Ecotoxicology of salinity tolerance in *Daphnia pulex*: interactive effects of clonal variation, salinity stress and predation

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Despite decades of research on the impacts of salinity in freshwater systems, the effects of salinity stress on planktivore–zooplankton interactions have received limited attention. We used laboratory-based experiments to examine *Daphnia pulex* responses to salinity stress and the lethal and non-lethal effects of *Chaoborus* (a dominant planktivore in fishless ponds). We also examined how *D. pulex* clonal variation mediates these responses using two clones known to differ in salinity tolerance. Presence of kairomone induced neckteeth formation, increased fertility and increased age and size at maturity relative to controls. As predicted, increasing salinity generally weakened life history responses to kairomone and reduced survivorship in the presence of lethal predation. While some of our results are suggestive of a moderating effect of clonal variation in salinity tolerance, clone effects on responses to increasing salinity were inconsistent. Our study demonstrates that non-lethal levels of salinity stress have the potential to impact *Daphnia* populations negatively by altering life history and behavioral responses to predators.

**KEYWORDS:** life history; phenotypic plasticity; predator–prey; salinity stress; zooplankton

**INTRODUCTION**

A large body of research has documented the increasing impacts of anthropogenic stressors, including toxins and pollutants, on natural populations. More recent work in this field has emphasized the importance of moving beyond the organismal level and considering ecological context when attempting to understand or predict the effects of stressors (Relyea and Hoverman, 2006; Schmitt-Jansen *et al.*, 2008; Gessner and Tili, 2016). Beyond the direct negative effects on fitness, indirect effects of stressors may be mediated by biotic interactions such as parasitism, predation and competition (Relyea and Hoverman, 2006; Schmitt-Jansen *et al.*, 2008). Historically, greater research emphasis has been placed on density-mediated indirect effects in which
effects of stressors on biotic interactions occur through alterations of species densities (e.g. via lethal effects on one or more interactors). More recent work in ecology and ecotoxicology has revealed the importance of trait-based effects in which non-lethal levels of environmental stressors alter species interactions by modifying behavioral, physiological, demographic or morphological traits (Weis et al., 2001; Werner and Peacor, 2003; Relyea and Hoverman, 2006). Uncovering such trait-based effects has important applied and management implications for they suggest that sublethal levels of stressors in the environment can still have significant negative impacts on populations when placed within a broader community context.

Of the wide range of anthropogenic stressors that may cause negative impacts in freshwater ecosystems, salinity stress resulting from road salt intrusion and land use change is of more recent concern (Kausal et al., 2005). Salts transported to road-adjacent freshwater systems, in particular chloride, can directly impact the fitness of aquatic organisms (Blasius and Merritt, 2002; Karraker et al., 2008; Fay and Shi, 2012) and indirectly impact populations by altering species interactions (Van Meter et al., 2011; Petranka and Francis, 2013). Especially vulnerable are small wetlands and ponds in which runoff may have larger impacts on water-column salt concentrations and resident biota when compared to larger or flowing surface waters (Fay and Shi, 2012). Understanding the ecological impacts of salinity stress in such ecosystems has been a focus of ongoing research efforts. However, much remains unknown regarding the role of ecological context in the ecotoxicology of salinity stress; especially how non-lethal effects of salinity can alter the traits and biotic interactions of planktonic taxa.

In the temperate zone, the plankton of fishless wetlands and ponds are generally dominated by large-bodied Daphnia species such as Daphnia pulex. Daphnia are commonly considered keystone species for their central role in trophic dynamics and ecosystem functioning (Gottingham and Schindler, 2000; Ives et al., 2003). They also face predation pressure from a diversity of planktivores of which Chaoborus larvae are a major component in fishless systems (Dodson, 1972; Pastorek, 1980). A large body of research has centered on Daphnia–Chaoborus interactions and documented several trait responses of Daphnia to this predator. These include behavioral responses such as enhanced swim speed (Swift, 1992); morphological defenses such as development of neckteeth (Tollrian, 1995a); and plasticity in life history traits including delayed maturation resulting in the production of larger neonates which are less prone to predation (Spitze, 1992; Tollrian, 1995b).

Numerous studies have revealed important effects of both natural environmental variation as well as anthropogenic stressors such as environmental contaminants on Daphnia trait responses to predation (Hanazato and Dodson, 1995; Barry, 2000; Coors and De Meester, 2008). However, few have addressed the effects of salinity stress on Daphnia–predator interactions and induced responses of Daphnia (Bezirci et al., 2012). Moreover, studies to date have largely failed to consider the role of intraspecific trait variation in the responses of prey to the combined presence of predators and environmental stressors. Prior work has revealed significant Daphnia clonal variation in salinity tolerance from habitats with naturally occurring salinity differences (Weider and Hebert, 1987; Teschner, 1995). The capacity to adapt evolutionarily to salinity stress has important implications not only for the stability and persistence of Daphnia populations but for the broader community given their functional importance in aquatic food webs.

Here we present a study in which we examined the interactive effects of salinity stress and intraspecific variation in salinity tolerance on D. pulex responses to the lethal and non-lethal effects of Chaoborus americanus, the dominant Chaoborus species in fishless ponds in the region (Garcia and Mittelbach, 2008). We first screened four D. pulex clones isolated from ponds that varied naturally in conductivity and assayed their tolerance to increasing salinity; we hypothesized that past exposure to higher conductivity would select for greater salinity tolerance. Of these clones, we chose two that showed strongly contrasting salinity tolerances for subsequent experiments. We predicted that the presence of Chaoborus chemical cues (kairomone) would induce both clones to develop neckteeth, mature later at larger sizes, and give birth to fewer but larger neonates. We further predicted that increasing salinity stress would weaken these responses as well as reduce survivorship in the lethal presence of Chaoborus predation. Clonal variation in salinity tolerance was predicted to mediate the effects of salinity stress, reducing the magnitude of effects on the more tolerant clone.

**METHOD**

We examined salinity tolerance of four D. pulex clones: two (P12 and P14) isolated from ponds at the Kellogg Biological Station Experimental Pond Facility (Hickory Corners, MI), one (OL2) isolated from a natural pond in the Barry State Game Area (Barry County, MI) and one (GR9) isolated from a natural pond in the E. S. George Reserve (Pinckney, MI). The source ponds represented a range of natural conductivities based on our prior field surveys (P14: 350.5 μS cm⁻¹; P12: 348.3 μS cm⁻¹; GR9: 65.2 μS cm⁻¹; OL2: 43.8 μS cm⁻¹). Cultures were established using a single female randomly isolated from pond samples and maintained as isogenic lines under controlled environmental conditions.
for numerous generations. These ponds were known to contain Chaoborus populations and prior pilot experiments showed that all four clones develop neckteeth when exposed to Chaoborus kairomone.

Chaoborus americanus larvae were obtained from natural ponds in the Kellogg Biological Station Lux Arbor Reserve (Delton, MI) in summer and autumn 2014. Third or fourth instar larvae were used as they have sufficient gape sizes to feed on juvenile D. pulex (Swift, 1992). A subset of the animals was maintained in the lab under constant temperature (20°C), 12 h light:12 h dark photoperiod and fed D. pulex juveniles. The rest of the Chaoborus larvae were used to extract kairomone (Hebert and Grewe, 1985). Extracted kairomone solution was stored in a −80°C freezer. This solution was effective in inducing neckteeth responses of D. pulex 9 months after extraction.

Salinity tolerance experiment
To determine salinity tolerance of the four candidate clones, the clones were first cultured under low density, high food, common garden conditions for three generations. At the start of the experiment, <24-h old neonates from the third generation were isolated from their stock cultures and transferred to beakers containing 200 mL of experimental medium; replicate beakers contained 15 cultures and transferred to beakers containing 200 mL from the third generation were isolated from their stock cultures and cultured for 2 days in medium whose salinities matched the experimental target concentrations. After this acclimation period, D. pulex individuals were transferred into beakers with 100 mL of 0 mM NaCl medium occupied by 20 starved Chaoborus. We chose to assay behavior in the absence of salinity stress to avoid salinity effects on Chaoborus. However, the transfer into low salinity conditions may have imposed additional osmotic stress on D. pulex individuals in the non-zero salinity treatments. This may have affected behavior responses; a caveat that should be kept in mind when interpreting our results. Escape behavior was determined in multiple, non-concurrent trials following Swift and Fedorenko (1975). For each clone and salinity level combination, five replicate trials were conducted with freshly starved Chaoborus. Trials and treatment combinations were randomized through time. For each replicate trial, 20 Daphnia were added into a beaker one by one after the previous one was ingested. The total numbers of strikes, escapes and ingestions occurring in each trial were recorded where a strike was defined as a predatory movement of Chaoborus directly towards the Daphnia individual, and an ingestion was a successful attack with the whole body of the Daphnia being ingested by the predator (Havel and Dodson, 1984). Escapes were the number of strikes which did not result in ingestion. The escape efficiency reflects the ability of Daphnia to escape from predation and was calculated by dividing the number of escapes by the number of strikes (Swift and Fedorenko, 1975). Daphnia individuals remained in the experimental beaker for <5 min before ingestion occurred. Hence, exposure to the low salinity environment was short relative to the 2-day pre-trial exposure period.

Behavior experiment
Escape behavior of clones P14 and GR9 was assessed in the presence of live Chaoborus after the clones experienced short-term exposure to one of four salinity levels (0, 12.0, 24.0 and 34.2 mM NaCl). A maximum level of 34.2 mM was chosen to minimize mortality and ensure an adequate number of individuals for the experiment. Chaoborus cultures were acclimated in the lab for at least one month before the initiation of experiments and were starved for 2 days before being used in the behavior experiment (Swift and Fedorenko, 1975). D. pulex cultures were cloned under common garden conditions for three generations (as described above) prior to the experiment. At the start of each experiment, <24-h old neonates were isolated from their stock cultures and cultured for 2 days in medium whose salinities matched the experimental target concentrations. After this acclimation period, D. pulex individuals were transferred into beakers with 100 mL of 0 mM NaCl medium occupied by 20 starved Chaoborus. We chose to assay behavior in the absence of salinity stress to avoid salinity effects on Chaoborus. However, the transfer into low salinity conditions may have imposed additional osmotic stress on D. pulex individuals in the non-zero salinity treatments. This may have affected behavior responses; a caveat that should be kept in mind when interpreting our results. Escape behavior was determined in multiple, non-concurrent trials following Swift and Fedorenko (1975). For each clone and salinity level combination, five replicate trials were conducted with freshly starved Chaoborus. Trials and treatment combinations were randomized through time. For each replicate trial, 20 Daphnia were added into a beaker one by one after the previous one was ingested. The total numbers of strikes, escapes and ingestions occurring in each trial were recorded where a strike was defined as a predatory movement of Chaoborus directly towards the Daphnia individual, and an ingestion was a successful attack with the whole body of the Daphnia being ingested by the predator (Havel and Dodson, 1984). Escapes were the number of strikes which did not result in ingestion. The escape efficiency reflects the ability of Daphnia to escape from predation and was calculated by dividing the number of escapes by the number of strikes (Swift and Fedorenko, 1975). Daphnia individuals remained in the experimental beaker for <5 min before ingestion occurred. Hence, exposure to the low salinity environment was short relative to the 2-day pre-trial exposure period.

Morphological and life history experiment
Daphnia pulex life history and morphological responses were assessed using a 4 × 2 × 2 factorial design: four salinity levels (0, 4.3, 8.6, 17.1 mM NaCl) crossed with presence/absence of Chaoborus kairomone crossed with clone identity (P14 versus GR9). Salinity levels were
chosen based on pilot experiments in which 17.1 mM NaCl was found to be close to the limit where eggs of both clones were able to develop into neonates. Clones were cultured in common garden conditions as described above. However, after F2 generation mothers gave birth to their first broods, they were transferred to beakers containing media that matched salinity concentrations used in the experiment. This ensured that the target animals experienced experimental conditions from the egg stage. At the start of the experiment, <3-h old neonates were isolated from their stock cultures and transferred into beakers (one individual per beaker) containing 100 mL of medium of appropriate salinity and 50 000 cells mL$^{-1}$ of *A. falcatus*. Each treatment combination received six replicates, though one or two replicates from some treatments were lost due to experimental error. Food and media were refreshed daily. At their second instar stage, individuals were checked for neatketeeth development using a dissecting microscope and returned to their source beaker. A neatketeeth scoring system was modified from (Tollrian, 1993). The base of the teeth was scored as 0% for a morphologically “normal” neck, 15% for a small bump on the neck, 30% for a medium bump and 50% for a “pedestal”. The teeth (up to five) were scored by their size and number where a large tooth scored 10% and a small tooth scored 5%. The final neatketeeth score was the sum of the scores of the base and teeth. To obtain maturation times, animals were monitored every 3 h one day before estimated maturation time until they matured (defined as when eggs first appeared in an individual’s brood chamber). At maturation, images of individuals were captured using a digital camera mounted on a dissecting microscope, and sizes were measured using NIS-Elements Documentation software (Nikon Instruments Inc., Melville, NY). Size was measured as the linear distance between the top of the head and the base of the tail spine. The first brood of neonates was produced, clutch sizes were recorded, and neonate body sizes were measured (as above) within 12 h of release from the brood chamber. The experiment continued until the third brood of neonates was produced in each replicate. The numbers of neonates in each brood and corresponding ages of the mothers at birth were recorded and used to calculate intrinsic population growth rates ($\lambda$) for each individual using the Euler equation:

$$1 = \sum e^{-r/m_j} l_j,$$

where $x$ is the age in days, $m_j$ represents the age-specific brood size, and $l_j$ is age-specific survivorship. To solve the equation for $\lambda$, we used the uniroot function in R Version 3.2.2 (R Development Core Team, 2011). Three broods are sufficient to calculate $\lambda$ as later broods add little precision (Riessen and Sprules, 1990).

**Statistical analysis**

All analyses were performed using R Version 3.2.2. For the first salinity tolerance experiment, we analyzed the effects of clone identity, salinity (as a continuous predictor) and their interactions on survivorship using GLM with binomial errors and a logit link. Data from the behavior experiments were analyzed using GLM with Gaussian errors, testing for effects of clone identity, salinity and their interaction. For the life history and morphology experiment, the effects of clone identity, salinity, kairomone and their interactions were analyzed for all response variables. Responses to salinity in the life history experiment were non-linear and idiosyncratic for some measures. Thus, we treated salinity as a fixed effect in these analyses. In cases where significant clone identity $\times$ salinity or clone $\times$ salinity $\times$ kairomone interactions were detected, we performed planned contrasts to test predictions that clone P14 would be less negatively affected by increasing salinity when compared to GR9. All other post-hoc tests were performed using pairwise comparisons with Tukey’s HSD test. We analyzed the size of the first brood to relate brood size to neonate size (which was only measured for the first brood) in addition to time-averaged brood size (averaging across the first three broods). For count data (maturation time and first brood size), GLM with Poisson errors and a log link was employed. For analyses of neatketeeth scores, $\log_{10}$ maturation size, $\log_{10}$ neonate size, time-averaged brood size and intrinsic population growth rate ($\lambda$), GLM with Gaussian errors was used. In cases of unbalanced data, we used type III sums of squares. Removal of non-significant effects and model selection for the GLM analyses were based on AIC using the step function in R. For analyses using GLM with Poisson or binomial errors, quasipoisson and quasi-binomial errors were used when over-dispersion of data was detected (Crawley, 2007). Assumptions of normality and homogeneity of variances for GLM with Gaussian errors were tested using Lilliefors and Levene’s tests, respectively, and by inspection of plots of residuals. Unless otherwise stated, data met model assumptions.

**RESULTS**

**Salinity tolerance experiment**

Survivorship was dependent on both salinity level and clone identity (Fig. 1A; clone $\times$ salinity effect: $F_{8, 94} = 3.22, P = 0.027$, GLM with quasibinomial errors). P14, which was isolated from the pond with the highest conductivity, tended to have the highest survivorship across salinity concentrations (Fig. 1A). In contrast, clones
GR9 and OL2 (both obtained from low conductivity ponds) showed low survivorship across the two highest salinities. For subsequent experiments, we chose P14 and GR9 which had strongly contrasting survivorships at the 34.2 and 51.3 mM NaCl concentrations ($P < 0.04$, two-sample $t$-test).

### Behavior experiment

Escape efficiencies generally declined for both clones P14 and GR9 as salinity increased (Fig. 1B; salinity effect: $F_{1,36} = 85.02, P < 0.001$, GLM with Gaussian errors). However, P14 exhibited more efficient escape behavior than GR9 across salinity levels; a significant effect of clone identity was detected ($F_{1,36} = 30.83, P < 0.001$, GLM with Gaussian errors). A weak but statistically insignificant interaction was found between the two factors ($F_{1,36} = 2.36, P = 0.099$, GLM with Gaussian errors), indicating that the slopes of the relationship between escape efficiency and salinity did not differ between clones. Despite this, differences in escape efficiencies between P14 and GR9 were stronger at the highest salinity level (Fig. 1B; $P < 0.001$, $t$-test) compared to the lowest salinity level (Fig. 1B; $P = 0.11$, $t$-test).

### Life history and morphology experiment

Neckteeth development was induced only when Chaoborus kairomone was present (Fig. 1C). There was a significant three-way interaction between clone identity, salinity and kairomone ($P < 0.001$, GLM with Gaussian errors, Table I). However, due to zero values in the absence of kairomone, errors were not normally distributed ($P < 0.001$, Lilliefors test) and variances were not homogeneous ($P < 0.001$, Levene’s test). Regardless, neckteeth scores were all significantly greater than zero for all treatments with kairomone present (all $P < 0.001$, Bonferonni adjusted, one-sample $t$-tests) while values were all zero in the absence of the chemical cue. Analysis of data from the kairomone-present treatments showed a significant two-way interaction between salinity and clone identity ($F_{3, 39} = 13.30, P < 0.001$, GLM with Gaussian errors). This interaction was driven by a significant difference in the neckteeth scores of the two clones at 4.3 mM NaCl ($P < 0.001$, Tukey’s HSD); no differences between clones were detected for the other salinity levels ($P > 0.30$, Tukey’s HSD).

Both salinity ($P = 0.002$, GLM with Poisson errors, Table I) and kairomone ($P = 0.0017$, GLM with Poisson errors, Table I) affected maturation time (Fig. 2A). There was no effect of clone identity ($P = 0.38$, GLM with Poisson errors) and no interactions were detected ($P > 0.10$, GLM with Poisson errors). Presence of kairomone delayed maturation when averaging across all clone and salinity combinations (Fig. 2A; $P < 0.01$, Tukey’s HSD). When analyzing the effects of salinity, mean maturation time in the 17.1 mM NaCl treatment was greater than all other salinity levels (all $P < 0.05$, Tukey’s HSD); all other pairwise
comparisons were not significant (all \( P > 0.80 \), Tukey’s HSD).

Kairomone and clone identity interactively affected size at maturation (Fig. 2B; \( P = 0.03 \), GLM with Gaussian errors, Table I); neither factor interacted with salinity (\( P > 0.40 \), GLM with Gaussian errors). The interaction was driven by increases in maturation size of clone P14 in the presence of kairomone (\( P = 0.005 \), Tukey’s HSD); GR9 showed no response to kairomone (\( P > 0.90 \), Tukey’s HSD). Salinity also affected maturation size (Fig. 2B; \( P < 0.001 \), GLM with Gaussian errors, Table I); there were significant differences among all salinity levels (\( P < 0.01 \), Tukey’s HSD) with the exception of the comparison between the 0 and 8.6 mM NaCl levels (\( P = 0.988 \), Tukey’s HSD). The largest and smallest mean maturation sizes were produced by the 4.3 and 17.1 mM treatments, respectively.

There was a significant three-way interactive effect of clone identity, kairomone and salinity on neonate size (Fig. 2C; \( P = 0.02 \), GLM with Gaussian errors, Table I). However, the assumption of homogeneity of variances was not met despite data transformation (\( P < 0.01 \), Levene’s test). Thus, \( P \)-values from the GLM should be viewed cautiously. Planned contrasts revealed that neonate size was smaller in the presence of kairomone for clone GR9 in the 8.6 mM NaCl treatment (Fig. 2C; \( P = 0.016 \), Bonferroni adjusted; \( P > 0.20 \) all other contrasts). Significant interclonal differences were only found at 8.6 mM NaCl in the absence of kairomone in which GR9 neonate size was larger than P14 (Fig. 2C; \( P < 0.01 \), Bonferroni adjusted, planned contrasts; \( P > 0.50 \) all other contrasts). Turning to effects of salinity, in the presence of kairomone, GR9 produced significantly smaller neonates in the 17.1 mM NaCl treatment when compared to 0 and 4.3 mM (Fig. 2C; \( P < 0.01 \), Tukey’s HSD; \( P > 0.19 \) all other comparisons). In the absence of kairomone, GR9 displayed significantly smaller neonate sizes under 17.1 mM NaCl when compared to 4.3 and 8.6 mM (Fig. 2C; \( P < 0.01 \), Tukey’s HSD; \( P > 0.11 \) all other comparisons). In the absence of kairomone, P14 had significantly larger neonate sizes under 0 mM NaCl when compared to the 4.3 and 17.1 mM treatments (Fig. 2C; \( P < 0.02 \), Tukey’s HSD; \( P > 0.60 \) all other comparisons). There were no significant differences in P14 neonate size among salinity levels in the presence of kairomone (all \( P > 0.60 \), Tukey’s HSD).

When analyzing first brood size (Fig. 2D), no significant interactions among the three factors were found (all \( P > 0.30 \), GLM with Poisson errors). Across salinity and clone treatments, brood sizes were greater in the presence versus absence of kairomone (Fig. 2D; \( P < 0.001 \), GLM with Poisson errors, Table I). Salinity also affected first brood size (\( P < 0.001 \), GLM with Poisson errors, Table I); sizes were significantly smaller at 17.1 mM NaCl when compared to the 4.3 and 8.6 mM treatments (Fig. 2D; \( P < 0.01 \), Tukey’s HSD; \( P > 0.10 \) all other comparisons). There was no effect of clone identity (\( P = 0.50 \), GLM with Poisson errors).

There was an interactive effect of salinity and kairomone when averaging brood size across the first three broods (Fig. 2E; \( P = 0.001 \), GLM with Gaussian errors, Table I). Kairomone increased brood size across all salinity levels (\( P < 0.001 \), Tukey’s HSD) but the magnitude of the effect decreased with increasing salinity (Fig. 2E). Salinity had non-linear effects on brood size. In the absence of kairomone, the largest brood sizes were produced in the 4.3 and 8.6 mM treatments (Fig. 2E);
responses in these two treatments were both significantly greater than responses in the 0 and 17.1 mM treatments \((P < 0.001, \text{Tukey's HSD}; P > 0.60 \text{ all other comparisons})\). Similarly, in the presence of kairomone, brood size was largest in the 4.3 mM treatment (Fig. 2E); responses were significantly greater in this treatment compared to all other levels \((P < 0.015, \text{Tukey's HSD})\). Brood size was also lower in the 17.1 mM treatment compared to all the other salinity levels in the presence of kairomone (Fig. 2E; \(P < 0.001, \text{Tukey's HSD}\)). There was some suggestion that responses also varied between clones (Fig. 2E), with P14 sustaining a stronger positive response to kairomone with increasing salinity (especially at the highest salinity level). Indeed, both clones exhibited significant positive responses to kairomone across salinity levels \((P < 0.05, \text{Tukey's HSD})\) with the exception of the 17.1 mM treatment in which only P14 showed a significant response \((P < 0.001, \text{Tukey's HSD});

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**Fig. 2.** Effects of salinity and *Chaoborus* kairomone presence/absence (+Kairomone and −Kairomone) on life history responses of *D. pulex* clones P14 and GR9. Responses included (A) maturation time, (B) size at maturation, (C) neonate size, (D) first brood size, (E) brood size averaged over the first three broods and (F) intrinsic population growth rate \((r)\). Shown are means \((±SE)\).
GR9 response: \( P = 0.99 \), Tukey’s HSD). While the three-way interaction term was retained using model selection, statistical confidence in the effect was weak (\( P = 0.10 \), GLM with Gaussian errors, Table I). Data met the assumption of normality but the assumption of homogeneity of variances was violated regardless of data transformations.

When analyzing intrinsic population growth rate (Fig. 2F), P14 exhibited higher growth rates compared to GR9 regardless of kairomone presence/absence or salinity level; a significant main effect of clone was detected (\( P = 0.005 \), GLM with Gaussian errors, Table I) but no clone \( \times \) treatment interactions (\( P > 0.20 \), GLM with Gaussian errors). Salinity and kairomone interactively affected population growth rate (Fig. 2F; \( P < 0.001 \), GLM with Gaussian errors, Table I). Kairomone induced a significant increase in \( r \) at 0 mM NaCl (\( P < 0.001 \), Tukey’s HSD) but had no effect at the other salinity levels (\( P > 0.90 \), Tukey’s HSD). Comparing responses across salinity levels in the absence of kairomone, growth rates in the 4.3 and 8.6 mM treatments were both significantly greater than responses in the 0 and 17.1 mM treatments (Fig. 2F; \( P < 0.001 \), Tukey’s HSD; \( P > 0.50 \) all other comparisons). In the presence of kairomone, population growth rate in the 17.1 mM treatment was significantly lower compared to the other three salinity treatments (Fig. 2F; \( P < 0.001 \), Tukey’s HSD); no differences were detected among the three lower salinity levels (\( P > 0.13 \), Tukey’s HSD). These results should be viewed with some caution as the assumption of normality was not met regardless of data transformations. As a supplement to the GLM analysis, we performed non-parametric one-way ANOVAs using Kruskal–Wallis (K–W) tests, testing for effects of clone identity, kairomone and salinity separately. Significant main effects of kairomone \( \left[ \chi^2(1, N=84) = 8.89, P = 0.003 \right] \) and \( \left[ \chi^2(3, N=84) = 44.2, P < 0.001 \right] \) were found on the ranks of \( r \).

**DISCUSSION**

Sodium chloride is known to cause physiological stress in freshwater invertebrates, driving allocation of energy to osmoregulation to prevent water loss. In *Daphnia*, this can negatively impact size-related swimming velocities (Baillieul et al., 1998) and body size due to reduced growth rates (Bezirci et al., 2012); both of which may increase vulnerability to *Chaoborus* predation. Consistent with this prediction, we found that increasing salinity was detrimental to the escape abilities of both clones. However, we predicted that greater salinity tolerance of clone P14 would give rise to a greater escape efficiency with increasing salinity concentrations when compared to clone GR9. In contrast, clone P14 displayed higher escape efficiency from *Chaoborus* across salinity levels and the interaction between clone identity and salinity was weak (\( P = 0.099 \)). Despite this, the interaction was retained following model selection. Moreover, P14 exhibited significantly higher escape efficiencies at the highest salinity level and a weaker, statistically insignificant difference with GR9 at the lowest salinity level, consistent with our predictions. The trend for a higher overall escape efficiency of P14 suggests a stronger background capability to escape from *Chaoborus* predation.

One possible explanation is that GR9 tended to produce slower-swimming juveniles compared to P14 regardless of salinity level (personal observation). Differences between our clones may reflect prior evolutionary responses to differences in predation pressure in their source environments or a correlated response to some other unknown selective pressure.

In addition to behavioral responses, *Daphnia* are known to exhibit morphological and life history plasticity in response to predators. Neckteeth are a known inducible defense against gape-limited predators such as *Chaoborus* (Swift and Fedorenko, 1975; Havel and Dodson, 1984). We predicted that increasing salinity would reduce neckteeth development, with P14 experiencing weaker reductions with increasing salinity stress. As predicted, both clones developed neckteeth in the presence of kairomone. However, a consistent effect of salinity or clone identity was not evident; P14 only displayed greater neckteeth development at 4.3 mM NaCl. The reason for this differential response is unclear. Maturation times tended to be shortest and maturation sizes largest at this salinity level for both clones. It is possible GR9 trades-off reduced neckteeth development for greater somatic growth under favorable salinity conditions.

Effects of predators on life history traits are also well documented in *Daphnia* (Lüning, 1992; Spitze, 1992; Tollrian, 1995b). Life history theory commonly predicts that delayed maturation is an optimal strategy for prey that experience predation on juvenile and early life stages (Law, 1979; Taylor and Gabriel, 1992). Moreover, larger maturation size has been cited as a defense against *Chaoborus* in previous studies of *Daphnia* (Lüning, 1992; Spitze, 1992; Tollrian, 1995b), a strategy that requires more time for somatic growth and delayed reproductive maturity. Consequently, we predicted that kairomone would induce delayed maturation and larger size at maturation in both clones and that P14 would show stronger and more sustained responses...
with increasing salinity. We found some support for this. Presence of kairomone delayed maturation as predicted, but effects were equal for both clones regardless of salinity level. This resulted in predicted increases in maturation size but only for clone P14, consistent with the view that this clone is more responsive to the presence of Chaoborus. Increasing salinity both increased maturation times and reduced maturation sizes at the highest concentrations, regardless of clone identity. Thus, increasing salinity stress in natural systems has the capacity to impair life history responses to predation risk, potentially increasing susceptibility to predators.

With larger size at maturity, prey may be able to produce more or larger neonates per reproductive bout. Prior studies have shown that larger neonate size contributes to survival from Chaoborus predation (Havel and Dodson, 1984), and as a trade-off, fewer neonates are produced (Lüning, 1992). Thus, we predicted that presence of kairomone would induce production of fewer but larger offspring, with clone P14 maintaining this response to a greater degree across salinity levels. In terms of reproductive output in the first brood, unexpected results were found. Chaoborus kairomone increased clutch sizes of both clones but had no consistent effect on neonate body size. As predicted, increasing salinity stress, at least at the highest NaCl concentration, reduced offspring size and offspring number of the salinity stress, at least at the highest NaCl concentration, reduced offspring size and offspring number of the first brood, but clone identity did not consistently mediate these effects. Results were similar when averaging fecundity across the first three broods. However, an interactive effect of clone, salinity and kairomone was suggested by our data and retained following model selection, though confidence in the effect was weak ($P = 0.10$). Post-hoc comparisons revealed that P14 was able to maintain significantly higher reproductive output in response to kairomone at the highest salinity level when compared to GR9. While our results did not match our general predictions, they are consistent with (Spitze, 1991) in which Chaoborus predation selected for larger maturation size and higher fecundity in experimental D. pulex populations. Enhanced reproductive output in the presence of kairomone could be a general adaptive response to predators if it translates into larger per capita population growth rates which may compensate for elevated mortality levels (Smith and Fretwell, 1974; Brockelman, 1975). We found limited support for this prediction; a strong effect of kairomone on $r$ was only evident in the 0 mM NaCl treatment.

A well-known and fundamental life history trade-off is positive effects of kairomone on brood size across clone and salinity treatments, a significant effect on neonate size was only detected for clone GR9 in the 8.6 mM NaCl treatment. When analyzing the relationship between log neonate size and brood size within each salinity treatment for each clone separately, a significant association was found only in this treatment combination ($r = -0.70$, $P = 0.023$, Pearson correlation; $P > 0.30$ all other comparisons). A possible explanation for the lack of covariation in offspring size and quantity is that algal resources were supplied at non-limiting concentrations which may have weakened expression of the trade-off. In natural settings, resource quantity and quality can vary greatly among and within systems. Thus, incorporation of such effects in future studies could provide greater insight into how clonal variation mediates trait responses to natural and anthropogenic stressors. Another interesting finding was that reproductive output and intrinsic growth rates were highest at intermediate salinity levels. Similar results have been found in prior studies of Daphnia (Weider and Hebert, 1987; Bezirci et al., 2012). It is possible that these concentrations better matched solute concentrations in the interstitial fluids of D. pulex, reducing osmotic stress.

**CONCLUSIONS**

How our results translate into realized fitness in natural systems (in the face of both salinity stress and active Chaoborus predation) is unknown. Chaoborus has been shown to possess high salinity tolerance and is capable of surviving most salt concentrations in impacted wetlands (Benbow and Merritt, 2004). Thus, these predators are a persistent biotic stressor in systems experiencing salt intrusion. Our experiments indicate that as salinity continues to increase to sublethal levels in freshwater systems, D. pulex will become more vulnerable to Chaoborus predation due to effects on escape behaviors and life history. Our results suggest that intraspecific variation in salinity tolerance can potentially mediate these effects, though the effects of clone identity we observed were generally weak or inconsistent. A potential explanation for the lack of strong clone effects is that our clones were obtained from ponds that were not impacted by salt pollution. The candidate clones used in our initial screening exhibited variation in salinity tolerances that corresponded to conductivity levels in their source ponds, consistent with adaptation to increasing osmotic stress. However, based on published conductivity-salinity regressions (Benbow and Merritt, 2004), estimated Cl$^{-1}$ concentrations in our source ponds were below 2.3 mM. In a
survey of highway-adjacent ponds in Michigan, *D. pulex* populations have been found to persist at Cl⁻¹ concentrations up to 10.2 mM (Liu, 2016). Moreover, assays of *D. pulex* clones obtained from these systems have revealed significantly higher salinity tolerances compared to those in the present study (Liu, 2016). Thus, the clones we utilized represented a small degree of the potential intraspecific variation present among natural populations. It is possible that the greater salinity tolerance observed in impacted systems may translate into a greater capacity to mediate the impacts of salinity stress on *D. pulex* interactions with *Chaoborus*. Further research with a broader range of clonal variation in salinity tolerance is warranted to further address the effects of intraspecific variation.

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