments. An increase in treatment number potentially increases variation within treatments, and thus residual error, making it less likely to detect treatment effects. This is particularly the case when examining an interaction between two effects, in this instance exposure window and stressor dose. For example, a power analysis in JMP (v.11, SAS), using $\alpha=0.05$, root of the mean square error, the effect size for the exposure window×salinity interaction term and the total sample size, indicated a 95% probability of detecting a significant interaction for mass. The probability of determining a significant interaction using the current sample size for the other variables ranged from 61% for total body length up to 91% for tail width. Power analysis indicated that increasing the sample size by 50%, from approximately 250 samples to 375 samples, resulted in the probability of a significant interaction increasing to at least 90% in all variables. Thus, treatment replicate number is an important consideration when designing future experiments that utilize the 3D critical window concept.

Adult *Artemia* have a remarkable ability to survive a wide range of salinities, reportedly up to 250 ppt (Van Stappen, 1996). The range of salinities used in this study (10–50 ppt) is relatively narrow in comparison to this extreme range, and this may have reduced potential treatment differences, discussed above. However, we chose the salinities based on our preliminary studies in which we examined survival of early nauplii in a range of salinities up to 150 ppt. These preliminary studies indicated that 50 ppt was the upper lethal limit for our particular population of cysts, and therefore our treatments were selected to fall below this range. Of course, different tolerances may exist between different strains and populations of *Artemia* (Clegg and Conte, 1980; Van Stappen, 1996). Alternatively, the ionic composition of the water may have also influenced the results presented in this study. *Artemia* live in neutral to alkaline waters and survival is above 90% for all developmental stages at pH 7 and 8 (Doyle and McMahon, 1995). However, we cannot rule out the possibility that *A. franciscana* may have experienced pH stress during this study, as pH 7 is at the lower permissive limit for first instar larvae. In addition, we added low amounts of sodium bicarbonate to buffer the water to pH 7, and this may have altered the ionic composition of the water. Future studies that examine the sensitivity to particular ions during windows of development may provide additional insight into salinity tolerance of *Artemia* during development.

Development and growth

Significant effects on morphometry resulted from exposure window and salinity as measured on day 15 in *A. franciscana*. These effects appeared to be due to a combination of both development rate and growth, and may be related to salinity effects on survival (Burggren and Mueller, 2015). On day 15, the

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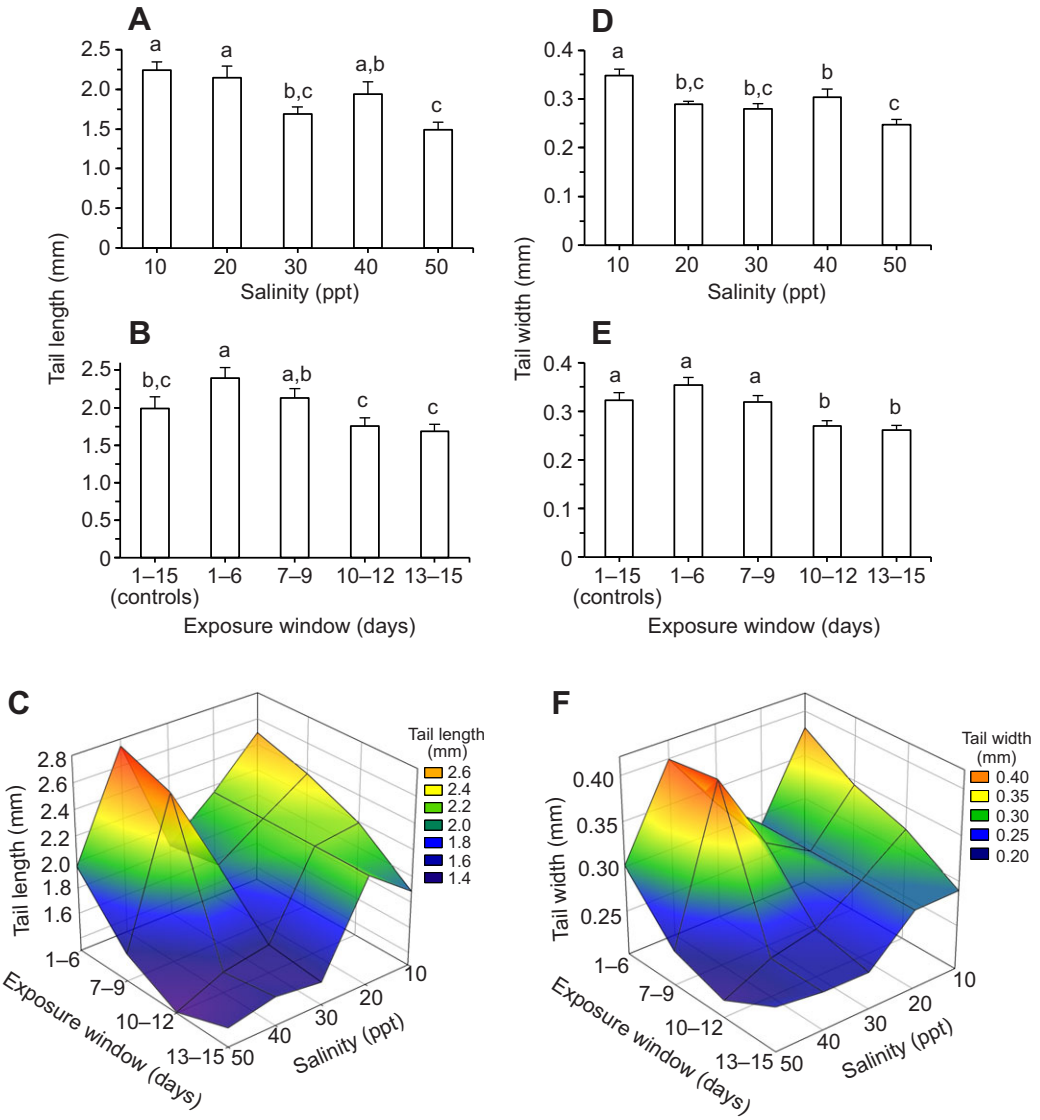

Fig. 4. Influence of salinity and exposure window on tail length and width. (A,D) Tail length and width of *A. franciscana* on day 15 following exposure to different salinities, and (B,E) following exposure to all salinities during different exposure windows. Data are presented as means+s.e.m. Different letters indicate significant differences between treatments (SNK multiple comparisons, $P<0.05$). *N* as in Fig. 3. (C) Tail length and (F) width of *A. franciscana* plotted against exposure window and salinity.

majority of individuals were easily identified as male or female, but the number of sexually mature individuals was highest following exposure to 40 ppt on days 1–6 and 7–9 (Fig. 1). Previous studies have indicated that the time to reach maturity and the length of the pre-reproductive period increase with increasing salinity up to 180 ppt (Dana et al., 1993; Abatzopoulos et al., 2003; Baxevanis et al., 2004). In contrast, we did not find a linear relationship between salinity and development stage on day 15. This may be because treatment salinities were below (10 ppt) and above (30, 40 and 50 ppt) the control (20 ppt), which may have created a 'hump-shaped' survival relationship. The discrepancy may also be attributed to our more narrow range of salinities (10–50 ppt) compared with other studies (35–180 ppt).

The higher percentage of mature animals following early (days 1–6, 7–9) exposure to 40 ppt was matched by greater mass on day 15 (Fig. 2). However, the effect of early exposure on maturity and mass was not seen across all salinities, indicating that variable effects can be induced when using different doses of a stressor. For instance, if *A. franciscana* had only been exposed to 30 ppt in our study, no effect of exposure window on mass would have been detected, leading to the erroneous conclusion that there were no critical windows for the effect of salinity on growth. Similarly, *A. franciscana* exposed to 40 ppt for all of development, from day 1 to day 15, were smaller than individuals exposed to the same salinity for days 1–6 or 7–9 only (Fig. 2A). The use of only chronic exposures, then, would have actually masked the effect of exposure on days 1–6 and 7–9 to this salinity, which actually resulted in larger, more mature *A. franciscana*. These findings illustrate how a stressor may induce different effects depending on when during development the actual exposure occurs. Moreover, this experimental approach has revealed why it is advantageous to use more than a single level of a stressor to assess stressor-induced phenotypic modification. The advantage of the 3D approach to examining these complexities lies in the ability to easily visualize these interactions between exposure window and stressor dose (Fig. 2B).

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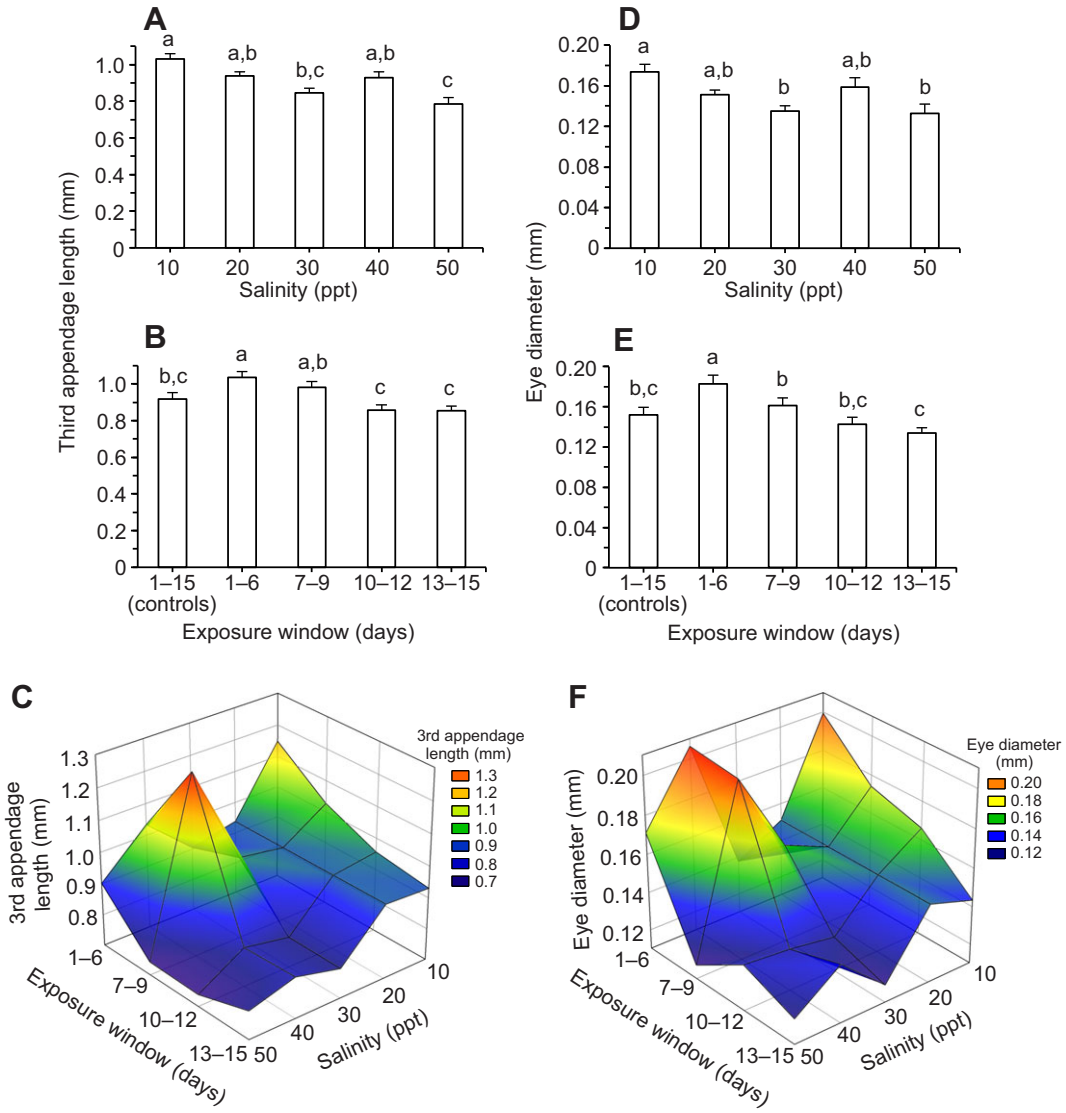

Fig. 5. Influence of salinity and exposure window on third appendage length and eye diameter. (A,D) Third appendage length and eye diameter of *A. franciscana* on day 15 following exposure to different salinities, and (B,E) following exposure to all salinities during different exposure windows. Data are presented as means+s.e.m. Different letters indicate significant differences between treatments (SNK multiple comparisons, $P<0.05$). *N* as in Fig. 3. (C) Third appendage length and (F) eye diameter of *A. franciscana* plotted against exposure window and salinity.

The effect of salinity on morphometry in this study, and the 3D diagrams generated, should be interpreted in the context of the significant effect of salinity on survival during exposure on days 1–6 and 7–9 (Burggren and Mueller, 2015). Based on these previous findings, we hypothesized a correlation between survival and morphometry of *A. franciscana*, such that if a treatment produced low survival, then the few *A. franciscana* that survived would be smaller than controls because of the suboptimal salinity levels. This hypothesis was consistent with a previous study, which reported that a slowing of growth in *A. franciscana* occurred in concert with high mortality at higher salinities of 120 and 140 ppt (Triantaphyllidis et al., 1995). Exposure to 10 and 50 ppt during days 1–6 in the present study resulted in larger and smaller *A. franciscana*, respectively, which matched the attendant high and low survival found previously in those treatments (Burggren and Mueller, 2015). Therefore, these findings supported our hypothesis. However, the effect of exposure to 40 ppt on days 1–6 and 7–9 on morphometry was the opposite of what was hypothesized based on the changes in survival of *A. franciscana*. We previously reported that survival immediately after exposure to 40 and 50 ppt was reduced in *A. franciscana* when compared with exposure to 10, 20 and 30 ppt during days 1–6 (Burggren and Mueller, 2015). An interesting selective effect occurred at 40 ppt during days 1–6, however, in which the few *A. franciscana* that survived before being transferred back to 20 ppt on day 6 ended up growing to a larger size on day 15. Therefore, the especially hardy individuals that survived the initial exposure to 40 ppt thrived and reached adult stages and larger sizes once they were returned to control salinity. This did not occur at 50 ppt, suggesting a threshold salinity exists between 40 and 50 ppt at which recovery from early exposure (days 1–6 and 7–9) to high salinities is possible and a selective effect is evident. Thus, in some instances, reduced survival and smaller individuals occurred together, but this is not consistent in this study. While we did not assess mass at the end of each exposure window in this study, we did observe larger individuals prior to the first salinity shift on day 6 in those treatments that produced larger individuals on

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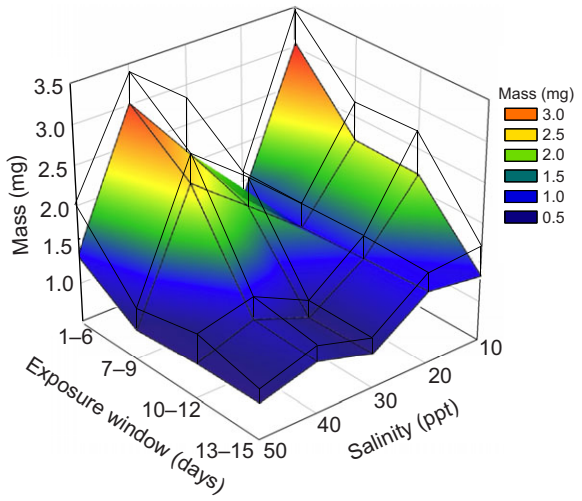

Fig. 6. 3D graph with error overlay. Mean mass of *A. franciscana* on day 15 as in Fig. 2B (colour) with an overlay (black lines) representing positive s.e.m. for each treatment.

day 15. These observations demonstrate that it was not the salinity shift alone that may have selected for the larger individuals, but rather the salinity *A. franciscana* were incubated in during each window.

Morphometric characteristics

The morphometric characteristics presented in this study must be examined with the caveat that there are differences in survival across the salinity treatments (Table 1; Burggren and Mueller, 2015). These survival differences and the salinity treatments may both contribute to the altered morphometric characteristics observed. Total body length, tail length and width, length of the third appendage and eye diameter were all larger following exposure to salinities above and below control during days 1–6 compared with exposures on days 10–12 or 13–15 (Figs 3B, 4B,E and 5B,E). These results, together with the changes in mass, indicate that growth of *A. franciscana* throughout development was particularly affected by salinity during days 1–6. This finding supports our hypothesis that salinity effects would be most pronounced during early developmental exposures. This period includes the nauplius stage and what are often referred to as metanauplii stages, which encompass the development of thoracic segments. During these stages, developing *Artemia* molt every 12–24 h (Weisz, 1946). Therefore, this period of 6 days encompasses probably at least the first six instars (Heath, 1924). However, the instar reached by *A. franciscana* on day 6 likely varies between treatments, as salinity can influence the number of molts during *Artemia* development (Weisz, 1946). A future study that closely examines the molting of *A. franciscana* exposed to different salinities during certain windows of development is warranted.

Salt sensitivity of *A. franciscana* on days 1–6 may be linked to the immaturity of the thoracic swimming appendages and absence of branchiae, which serve as important ion-secreting organs in adults (Croghan, 1958; Conte, 1984). Likewise, the earliest instars of *A. franciscana* also show greater sensitivity to an acidic environment compared with adults, and this may be linked to developmental changes in ionoregulatory organs (Doyle and McMahon, 1995). Thus, this early window is potentially a period of development that could be of particular focus for future studies attempting to tease apart how developing *Artemia* respond to changes in salinity. The earliest stages of *Artemia* represent an interesting period of development in terms of sensitivity to salinity and osmoregulatory ability. The number of cysts that hatch is generally unaffected by salinity, but hatching is delayed and there is an increase in an oval phenotype of prenauplii at salinities above 35–44 ppt (Jennings and Whitaker, 1941; Neumeyer et al., 2015). Newly hatched nauplii have a 'neck organ' that is thought to serve as the main osmoregulatory organ (Croghan, 1958; Conte, 1984). Following the first molt, second instars use a renal maxillary gland and their gut to maintain water and ion balance (Conte, 1984). Despite these osmoregulatory organs, it is unlikely that early developmental stages have the same ionoregulatory ability as later stages and adults.

In addition to the differences in salinity tolerance between exposure windows, 30 and 50 ppt were generally detrimental for *A. franciscana* growth, while 10 ppt was beneficial. For example, total length and tail length were reduced following exposure to 30 and 50 ppt during any exposure window (Figs 3A and 4A). Tail width and third appendage length were decreased following exposure to 50 ppt, and tail width increased following exposure to 10 ppt (Figs 4D and 5A). These results indicate that each salinity can have unique effects on morphometry, which contrasts starkly with other studies that found a steady decrease in the size of *Artemia* as salinity increased (Gilchrist, 1960; Dana and Lenz, 1986; Dana et al., 1993; Triantaphyllidis et al., 1995). The salinity-specific effects on morphometry are similar to the changes in maturation with salinity (Fig. 1), indicative of how growth and development progress via molting.

Molting of the exoskeleton is an important developmental process in *A. franciscana*. A previous study of critical windows in another crustacean that molts, the Japanese tiger shrimp (*Penaeus japonicus*), found that sensitivity to ammonia decreased from nauplius to juvenile stages (Lin et al., 1993). However, when exposures occurred during molting, mortality was higher than during inter-molt exposures at all developmental stages. Other studies have illustrated increased sensitivity of crustaceans to environmental toxicants during molting, and this is most likely due to increased permeability of the cuticle (Armstrong et al., 1976; Conklin and Rao, 1978; Wajsbrot et al., 1990). Thus, critical window studies that incorporate exposures during the inter-molt and molt periods of development are important for detecting those periods when the animal is most sensitive to the environment. It is possible that *A. franciscana* may also have increased sensitivity to changes in salinity during molting. In fact, the higher sensitivity during days 1–6 to salinity, discussed below, may be due to molting occurring every 12–24 h compared with 24–30 h in later developmental stages (Weisz, 1946). Examining sensitivity during molting and inter-molting periods in *A. franciscana* would be difficult because of this relatively short interval between molting. Other crustaceans with longer molting intervals would provide better opportunities to further this interesting area of research.

Defining the critical window

The current study used four non-overlapping exposure windows, but a limited number of studies have employed overlapping exposure windows in an attempt to define the boundaries of a critical window (Olmstead and Leblanc, 2002; Hogan et al., 2008; Tate et al., 2015). While the period from day 1 to 6 appears to be a critical window for the effect of salinity on the morphometry of *A. franciscana*, this window is defined by the exposure period. This was also a longer exposure window (6 days) compared with the others used in the study (3 days), and it cannot be dismissed that the longer duration may have amplified the salinity effects observed. It

is possible that the window during which morphometry is particularly influenced by salinity is slightly shorter or longer than days 1–6. The critical window may actually be better defined as days 3–6 or 1–8, for example. Consequently, only by using exposure windows that overlap is it possible to tease apart the boundaries of the period of susceptibility or plasticity. If changes in morphometry on day 15 are consistent following exposures during days 1–6, 1–8, 3–6 and 4–8, then the critical window can be defined as days 1–8 with a reasonable level of confidence. However, if morphometry on day 15 is altered following exposure during days 1–6 and 1–8, but not 3–6 or 4–8, then it may actually be exposure during days 1–2 that is having the greatest effect on morphometry and this is the true critical window. The benefit of the 3D construct is that if we know a developmental period during which a stressor-induced effect occurs, and when the magnitude of the phenotypic change is greatest, overlapping exposure windows may be used to better define the onset and cessation of phenotypic modification.

In conclusion, using the morphometry of *A. franciscana* as an example, we have shown that a 3D critical window construct can be used to visualize how the time of exposure in development and dose of a stressor interact to alter the phenotypic characteristic of interest. Salinity had the greatest influence on morphometry during days 1–6, and thus this is potentially a critical window for when salinity induces the greatest phenotypic plasticity during development of *A. franciscana*. This study can be seen as a starting point for understanding how salinity interacts with other developmental parameters in *A. franciscana*. Future studies that measure physiological parameters of *A. franciscana* following the same salinity exposures may be used to assess whether the shape of the 3D construct for physiological performance matches that for morphometric characteristics. For example, salinity, which alters the solubility of oxygen, influences oxygen consumption rate in developing *Artemia* (Engel and Angelovic, 1968; De Wachter and Van Den Abbeele, 1991; El-Gamal, 2011). Thus, it would be interesting to examine whether the shape of the physiological 3D interactions between exposure window, salinity dose and oxygen consumption differs from morphometry, or whether they reflect each other. Assessing physiology as well as morphology in this system, and in other organisms of interest, will contribute to our understanding of how all components of an organism or system are influenced by a stressor during development, hence providing a ‘system approach’ to critical windows (Burggren et al., 2014; Burggren and Mueller, 2015).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
C.A.M. and W.W.B. designed the study; C.A.M. and E.W. performed the experiments; C.A.M. analyzed the data and wrote the manuscript; C.A.M., E.W. and W.W.B. revised the manuscript.

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