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Evolutionary Ecotoxicology Of Salinity Tolerance In Daphnia Pulex: Interactive Effects Of Clonal Variation, Salinity Stress, And Predation

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EVOLUTIONARY ECOTOXICOLOGY OF SALINITY TOLERANCE IN *DAPHNIA PULEX*: INTERACTIVE EFFECTS OF CLONAL VARIATION, SALINITY STRESS, AND PREDATION

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Introduction

Ecotoxicology of environmental stressors, especially anthropogenic pollutants, has experienced significant growth since the 1960s (Relyea and Hoverman, 2006). Its first scientific definition was proposed by Truhaut (1977), considering it as a branch of toxicology. Ecotoxicology addresses effects of toxicants on ecosystem components in an integrated ecological context (Truhaut, 1977). Thus, instead of simply investigating the direct toxicity of pollutants on single species, emphasis is also placed on understanding the indirect effects of stressors on species as mediated by their biotic interactions such as predation and competition (Relyea and Hoverman, 2006). Two types of indirect effects, density-based and trait-based indirect effects (Abrams, 1995), may underlie the ecological impacts of pollutants. While both types of indirect effects can occur simultaneously, historically, ecology has emphasized density-based effects of endogenous and exogenous perturbations in natural communities (Peacor and Werner, 2004; Preisser et al., 2005). More recent work in ecology and ecotoxicology has highlighted the importance of trait-based effects in which non-lethal impacts of environmental stressors alter species interactions by modifying behavioral, physiological, demographic or morphological traits (Weis et al., 2001; Peacor and Werner, 2004). The trait-based effects of multiple environmental stressors (such as the presence of toxins and antagonistic interactions) are an area of ongoing research. In this study, I examined trait-based effects of salinity stress on *Daphnia pulex* in response to lethal and non-lethal presence of its predator, *Chaoborus*.

Of the wide range of environmental factors that may cause negative impacts on freshwater ecosystems, salinity stress resulting from road salt intrusion is of more recent concern (Kaushal et al. 2005; Mullaney et al., 2009). Road salt is mainly composed of NaCl and often contains $MgCl_2$,

KCl, and $CuCl_2$. Chloride may be transported to road-adjacent freshwater systems by rainfall and melted snow and ice, and may thereby directly impact somatic growth, reproduction, and other physiological traits of freshwater organisms (Camargo, 2003; Soucek and Kennedy, 2005). In a study of road salt toxicity in 43 highway-adjacent wetlands in Michigan, Benbow and Merritt (2004) found summer chloride levels between 18 to 2700 mg/l, with 25% of the levels more than 334 mg/l, a level that may not cause significant damage to macroinvertebrates (Blasius and Merritt, 2002) but can seriously harm freshwater zooplankton (Sarma et al., 2006) in lentic freshwater systems.

Compared to lotic freshwater systems such as streams, impacts from road salt pollution are considered to be greater in lentic ecosystems which may have little or no inflows and outflows (Moore et al., 1999). Chloride from road salt has a greater retention time in standing than moving waters (Rosenberry et al., 1999), causing stronger and longer negative impacts on organisms in them (Van Meter et al., 2011; Gallagher et al., 2011). Among lentic systems, wetlands and ponds are common roadside features and of general conservation concern due to their potentially high levels of freshwater vertebrate and invertebrate biodiversity (Denny, 1994; De Meester et al. 2005; Gopal, 2009). *Daphnia* are often the dominant primary consumers in temperate zone systems and are recognized as keystone species in freshwater communities (Martin-Creuzburg et al., 2005; Sarnelle, 2005; Persson et al., 2007). *Daphnia pulex*, a well-studied model organism (Garda et al., 2009; Lampert, 2006), plays an important role in the biological structure and functioning of many small freshwater systems (Steiner, 2004). In wetlands and small lakes in temperate and boreal regions, *Daphnia* generally face predation pressure from 3rd and 4th instars of *Chaoborus* larvae (Moore, 1986; Pastorok, 1980). In fishless ponds in Michigan, *Chaoborus americanus* is the dominant taxon among the four most common *Chaoborus* species (Garcia and Mittelbach, 2008;

Von Ende, 1979). *Chaoborus americanus* species selectively feeds on small (< 1mm) crustacean zooplankton (Von Ende and Dempsey, 1981; Cooper, 2011) including *D. pulex*, especially when it is more available (Spitze, 1985).

Daphnia have developed behavioral defensive mechanisms against *Chaoborus*, ambush predators that normally stay still and strike when prey are in close proximity. *Daphnia* may swim fast to escape from *Chaoborus* strikes (Swift, 1992). *D. pulex* can display other responses that assist with defense against *Chaoborus*. Morphologically, *Daphnia pulex* may develop an additional neckteeth structure on the dorsal margin in juvenile instars in presence of *Chaoborus* kairomone, a predator cues that may be detected by preys (Krueger and Dodson, 1981; Parejko and Dodson, 1991). This structure is believed to contribute to successful escapes of *D. pulex* by reducing the abilities of *Chaoborus*, which are gape-limited predators, to handle and ingest the prey (Tollrian, 1995a; Spitze, 1992; Havel and Dodson, 1984). In addition, *D. pulex* exhibits plasticity in life history traits in response to *Chaoborus*. Black (1993) found that *D. pulex* experienced a slower maturation rate with larger maturation sizes and gave birth to larger but fewer neonates in response to *Chaoborus* kairomone. It was suggested by Black (1993) that larger maturation and neonate sizes assisted with defense to the gape-limited predators. As trade-offs, longer time was required for maturation, and fewer neonates were produced.

How the defense behaviors and morphological and life history responses are affected by a variety of environmental factors has been extensively studied. Natural factors such as light intensity (Dodson et al., 1997), food availability (Van Gool and Ringelberg, 1995), and water temperature (Ziarek et al., 2011) have been shown to influence the swimming behavior and depth distribution of *Daphnia*. Not only natural but anthropogenic factors such as nanoparticles (Lovern et al., 2007) and insecticides (Dodson and Hanazato, 1995) can also affect defensive swimming

behaviors of *Daphnia*. Neckteeth of *D. pulex* was negatively impacted by a pesticide endosulfan (Barry, 2000). Coors et al., (2008) found additive effects of parasites, pesticides and *Chaoborus* kairomone on the sizes and ages at maturity of *Daphnia magna*. Inorganic substances such as metal ions (Hunter and Pyle, 2004) and oxygen concentration (Hanazato and Dodson, 1995) were shown to affect the responses of *D. pulex* to *Chaoborus* kairomone.

However, very limited studies have addressed the effects of salinity stress on *Daphnia-Chaoborus* interactions and induced responses of *Daphnia*. 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 (Grzesiuk and Mikulski, 2006; Teschner, 1995), including *D. pulex* from habitats with naturally incurred salinity differences (Weider and Hebert, 1987; Weider 1993; Latta et al., 2012). In the light of local adaptation, under the selection pressure of high salinity, *Daphnia* clones with higher salt tolerance may outcompete the ones with lower tolerance and eventually dominate the population (Teschner, 1995; Liao et al., 2015; Kawecki and Ebert, 2004). This adaptation may have significance in contributing to the resilience and stability of the population in face of salinity stress (Liao et al., 2015; Gonzalez et al., 2013). Providing the important ecological functions of *Daphnia* in their food webs, the significance of the adaptation may extend to community levels (Fussmann et al., 2007). Unfortunately, how genetic variation and local adaptation of *Daphnia* to salinity stress mediate its interactions with predators largely remains unexplored (but see Bezirci et al., 2012).

In natural ponds in southern Michigan, ionic concentrations, as measured by conductivity, vary greatly in the absence of anthropogenic salt inputs (Steiner, unpublished data). In my study, adaptation of *D. pulex* clones to the ionic concentrations in their habitats was tested. I hypothesized

that past exposure to higher conductivity would select for tolerance to higher ionic concentrations which in turn would translate into greater salinity tolerance. Of the tested clones, I chose two that showed strongly contrasting salinity tolerances for the following study. To investigate if salinity tolerance can mediate the escape behavior of *D. pulex* in face of lethal predation of *Chaoborus*, I tested escape efficiencies of the two clones. I hypothesized that the stronger salt-tolerant clone would display higher escape efficiencies than the less tolerant clone after receiving different levels of salt stress. Responses of *D. pulex* to *Chaoborus* kairomone, effects of salinity stress on the responses, and mediation of salinity tolerance in salinity effects were then investigated. I hypothesized that both clones would exhibit phenotypic plasticity and respond to predator cues by developing neckteeth and shifting their life histories towards larger maturation sizes at older ages along with reproducing fewer but larger offspring. I further hypothesized that salinity stress would negatively impact all the responses while the clone with stronger salinity tolerance would display stronger neckteeth development and life history shifts across increasing levels of salinity stress when compared to the less tolerant clone.

Methods

Target animals

I examined salinity tolerance of four *Daphnia pulex* clones: two (P12 and P14) isolated from ponds at the Kellogg Biological Station (KBS), 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 (Pinkney, MI). 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 exhibit morphological defenses (neckteeth formation) when exposed to *Chaoborus* kairomone. The source ponds also represented a range of natural conductivities based on prior field surveys (P14: 350.5μS/cm; P12: 348.3μS/cm; GR9: 65.2μS/cm; OL2: 43.8μS/cm; Steiner, unpublished data).

Chaoborus spp., were sampled from natural ponds in KBS Lux Arbor Reserve, Delton, Michigan in summer and fall 2014. *Chaoborus americanus* is the dominant *Chaoborus* species in ponds in the region (Garcia and Mittelbach, 2008; Personal Observation and Asgari, pers. Comm.) Only the 3rd or 4th instar larvae of this genus were used because only they have sufficient gape sizes to feed on juvenile *D. pulex* (Moore, 1986; Swift, 1992). Some of the animals were maintained in the lab under constant temperature (20 °C) and photoperiod (12h light: 12h dark). They were fed with *D. pulex* juveniles to match the experimental conditions, while the rest were used to extract *Chaoborus* kairomone. The extraction method was modified from Hebert and Grewe (1985). Twenty-five grams (about 6250 individuals) of *Chaoborus* were boiled in 1.5 L distilled water for 10 minutes. The liquid was then filtered using a 30- were boiled in 1 by vacuum filtration using Whattman GF/B (1.0 μm pore size), Whattman GF/F (0.7 μm pore size), and Callman (0.2 μm pore size) glass fiber filters in sequence. Extracted kairomone solution was stored in -80 °C freezer. This kairomone 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, maternal effects were first removed by culturing the animals in common garden conditions for three generations (Tollrian, 1995b). Less than 24 hour old neonates from the third generation were isolated from their stock cultures and transferred to beakers containing 200mL of medium; replicate beakers contained 15

neonates of a given clone. The experimental medium consisted of aged tap water at one of four salinity concentrations (0, 1, 2, 3 g/l NaCl). The average chloride level in Detroit tap water was 0.011 g/L, a negligible level compared to the experimental salinity levels (2014 Water Quality Report, Detroit Water and Sewage Department). These salt concentrations are comparable to the range observed in natural roadside waterbodies in Michigan (Benbow and Merritt 2004; see also Chapter 2). Clones GR9, P12, P14, and OL2 had 8, 4, 6, and 5 replicates per salinity level, respectively. They were all fed with *Ankistrodesmus falcatus* at a non-limiting concentration (100,000 cells/ml). Food and culture media were refreshed daily. After 96 hours, the numbers of surviving juveniles in each replicate were counted, and survivorship was calculated by dividing the number of surviving individuals by the total number at the beginning. The two clones with the highest and lowest survivorship (clones P14 and GR9) were used for subsequent experiments examining behavioral, morphological and life history responses.

Behavior experiment

Escape behavior of clones P14 and GR9 was assessed for clones that were given short-term exposure to four salinity levels (0, 0.7, 1.4, and 2.0 g/l NaCl). *Chaoborus* cultures were acclimated in the lab for at least one month before the initiation of experiments. Individuals were starved for 2 days (Swift and Fedorenko, 1975) before being employed in the behavior experiment. To remove maternal effects, *D. pulex* clones were cultured under common garden conditions (as described above). Neonates from the third generation were employed in the experiment. Before the start of each experiment, less than 24 hour old *D. pulex* 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 g/L NaCl, aged tap water occupied by 20 starved *Chaoborus* (modified from

Swift and Fedorenko, 1975). Escape behavior was determined in multiple, non-concurrent trials. For each clone and salinity exposure level combination, 5 replicate trials were used, and freshly starved *Chaoborus* were used in each replicate. 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 of *Daphnia* was calculated by dividing the number of escapes by the number of strikes, and it reflects the ability of *Daphnia* to escape from predation as a defense behavior (Swift and Fedorenko, 1975).

Morphological and Life history experiment

D. pulex life history and morphological responses were assessed in a 4 x 2 x 2 factorial design: 4 salinity levels (0, 0.25, 0.5, 1 g/l 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 1g/l NaCl was found to be close to the limit where eggs of both clones were able to develop into neonates (personal observation). Maternal effects were removed using similar methods to those 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, less than 3 hour old neonates were isolated from their stock cultures and transferred into individual beakers (one *Daphnia* per beaker) containing 100 ml of aged tap water of appropriate salinity and algal food at 10,000 cells/ml. Each treatment

combination received 6 replicates, though one or two replicates from some treatments were lost due to experimental error. Food and media were refreshed daily. At their 2nd instar stage, the target animals were checked for neckteeth development using a dissecting microscope and returned to their source beaker. A neckteeth scoring system was used, modified from Tollrian (1993). In terms of the base for the teeth, a morphologically "normal" neck = 0%, a small bump on the neck = 15%, a medium bump = 30%, and a "pedestal" = 50%. 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 neckteeth score is sum of the scores of the base and teeth. The animals were monitored every 3 hours from the start of the experiment until maturation to obtain maturation times in hours. Maturation of *Daphnia* was defined as when their egg chambers were filled with eggs. At maturation, images of individuals were captured using a Photometrics CoolSNAP EZ digital camera mounted on a Nikon SMZ 1000 dissecting scope, and maturation sizes were measured using NIS-Elements Documentation software. Size was measured as the linear distance between top of the head and base of the spine. When the first brood of neonates from the target animals were produced, clutch sizes were recorded, and neonate body sizes were measured in the same manner. The birth events of the first broods were monitored every 12 hours so that the neonates were photographed at their 1st instar, a stage when the size of *Daphnia* stays the same as when they were born. 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 (r) using the Euler equation: $1 = \sum e^{-rx} l_x m_x$, where x is the age in days, m_x represents the age-specific brood size, and l_x is age-specific survivorship. To solve the equation for r, I used uniroot function in R Version 3.2.3 with defining a value range provided by an

estimated r value using log (net reproduction rate/generation time). Three broods is sufficient to calculate r as later broods add little incremental precision (Riessen and Sprules, 1990).

Statistical analysis

General Linear Model (GLM) with binomial errors and a logit link was used to analyze the effects of clone identity, salinity (as a continuous predictor) and their interactions on survivorship in the first salinity tolerance experiment. Results were highly overdispersed, thus, I present results using quasibinomial errors. Non-significant interactions and model selection was performed based on AIC using the step function in R (with forward and backwards model selection). In cases of unbalanced data, I used type III sums of squares. Data from the behavior experiments were analyzed using GLM with Gaussian errors and an identity link, testing for effects of clone identity, salinity (as a continuous covariate) 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 were highly non-linear and sometimes idiosyncratic for some measures. Thus, I treated salinity as a fixed effect (rather than a continuous predictor) in these analyses. For neckteeth scores, maturation sizes, and neonate sizes, GLM with Gaussian errors was used. For count data (maturation time and first brood sizes), GLM with Poisson errors and a log link was employed. For effects on intrinsic growth rate, GLM with Gaussian errors and Kruskal-Wallis test were both used. Assumptions of normality and homogeneity of variances for GLM with Gaussian errors were tested by Kolmogorov–Smirnov (K-S) and Levene's tests, respectively. Pairwise comparisons were performed using Tukey's HSD in SYSTAT 13 and R (using the ghlt function in the multcomp package). GLM and Kruskal-Wallis was performed using R Version 3.2.3. Kolmogorov–Smirnov and Levene's were performed using SYSTAT 13.

Results

Salinity tolerance experiment

Survivorship was dependent on both salinity level and clone identity as indicated by a significant interaction (Fig. 1; $F_{3, 88} = 3.22$, $p = 0.027$, GLM with quasibinomial errors). P14 tended to have the highest survivorship across salinity concentrations (Fig. 1). For subsequent experiments I chose P14 and GR9 which had strongly contrasting survivorships at the 2g/l NaCl concentration, ($p = 0.003$, two-sample t-test) and 3g/L NaCl concentration ($p = 0.041$, two-sample t-test).

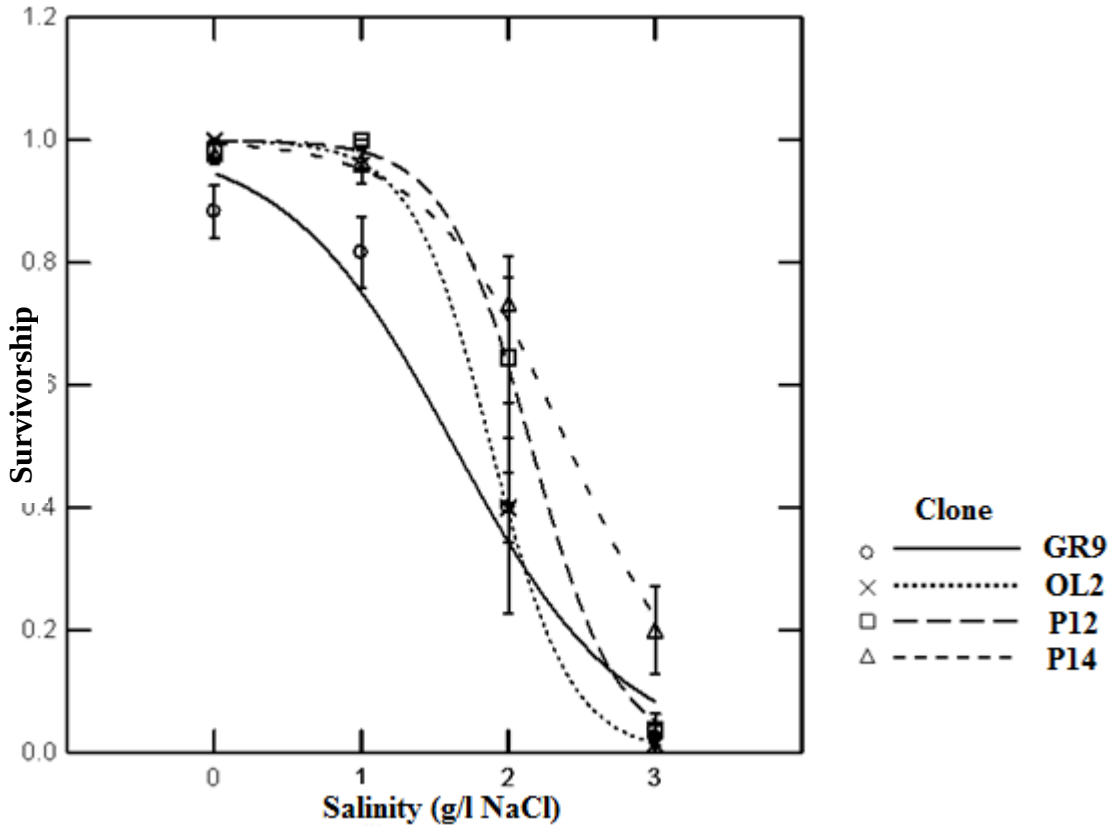

Figure 1: Relative survivorships of juveniles of the two *D. pulex* clones GR9 and P14 under exposure to different salinity levels for 96 hours. Shown are fits from the GLM analysis.

Behavior experiment

Escape efficiencies generally declined for both clones P14 and GR9 as salinity increased (Fig. 2); significant effects of salinity ($F_{1, 38} = 85.02$, $p < 0.001$, GLM with Gaussian errors) and clone identity ($F_{1, 38} = 30.85$, $p < 0.001$, GLM with Gaussian errors) on escape efficiency was detected, P14 appeared to exhibit more efficient escape behavior than GR9 across salinity levels. No interaction was found between the two factors ($F_{1, 38} = 2.86$, $p = 0.099$, GLM with Gaussian errors), indicating that the slopes of the relationship between escape efficiency and salinity did not differ between clones. Despite this, escape efficiency of P14 was significantly greater than GR9 at the highest salinity level (Fig. 2; $p < 0.001$, ANOVA) but did not differ at the lowest salinity level (Fig. 2; $p = 0.11$, ANOVA). Normality and homogeneity of variances of the data were analyzed using K-S Test and Levene's Test, respectively, and the assumptions were met.

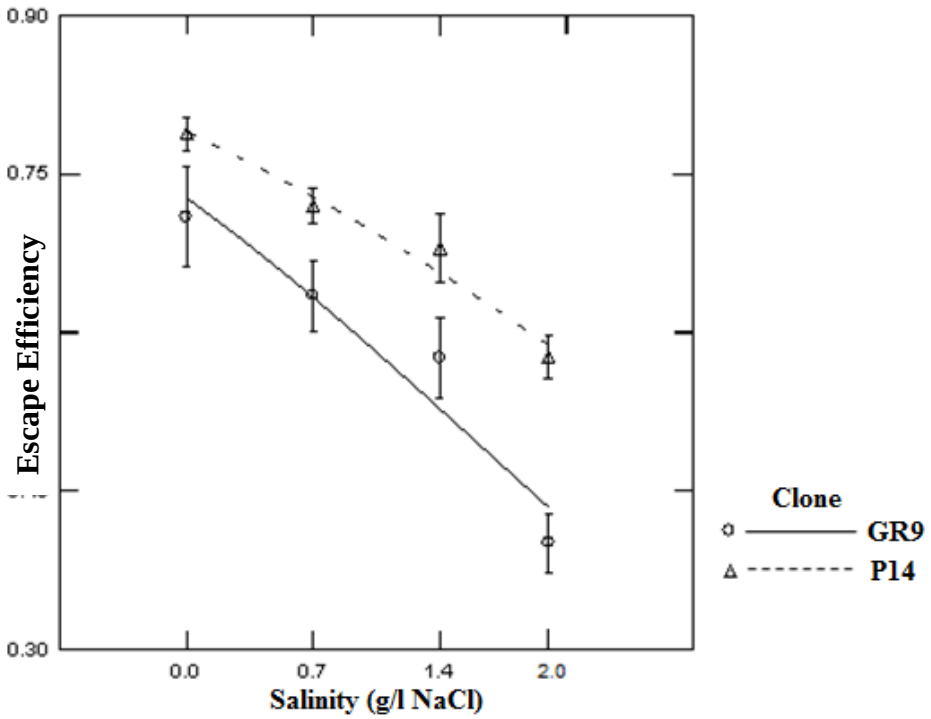

Figure 2: Escape efficiencies of the 3rd instar *D. pulex* juveniles from *Chaoborus* predation after exposure to different salinity levels for 72 hours. Shown are linear regression fits for both clones.

Morphological and Life history experiment

Neckteeth development was induced only when *Chaoborus* kairomone was present (Fig. 3). There was a significant three-way interaction between clone identity, salinity and kairomone ($F_{3, 79} = 1231$, $p < 0.001$, GLM with Gaussian errors). However, due to zero values in the absence of kairomone, errors were not normally distributed ($p < 0.001$, K-S Test) and variances were not homogeneous ($p < 0.001$, Levene's Test). Thus, p-values must be interpreted with caution. Analysis of data from the kairomone-present treatments showed a significant 2-way interaction between salinity and clone identity ($F_{3, 39} = 13.3$, $p < 0.001$, GLM with Gaussian errors). This interaction was driven by a significant difference in the neckteeth scores of the two clones under 0.25 g/l NaCl ($p < 0.001$, Tukey's HSD); no differences between clones were detected for the other salinity levels ($p > 0.05$, Tukey's HSD). Data met did not meet homogeneity of variances regardless of data transformation ($p < 0.001$, Levene's test). Thus, p-values from the GLM should be viewed with caution.

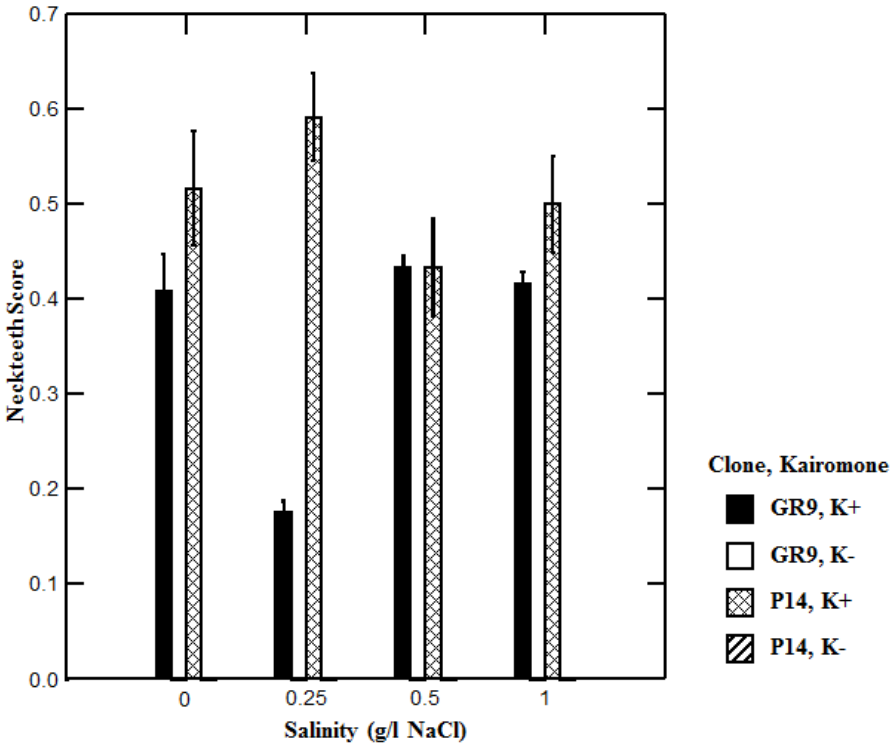

Figure 3: Neckteeth scores of the two *D. pulex* clones under the treatments of different combinations of salinities and *Chaoborus* kairomone conditions (K+: kairomone present, K-: kairomone absent).

Both salinity (Fig. 4; $\chi^2(3, N = 90) = 14.85$, $p = 0.002$, GLM with Poisson errors) and kairomone (Fig. 4; $\chi^2(1, N = 90) = 9.89$, $p = 0.0017$, GLM with Poisson errors) affected maturation time. There was no effect of clone identity ($p = 0.38$, GLM with Poisson errors). No interactions among the three treatments were detected ($p > 0.05$, GLM with Poisson errors). Presence of kairomone delayed maturation when averaging across all clone and salinity combinations (Fig. 4; $p < 0.01$, Tukey's HSD). When analyzing the effects of salinity averaged across the other treatments, 1 g/l NaCl had the highest maturation times when compared to all other salinity levels (Fig. 4; all $p < 0.05$, Tukey's HSD).

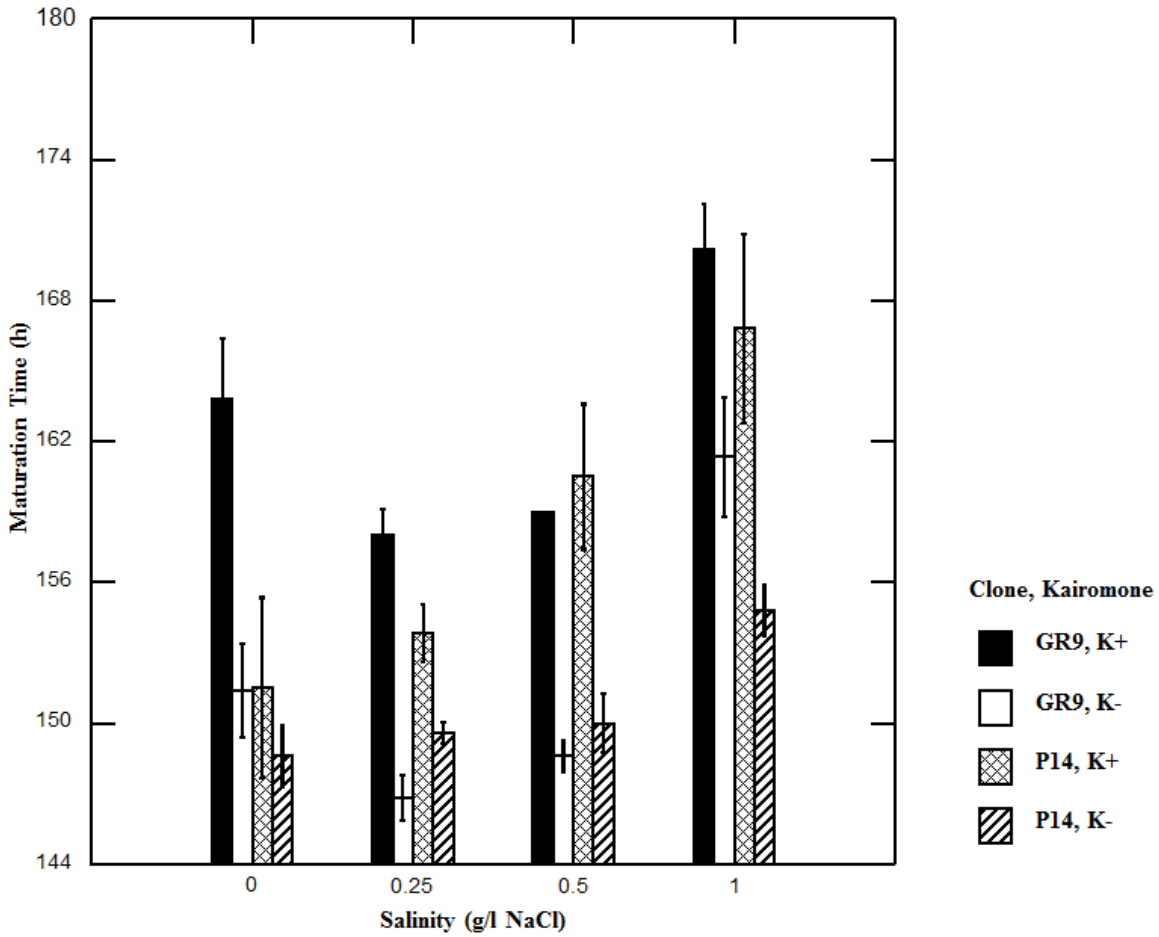

Figure 4: Maturation time of the two *D. pulex* clones under the treatments of different combinations of salinities and *Chaoborus* kairomone conditions. (K+: kairomone present, K-: kairomone absent)

Kairomone and clone identity interactively affected maturation size (Fig. 5; $F_{3, 73} = 5.21$, $p = 0.025$, GLM with Gaussian errors); neither factor interacted with salinity ($p > 0.05$, GLM with Gaussian errors). Pairwise comparisons indicated that this interaction was solely 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 ($p > 0.05$, Tukey's HSD). Salinity also affected maturation size (Fig. 5; $F_{3, 73} = 33.27$, $p < 0.001$, GLM with Gaussian errors). Pairwise comparisons revealed significant differences among all salinity levels ($p < 0.01$, Tukey's Test) with the exception of the comparison between the 0 and 0.5 g/l NaCl levels ($p = 0.988$, Tukey's Test). As shown in Fig. 5, 0.25 g/l NaCl produced the largest mean maturation size while 1 g/l NaCl reduced mean maturation size the most.

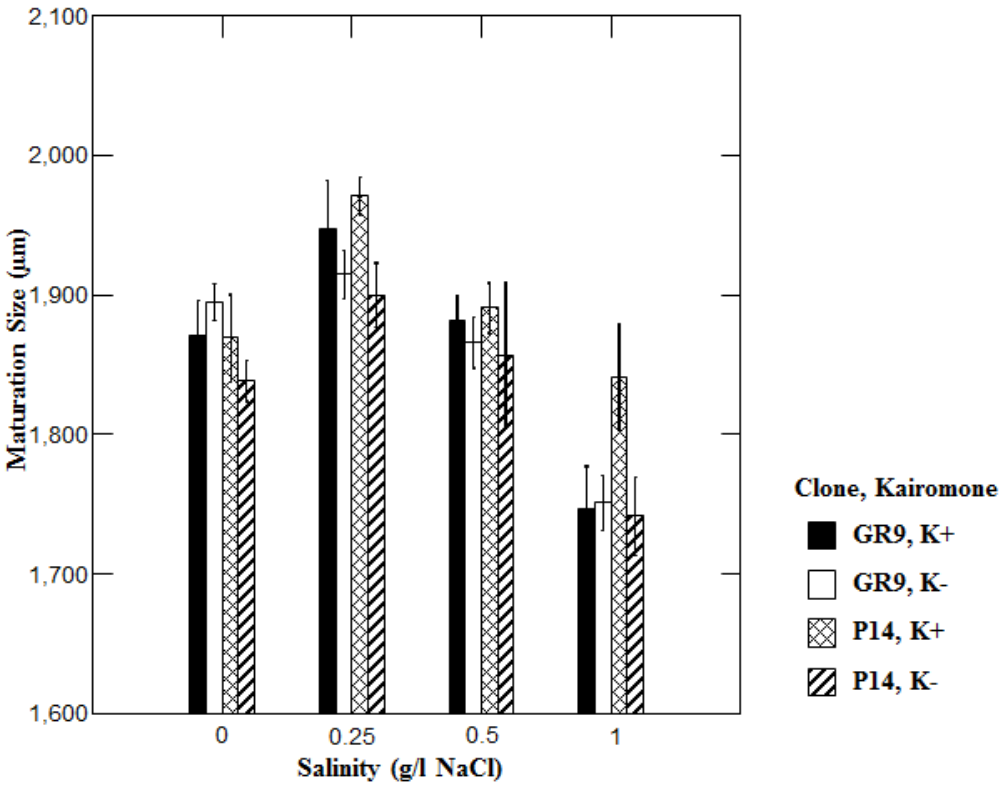

Figure 5: Maturation sizes of the two *D. pulex* clones under the treatments of different combinations of salinities and *Chaoborus* kairomone conditions. (K+: kairomone present, K-: kairomone absent)

There was a significant 3-way interactive effect of clone identity, kairomone and salinity on neonate size (Fig. 6; $F_{3, 66} = 3.52$, $p = 0.02$, GLM with Gaussian errors). Pairwise comparisons indicated that the kairomone treatment did not have any effects on neonate size within every combination of salinity and clone identity ($p > 0.05$, Tukey's HSD). In the presence of kairomone, GR9 produced significantly smaller neonate sizes under 1 g/l NaCl when compared to 0 g/l NaCl ($p = 0.009$, Tukey's HSD) and 0.25 g/l NaCl ($p = 0.001$, Tukey's HSD). In the absence of kairomone, GR9 displayed significant smaller neonate sizes under 1 g/l NaCl when compared to 0.25 g/l NaCl ($p = 0.004$, Tukey's HSD) and 0.5 g/l NaCl ($p = 0.002$, Tukey's HSD). In the absence of kairomone, P14 had significantly larger neonate sizes under 0 g/l NaCl compared to 0.5 g/l NaCl ($p = 0.025$, Tukey's HSD) and 1 g/l NaCl ($p = 0.011$, Tukey's Test). No significant difference ($p > 0.05$, Tukey's HSD) was present in the neonate sizes in P14 under different salinity levels in the presence of kairomone. Significant interclonal differences ($p = 0.006$, Tukey's HSD) were only found under 0.5 g/l NaCl without kairomone treatment in which GR9 neonate size was larger than P14.

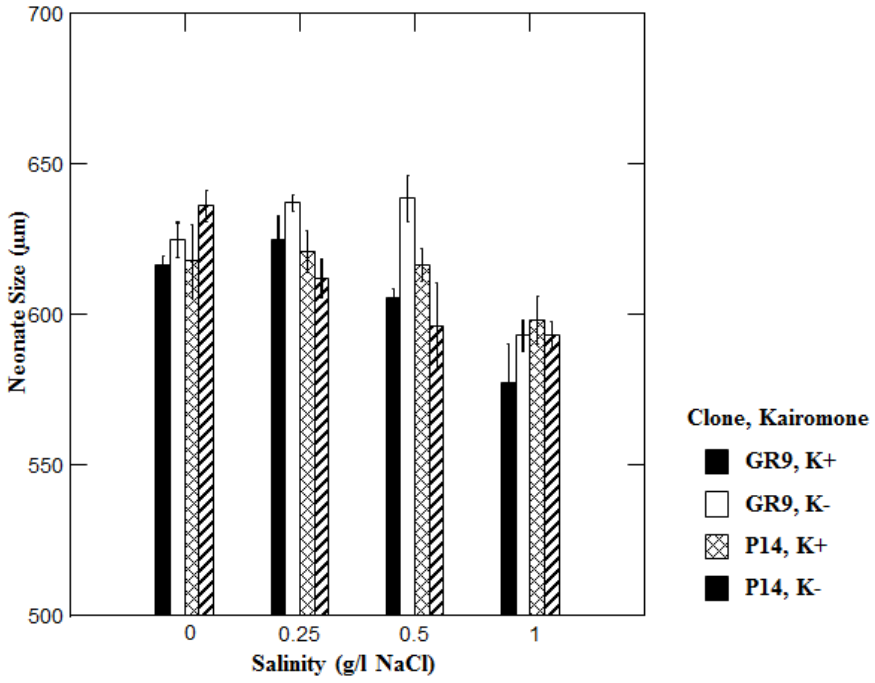

Figure 6: Neonate sizes (body length) of the two *D. pulex* clones under the treatments of different combinations of salinities and *Chaoborus* kairomone conditions. (K+: kairomone present, K-: kairomone absent)

For the first brood sizes (Fig. 7), no significant interactions among the three factors was found (all $p > 0.05$, GLM with Poisson errors). Significant main effects of salinity ($\chi^2(3, N = 83) = 17.36$, $p < 0.001$, GLM with Poisson errors,) and kairomone ($\chi^2(1, N = 83) = 18.87$, $p < 0.001$, GLM with Poisson errors,) were detected. No effect of clone identity was present ($p > 0.05$, GLM with Poisson errors). Presence of kairomone significantly induced more neonates in the first broods across the other treatments (Fig. 7; $p < 0.001$, Tukey's HSD). When analyzing the effects of salinity averaged across the other treatments, 1 g/l NaCl had the smallest brood size when compared to 0.25 and 0.5 g/l NaCl (Fig. 7; both $p < 0.05$, Tukey's HSD).

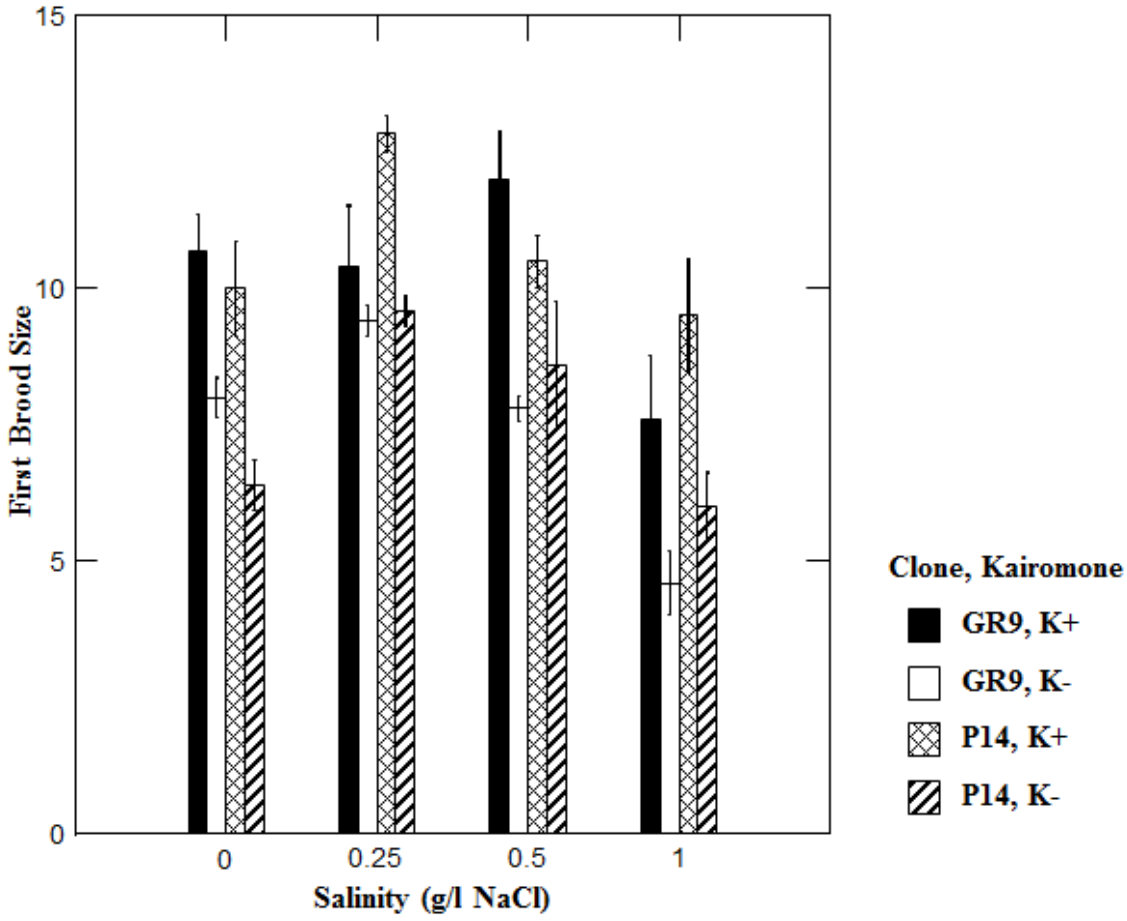

Figure 7: First brood sizes of the two *D. pulex* clones under the treatments of different combinations of salinities and *Chaoborus* kairomone conditions. (K+: kairomone present, K-: kairomone absent)

When analyzing intrinsic population growth rate, r (Fig. 8), P14 exhibited higher growth rates compared to GR9 regardless of kairomone presence/absence or salinity level; a significant main effect of clone was detected ($F_{3, 68} = 9.12$, $p = 0.003$, GLM with Gaussian errors,) but no clone x treatment interactions ($p > 0.05$, GLM with Gaussian errors). Salinity and kairomone interactively affected population growth rate (Fig. 8; $F_{3, 68} = 13.83$, $p < 0.001$, GLM with Gaussian errors). Kairomone only induced a significant increase in r under 0 g/l NaCl across both clones ($p < 0.001$, Tukey's HSD). 1 g/l NaCl induced the lowest r among all salinity levels across both clones regardless of kairomone presence/absence ($p < 0.001$, Tukey's HSD). Population growth rate under 0 g/l NaCl across the two clones was significantly smaller than 0.25 and 0.5 g/l NaCl in the absence of kairomone ($p < 0.001$, Tukey's HSD). The assumption of normality was not met (p = 0.001, K-S Test) regardless of data transformations. Thus, p-values must be viewed with caution. As a supplement to the GLM analysis, I performed non-parametric one-way ANOVAs using the Kruskal-Wallis (K-W) Test testing for effects of clone identity, kairomone and salinity separately. Significant main effects of kairomone ($\chi^2(1, N = 84) = 8.89$, $p = 0.003$, K-W Test), salinity ($\chi^2(3, N = 84) = 44.2$, $p < 0.001$, K-W Test), and clone ($\chi^2(1, N = 84) = 5.71$, $p = 0.017$, K-W Test) were found on the ranks of r.

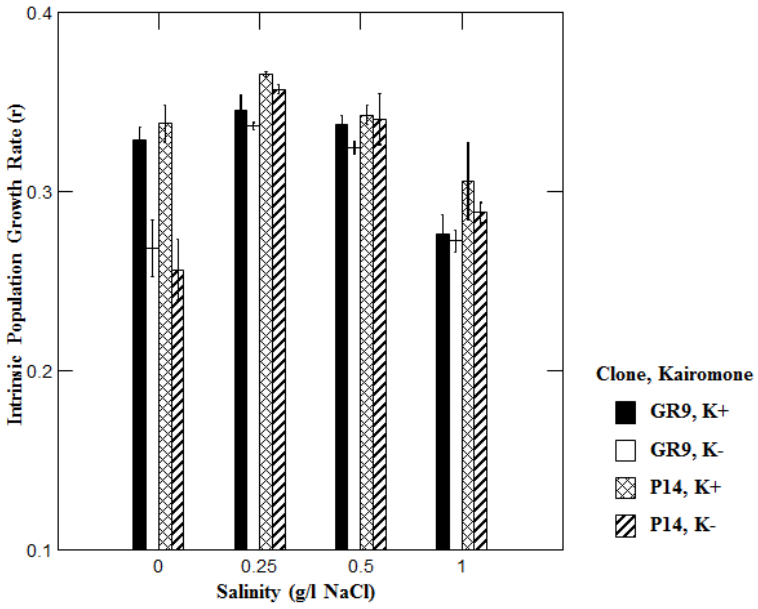

Figure 8: Intrinsic population growth rate (r) of the two *D. pulex* clones under the treatments of different combinations of salinities and *Chaoborus* kairomone conditions. (K+: kairomone present, K-: kairomone absent)

Discussion

Physiological responses play significant roles in population and community dynamics when organisms experience external stressors. Stress can lead to reduced somatic growth, reproduction, and/or survival, potentially altering population abundances and persistence. Given that *Daphnia* and *Chaoborus* are common species in freshwater ponds, it is important to reveal the mechanisms of how stressors can impact the interspecific interactions. Missing from many prior studies of ecotoxicology is explicit consideration of the effects of intraspecific variation on population and community responses to environmental perturbations. Heritable phenotypic variation is a potential correlate of the adaptive capacity of a species and thus its ability to persist under environmental changes. As human activities and environmental impacts continue to increase in scale and magnitude, it has become urgent to assess the degree to which intraspecific variation and adaptive evolution can mitigate biotic responses to anthropogenic stressors. Prior work has revealed inter-clonal differences in the salinity tolerance of *D. pulex* that were associated with salinity levels in their source habitats (Weider and Hebert, 1987; Latta et al., 2012), an indicator of past selection pressure and adaptive evolution. The four clones I used originated from ponds with different conductivities (P14: 350.5μS/cm; P12: 348.3μS/cm; GR9: 65.2μS/cm; OL2: 43.8μS/cm; Steiner, unpublished data) and thus potentially different levels of osmotic stress. Survivorship of the four clones generally fit my prediction that prior exposure and adaptation to higher conductivities would confer stronger capabilities to tolerate NaCl stress. Clone P14 was isolated from a pond with the highest conductivity of the four and exhibited the highest tolerance to elevated salinity. In contrast, clones GR9 and OL2 (both obtained from low conductivity ponds) exhibited low survivorship across the two highest salinity treatments relative to the other clones.

With greater salinity tolerance, P14 predictably displayed higher escaping efficiency from *Chaoborus* compared to GR9 under all salinity levels. Increasing salinity was also found to be detrimental to the escape abilities of both clones. NaCl is known to cause physiological stress in freshwater organisms through osmosis which drives organisms to allocate more energy to prevent water loss from their bodies to the surrounding environment. This energy allocation can negatively impact somatic growth (Bezirci et al., 2012) and size-related swimming velocities (Baillieul et al., 1998). The adverse effects of salinity on escape behavior that I observed could be due to such weakened responses and swimming rates as well as reduced body sizes from the 2 day exposure period (personal observation) which may have made the daphniids more vulnerable (Spitze, 1985). Some results deviated from general predictions. I predicted that greater salinity tolerance of clone P14 would give rise to a greater capacity to escape predation with increasing salinity concentrations when compared to clone GR9. In contrast, a non-significant interactive effect of clone identity and salinity indicated that both clones exhibited similarly reduced escape abilities with increasing salinity. However, P14 did exhibit significantly higher escape efficiencies at the highest salinity level and a weaker, statistically insignificant difference with GR9 at the lowest salinity level, consistent with my predictions. Although there was no significant difference between the clones in 0 g/l NaCl treatment, there was still a trend for higher escape efficiency of P14 in this treatment, suggesting a stronger background capability of P14 to escape from *Chaoborus* predation. One possible explanation is that GR9 tended to produce smaller and slower-swimming juveniles compared to P14 (personal observations). Differences between the 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. Clonal

differences in responses to *Chaoborus* have been previously demonstrated for *D. pulex* (Boeing et al., 2005; Krueger and Dodson, 1981; Repka et al., 1995).

One limitation of the behavioral experiment was that predation and escape behaviors were tested under 0 g/l NaCl conditions. Even though this was different from the conditions in which some *Daphnia* juveniles were treated, it avoided introducing potential effects of salinity on the ability of *Chaoborus* to attack their preys. Moreover, each replicate round took one hour to 90 minutes, a short amount of time relative to the two day salt exposure period.

In addition to behavioral responses, *Daphnia* are known to exhibit phenotypic plasticity in response to predators; a suite of morphological and life history responses to predators are well documented. Neckteeth is a common defensive structure against gape-limited predators like *Chaoborus* (Swift and Fedorenko, 1975; Havel and Dodson, 1984). This structure is usually inducible and only develops with the stimulation of predator cues as an energy saving strategy (Black, 1993; Lüning, 1992). This pattern was clearly shown in my experiment with neckteeth induction occurring only in the kairomone-present treatments. I predicted that P14 would show stronger neckteeth development with increasing salinity when compared to GR9 because of mediation of salinity tolerance. This was not supported by my results. The only significant difference in neckteeth development between the two clones was at 0.25 g/l NaCl with P14 displaying higher scores compared to GR9; responses did not differ at the other salinity levels. The reason for this differential response is unclear. The 0.25 g/l treatment produced the highest fitness levels (as measured by r) for both clones (see below), suggesting that GR9 may trade-off neckteeth production for greater growth and reproduction when salinity conditions are favorable.

Effects of kairomone were variable among life history responses. I 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. I found limited support for this. Presence of kairomone delayed maturation, but effects were equal for both clones regardless of salinity level. This resulted in larger maturation sizes, as predicted, but only for clone P14, supporting the view that this clone is more responsive to *Chaoborus* presence. Increasing salinity increased maturation times and reduced maturation sizes at the highest concentrations, regardless of clone identity. This supports the prediction that this stressor impairs life history responses and potentially increases susceptibility to predators. Larger maturation size has been cited as a defense mechanism of *D. pulex* against *Chaoborus* in previous studies (Lüning, 1992; Spitze, 1992; Tollrian, 1995b); a strategy that requires more time for somatic growth and delayed reproductive maturity. It is also possible that neckteeth development might have also delayed maturation due to a trade-off between energy allocations to somatic growth versus reproduction.

With larger size at maturity, *Daphnia* are able to produce more or larger neonates. Prior studies have shown that larger neonate size contributes to survival from *Chaoborus* predation, and as a trade-off, fewer neonates are produced (Krueger and Dodson, 1981; Havel and Dodson, 1984; Riessen and Sprules, 1990; Spitze, 1991). Thus, I predicted that presence of kairomone would induce production of fewer but larger offspring, with P14 maintaining this response to a greater degree across salinity levels when compared to GR9. In terms of the reproductive output in the first brood, unexpected results were found. *Chaoborus* kairomone increased clutch sizes for both clones but had no effect on body sizes of the neonates. These results could be explained by a bet hedging strategy (Reznick and Bryga, 1987). Predation risk was spread across a larger number of offspring to improve survivorship of individual neonates. Increases in offspring number in the presence of kairomone resulted in significant increases in r. In natural populations, faster population growth rates could serve as an anti-predator strategy by allowing populations to

compensate for elevated mortality (Smith and Fretwell, 1974; Brockelman, 1975). As predicted, increasing salinity stress reduced offspring size and number. However, contrary to my predictions, clone identity and salinity tolerance did not mediate this effect.

An interesting finding was that neonate size and brood size did not strongly covary within salinity treatments as predicted (cor = 0.24, p = 0.98, Pearson product-moment correlation coefficient test). A well-known and fundamental life history trade-off is the negative relationship between offspring size and number (Guisande and Gliwicz, 1992; Charnov and Ernest, 2006; Roff, 1992). A possible explanation is that algal resources were supplied at non-limiting concentrations which weakened expression of the trade-off. Unfortunately, only a few studies have incorporated this factor into their life history experiments (e.g., Tollrian, 1995b), and most other studies have employed relatively high food levels (Black, 1993; Boeing et al., 2005; Lüning, 1992). This might be the reason why a significant number of contradictory results can be found in the literature as trade-offs may not emerge under unrealistically high resource levels. Another interesting finding was that performance of both clones was highest in the 0.25 g/l NaCl treatment. Across all clone identity and kairomone treatment combinations, *Daphnia* reached reproductive maturity the fastest, obtained their largest maturation sizes, and produced the most offspring at this salinity level. This resulted in the largest intrinsic population growth rates (r) among salinity treatments, an important index of overall fitness. It is possible that this salinity level better matched solute concentrations in the interstitial fluids of *D. pulex*, reducing osmotic pressure and the amount of energy required to maintain their osmotic balance. Prior studies have also found different non-zero optimal salinity levels for *Daphnia* clones (Weider and Hebert, 1987; Latta et al., 2012; Bezirci et al., 2012).

How my results translate into realized fitness in natural systems (in the face of both salinity stress and active *Chaoborus* predation) is unknown. *Chaoborus americanus* has been shown to possess high salinity tolerance and is capable of surviving most salt concentrations in impacted Michigan wetlands (Benbow and Merritt, 2004). The experimental salinities used in my behavior and life history experiments were lower than the ones (3 to 10 per mL) found in *Chaoborus* habitats in temperate zones (Topping, 1971; Hammer, 1986). Thus, my results have implications for natural systems. My behavior experiment suggests that as salinity continues to increase to sub-lethal levels in freshwater systems receiving road salt, *D. pulex* will become more vulnerable to *Chaoborus* predation. Significant community structural changes can be predicted as a result of density-mediated indirect effects. *D. pulex* populations are expected to decline in more salt-polluted ponds because of higher predation rates from *Chaoborus*. Declines in *D. pulex* abundance may result in eventual declines in *Chaoborus* populations because of low food levels or shifts in predation to non-*Daphnia* prey. In addition, competitors of *Daphnia* may experience advantages from the decline of *D. pulex*, further altering community dynamics and structure. These effects are potentially mitigated by *D. pulex*'s morphological and life history responses to the presence of predators, though the magnitude of the effect is unknown. Furthermore, my results indicate that intraspecific variation can potentially mediate these responses (see also Chapter 2). If ponds are naturally high in conductivity before road salt intrusion, "pre-adapted" *D. pulex* phenotypes may exist in these systems, reducing impacts and enhancing ecosystem stability. At a landscape scale, dispersal of pre-adapted clones from high conductivity ponds into road salt-impacted systems could rescue those extinction-prone populations and contribute to ecosystem recovery and resilience. Rapid adaptive responses of populations to increasing salinity stress is also possible if rates of environmental change do not greatly exceed rates of evolutionary change.

CHAPTER 2 Field survey of road adjacent ponds

Introduction

Extensive field research has been done to monitor the chloride levels of freshwater systems and uncover the environmental factors that are responsible for their variation (Environment Canada and Health Canada, 2001; Mullaney et al., 2009). Road salt is considered to be a major cause of increasing chloride levels in roadside systems (Williams et al., 2000; Kaushal et al., 2005). Mullaney et al. (2009) found that major road densities explained a large portion of the variation in the chloride concentrations in 83 surface water monitoring stations in Northern United States. Increasing chloride loads and yields in forested, agricultural, and urban basins were estimated, suggesting that land use also plays an important role in road salt pollution. Provided that urbanization is generally associated with higher road densities, it is not surprising that more road salt is brought into water bodies in urban basins.

Evolutionary adaptation of organisms to environmental factors in their natural habitats is well documented in freshwater systems (Bagarinao, 1992; Crozier and Hutchings, 2014; Latta et al., 2012; Miyakawa et al., 2010). For example, *Daphnia* from ponds and lakes with high salinities have been shown to exhibit higher salinity tolerances (Latta et al., 2012; Weider and Hebert, 1987; Weider, 1993). Adaptive responses of organisms to environmental change is important because they have the potential to buffer populations and communities. Evolutionary ecotoxicology has widely considered the adaptation of organisms to stressors and how it may contribute to the stability and resilience of ecosystems in the face of environmental pressures (Bickham and Smolen, 1994; Bourret et al., 2008; Jansen et al., 2011; Meyer and Di Giulio, 2003; Hoffmann and Willi, 2008). In Chapter One, I provided evidence of heritable phenotypic variation in salinity tolerance among *D. pulex* clones that was related to the ionic concentrations (conductivities) in

their source habitats. However, chloride concentrations in their source ponds when they were sampled were unknown. Thus, salinity tolerance cannot be directly associated with exposure of the clones to NaCl. Moreover, the systems they were sampled from were far from salted roads, thus received very limited, if not no road salt input. Therefore, testing salinity tolerance of *D. pulex* clones from habitats with different NaCl concentrations and levels of road salt pollution would provide stronger insight into the capacity for *Daphnia* populations to adapt to this anthropogenic stressor.

At the community level, effects of salinity on aquatic community structure have been mainly studied in controlled microcosms or mesocosms (Searle et al., 2015; Rogell et al., 2009; Thompson and Shurin, 2012). While some salt-tolerant species such as Calanoid copepods and benthic cladocera might benefit from relatively high salinities because of the decline of their competitors (Jensen et al., 2010; Horváth et al., 2014), elevated salinity is generally negatively associated with zooplankton densities (Searle et al., 2015; Sarma et al., 2006), species richness, and diversity (Jensen et al., 2010; Horváth et al., 2014). However, more studies on this topic in natural systems are required to achieve a better understanding of this issue in real-world settings. Of particular concern is determining how salinity stress impacts populations of keystone species such as *Daphnia pulex*. Suppression or loss of such species may have effects that cascade through food webs, altering the structure, functioning and stability of these ecosystems.

In the previous chapter, I demonstrated significant negative effects of salinity on *Daphnia-Chaoborus* interactions and *D. pulex* life history and fitness at short time scales, I also documented significant clonal variation in response to salinity stress. Whether my results can be extrapolated to more natural settings requires further investigation. Here I present results of a field survey of planktonic communities in roadside ponds in southeast Michigan. Ponds varied in their proximity

to roads and salinity levels. Primary objectives of this survey were: 1) determining the relationship between pond salinity levels and proximity to major roads; 2) elucidating the effects of salinity on the relative abundance of *D. pulex* (the dominant *Daphnia* species in these systems), and 3) assessing *D. pulex* intraspecific variation in salinity tolerance among ponds that differed in salinity concentration. I predicted that increasing pond salinity would be related to closer proximity to major roads, decreasing relative abundance of *D. pulex*, and increasing salinity tolerance among *D. pulex* clones.

Methods

A field survey was conducted of 21 ponds located in southeast Michigan (Figures 9 and 10). The ponds were chosen to represent a range of distances from major highways or roads and thus different levels of potential exposure to road salt pollution. Ponds were sampled once in mid-April, 2015 when road salt intrusion to the ponds by spring run-offs possibly peaked and when many aquatic organisms started to grow and reproduce. Temperature, pH, conductivity, and NaCl concentration were measured at mid-depth at a single sampling location near the center of each pond using a Hydrolab MS5 and Horiba Laqua Twin B-721 Salt Meter. The Salt Meter measures sodium ions and calculates these values into NaCl levels. Road salt applied in Michigan is composed of NaCl (Road Salt 2014-2015 Winter Season Pricing Report, Department of Attorney General, State of Michigan, January 2015). Thus, the measured NaCl levels should largely represent road salt input to the ponds. Maximum depths of the ponds were categorized into three levels with 1 representing < 1 m, 2: 1 – 2 m, and 3: > 2 m. 500 mL of pond water from open areas

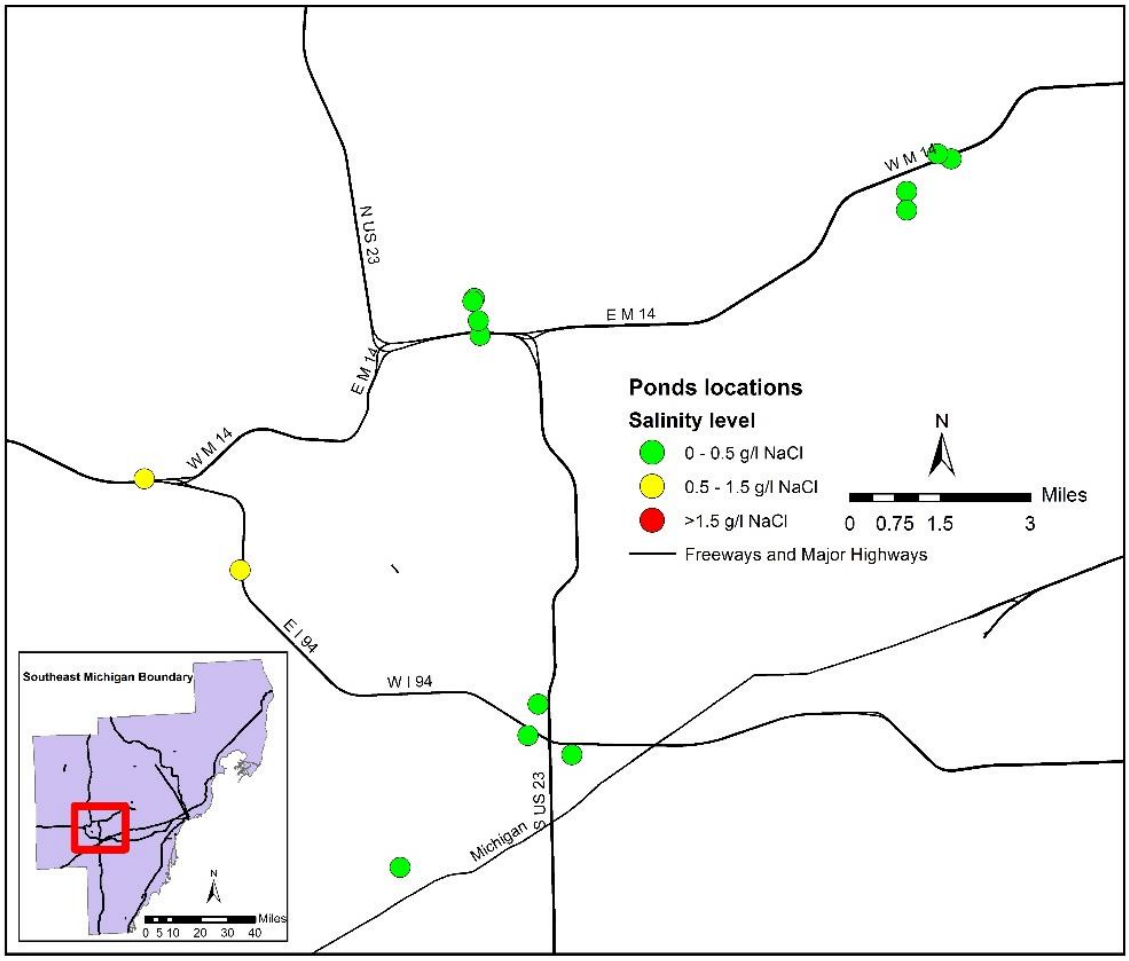

Figure 9: Locations of the sites 1 to 14 in the spring pond survey and salinity category of each pond

close to the middle of each pond was collected using 2-L pitchers and stored on ice, in the dark. Upon arrival to the lab, 20 to 50 ml of water from the sample was filtered onto a Whatman GF/F (0.7 µm pore size) glass microfiber filter to measure total chlorophyll *a* as a correlate of total algal biomass. A second sample was first filtered through a 30 µm mesh before being filtered onto a GF/F filter in order to measure chlorophyll *a* in the edible size range for cladocera. Chlorophyll *a* concentration was quantified following ethanol extraction using narrow-band fluorometry and a Turner Trilogy Fluorometer (sensu Welschmeyer, 1994). A sub-sample of water was frozen at -20 ^{o}C and later analyzed for total phosphorus (TP) content, as a measure of pond productivity, using

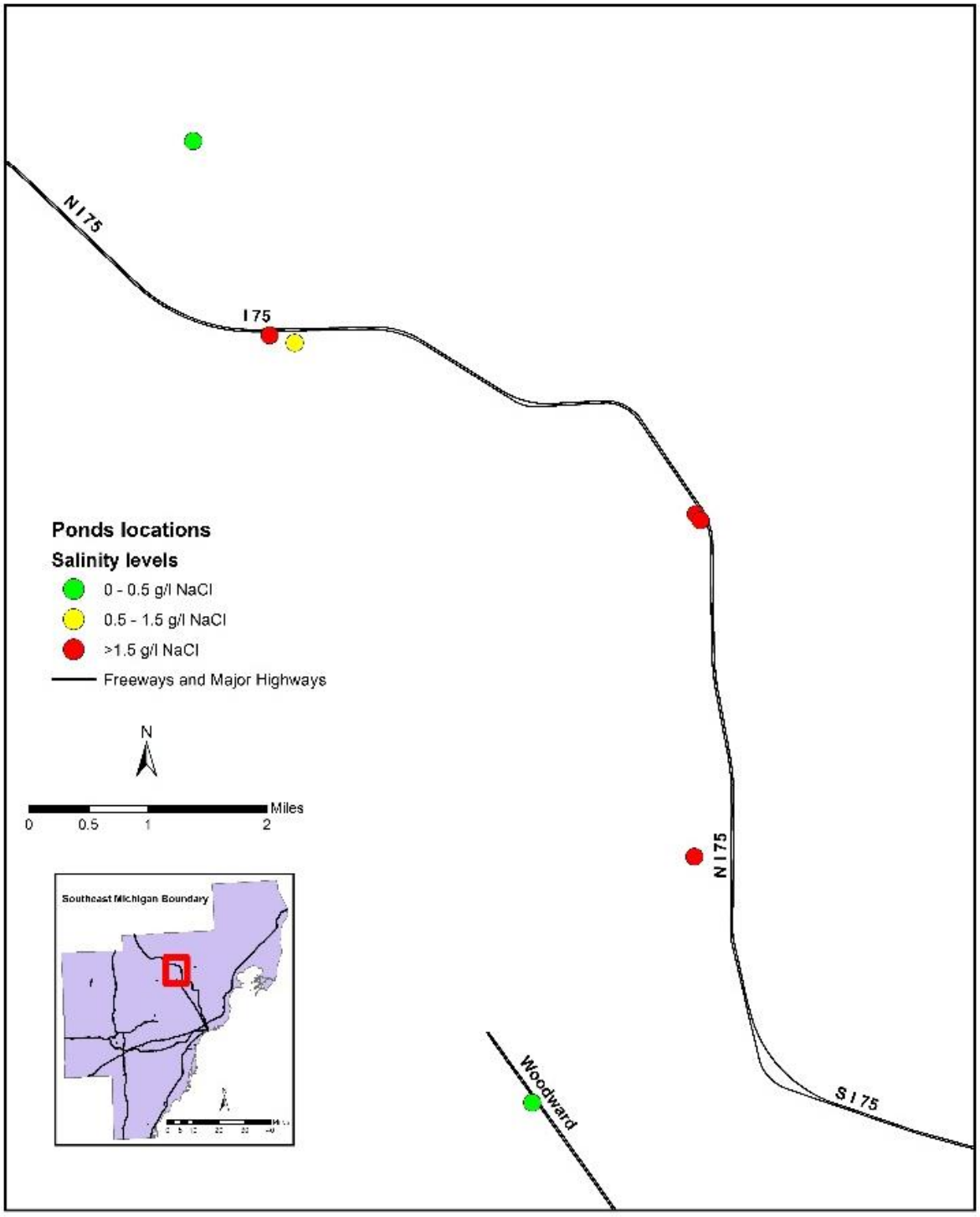

Figure 10: Locations of the sites 15 to 21 in the spring pond survey and salinity category of each pond

the ammonium molybdate method following persulfate digestion (Wetzel and Likens, 2000). Total phosphorus analyses were performed on a Shimadzu UV-1800 UV spectrophotometer with 885

nm light exposure. In addition to water samples, two 40 L zooplankton samples were taken from the littoral and open water zones of each pond using 2-L pitchers. One sample was filtered through an 80-μm sieve and preserved immediately in acid Lugol's solution for later enumeration of zooplankton composition. The second sample was filtered through a 150-μm sieve and the collected zooplankton were stored in a small amount of pond water in a cooler. The second sample was later used to isolate *Daphnia pulex* individuals and establish cultures for the salinity tolerance assays.

Preserved zooplankton were identified to genus or sub-family level for cladocera and species level for *Daphnia* and counted using a dissecting scope. This taxonomic resolution was adequate as I was only interested in quantifying *D. pulex* abundance and its abundance relative to total cladoceran abundance. Live *D. pulex* were obtained from four ponds (P4, P6, P8, and P12).While *D. pulex* was found in the preserved sample from pond P1, no individuals were found in the live sample from this pond. Isogenic cultures were established by haphazardly choosing a single female from each pond sample to initiate each culture line (care was taken to avoid females carrying ephippia). Cultures were maintained at 20 °C with a 12 h light: 12 h dark light cycle.

Once culture lines were established, 48 individuals from each monoclonal culture were preserved in 95% ethanol and genetically fingerprinted using four microsatellite loci: Dp3, Dp27, Dp496, and Dp502 (Colbourne et al. 2004). Individuals from the ethanol-preserved samples were rinsed with ultrapure water to remove debris and then incubated in sterile Tris-EDTA (TE) buffer for 2 hours at room temperature. DNA extraction was then performed in 250 μl of a solution containing 10% Chelex (Sigma-Aldrich) and 0.1 μg/μl proteinase K. Samples were autoclaved for 15 minutes at 120 ^{o}C, centrifuged and the supernatant then stored in a -80^{o}C freezer. PCR amplification of microsatellite loci was performed using multiplexed reactions with forward

primers of each marker labelled with a fluorescent dye: 6-FAM, VIC, NED and PET (Life Technologies, Grand Island, NY). Reactions were performed in 25-µl volumes containing 4µL of extracted DNA, 2.5 µl of GoTaq Flexi Buffer (Promega, Madison, WI USA), 0.33 µl of 25 mM deoxyribonucleotide triphosphates, 0.5 µl of 25 mM $MgCl_2$, 1.0 µl each of 10 mM forward and reverse primers of Dp3, Dp27, Dp496, 0.25 µl each of 10mM forward and reverse primer of Dp502, 1 unit of Taq DNA polymerase and 6.17 µl ultrapure water. PCR conditions were as follows: 95 ^oC for 2 min, 39 cycles of 95 ^oC for 30 s, 56^oC for 20 s, and 72^oC for 30 s, with a final extension at 72 ^oC for 7 min. DNA fragment analysis was performed at the University of Illinois, Roy J. Carver Biotechnology Center (Urbana, IL USA). Fragment lengths were determined using Peak Scanner Software (Life Technologies, Grand Island, NY USA). Clones were identified as multi-locus genotypes based on unique combinations of alleles for the four loci.

To determine salinity tolerance of the four clones, maternal effects were first removed by culturing the animals in high food, low density common garden conditions for two generations (Tollrian, 1995b). Less than 24 hour old neonates from the third generation were isolated from their stock cultures and transferred to beakers containing 100mL of medium; replicate beakers contained seven neonates of a given clone. The experimental medium consisted of aged tap water at one of four salinity concentrations (0, 1, 2, 3 g/l NaCl). Clones P12 and P8 had four replicates while P4 and P6 had five replicates per salinity level, all fed with *Ankistrodesmus falcatus* at a non-limiting concentration (100,000 cells/ml). Food and culture media were refreshed daily. After 48 hours, the numbers of surviving juveniles in each replicate were counted, and survivorship was calculated by dividing the number of surviving individuals by the initial total number.

Statistical analysis

Pond surface areas and distances from the closest location of each pond to the nearest major road were measured using the polygon function and ruler function in Google Earth 7.1.5, respectively. *D. pulex* relative abundance for each pond sample was calculated by dividing the density of *D. pulex* by the total density of cladocera. I chose to calculate this value relative to total cladocera only as these taxa are known to have similar feeding ecologies and thus potentially compete for shared resources. *D. pulex* relative abundance appeared to vary with pond depth category, with category 3 ponds having no *D. pulex* (see Results). To remove this potential depth effect, I first ran an ANOVA on *D. pulex* relative abundances including depth category as a fixed effect. Residuals from the ANOVA were then used as the dependent variable in a partial least squared regression analysis (PLSR) which tested the effects of environmental factors. Predictors in the model included temperature, pH, conductivity, salinity, chlorophyll a (total and < 30 μm), total phosphorus, and pond surface area. Spearman rank correlation was employed to analyze the relationship between salinities and distances to major roads of the surveyed ponds. Survivorship was analyzed testing for the effects of clone identity and salinity (as a categorical variable) using GLM with binomial errors and a logit link. The data were highly overdispersed, thus I present GLM results using quasibinomial errors. Tests were based on type III sums of squares due to unequal replication among treatments. Post hoc pairwise comparisons were performed using Tukey's HSD. All tests were performed in R (version 3.2.3) with the exception of PLSR which was performed in SYSTAT Version 13.

Results

Pond	GPS Coordinates	Surface area (m^2)	Depth Cat.	D (m)	Salinity (g/l NaCl)	pH	Cond. (ms/cm)	Temp. (ºC)	Total Chl a (µg/l)	<30 Chl a (µg/l)	TP (mg/l)	*D. pulex*/Clad. (%)
1	42°14'0.05"N 83°41'29.52"W	7200	2	120	0.4	7.21	0.98	10.63	9.64	8.08	0.089	50
2	42°13'36.94"N 83°41'32.35"W	15865	3	63.2	0	7.01	0.13	10.98	8.98	5.89	0.063	NA
3	42°13'19.55"N 83°40'41.87"W	4236	1	75.77	0.2	6.65	0.67	12.23	10.66	8.91	0.244	0
4	42°11'43.46"N 83°44'2.76"W	1846	1	37	0	6.87	0.08	13.53	31.76	31.29	0.198	89.47
5	42°16'1.97"N 83°47'7.32"W	3065	1	9.4	1.1	7.62	2.53	14.98	2.10	1.59	0.037	NA
6	42°17'21.64"N 83°48'58.59"W	2872	2	14.5	0.6	7.69	1.43	15.23	2.31	0.80	0.02	84
7	42°21'53.08"N 83°33'10.19"W	3789	2	67.59	0.3	6.93	0.86	17.6	6.82	4.84	0.045	0
8	42°21'55.83"N 83°33'28.11"W	1603	1	19.32	0.3	7.4	0.90	11.79	1.17	1.01	0.045	91.67
9	42°21'25.66"N 83°34'4.70"W	2106	3	18.8	0.1	7.9	0.69	12.34	9.37	3.96	0.033	0
10	42°21'9.45"N 83°34'4.91"W	2857	3	10.98	0.1	7.81	0.55	13.97	13.14	12.69	0.087	0
11	42°19'23.88"N 83°42'25.24"W	5763	2	15	0.4	7.71	1.07	14.8	4.74	4.04	0.034	NA
12	42°19'36.88"N 83°42'26.77"W	3145	1	17	0.1	7.94	0.50	16.17	0.99	0.84	0.082	75.25
13	42°19'55.86"N 83°42'31.61"W	3899	2	5	0.1	7.8	0.48	14.48	10.58	9.35	0.076	NA
14	42°19'54.11"N 83°42'32.15"W	1337	1	6	0	7.68	0.44	16.66	20.28	16.21	0.069	NA
15	42°36'38.76"N 83°16'18.64"W	13099	3	10.29	0.3	8.07	0.89	15.8	4.99	4.45	0.026	0
16	42°38'26.41"N 83°14'41.38"W	11778	1	42	3.2	7.82	5.92	15.67	2.30	1.94	0.011	NA
17	42°40'56.00"N 83°14'37.09"W	3093	2	38	2	7.61	3.90	17.78	7.70	6.53	0.03	NA
18	42°40'53.18"N 83°14'34.33"W	1343	1	26	2.3	7.34	4.11	18.74	9.10	8.20	0.138	NA
19	42°42'13.51"N 83°18'34.60"W	3102	1	32	0.8	7.67	2.02	23.66	3.58	2.61	0.057	NA
20	42°42'16.46"N 83°18'48.99"W	770	1	15	2.3	7.44	4.17	22.08	4.14	3.89	0.051	0
21	42°43'41.99"N 83°19'33.07"W	751	3	7.5	0.4	7.22	0.91	18.58	1.65	1.18	0.021	0

Table 1: Physical, chemical, and biological characteristics of 21 ponds in a mid-April 2015 field survey. D: distance to nearest major roads; Depth Cat.: Depth Category; Cond.: conductivity; Temp.: Temperature; Chl. a: Chlorophyll a; TP: total phosphorus; *D. pulex*/Clad.: percentage of *D. pulex* density to cladoceran density, NA: due to no cladocera. Depth Category: 1: < 1 m; 2: < 1-2 m; 3: > 2m.

Physical and chemical properties and *D. pulex* percent relative abundances are listed in Table 1. Salinities varied greatly among the ponds, ranging between 0 and 3.2 g/l NaCl (mean = 0.71 g/l NaCl), as did *D. pulex* relative abundances. The majority of ponds had no *D. pulex* in their samples. Salinities appeared to vary with locality with high salinity ponds (> 1.5 g/l NaCl) only being found along the I75 corridor (Fig. 10). Spearman rank correlation did not reveal a significant relationship between distances to major roads and salinities of the surveyed ponds (Fig. 11; rho = 0.11, p = 0.62). Microsatellite analysis identified three multi-locus genotypes (unique allele combinations) among the four *D. pulex* clones isolated from the survey ponds. Clones from ponds P4 and P8 had identical alleles and could not be differentiated based on the loci tested. However, this does not negate the possibility that these two clones differ at other loci.

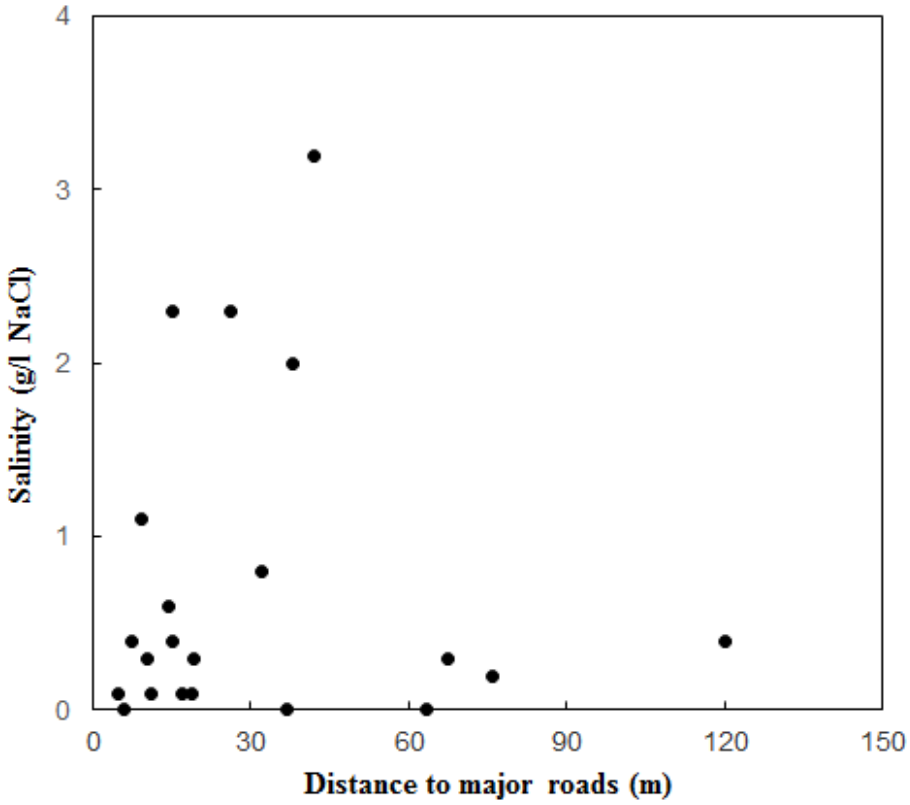

Figure 11: Distances to the closest major roads and salinities of the surveyed ponds

Clone identity and salinity interactively affected survivorship (Fig. 12; $F_{9, 56}$ = 3.03, p = 0.005, GLM with quasibinomial errors). When performing pairwise comparisons within salinity treatments, significant differences in survivorship among clones only emerged at the 3g/l NaCl

salinity, with *Daphnia* from P6 and P8 both displaying higher survivorship than those from P12 and P4 ($p < 0.01$, Tukey's HSD).

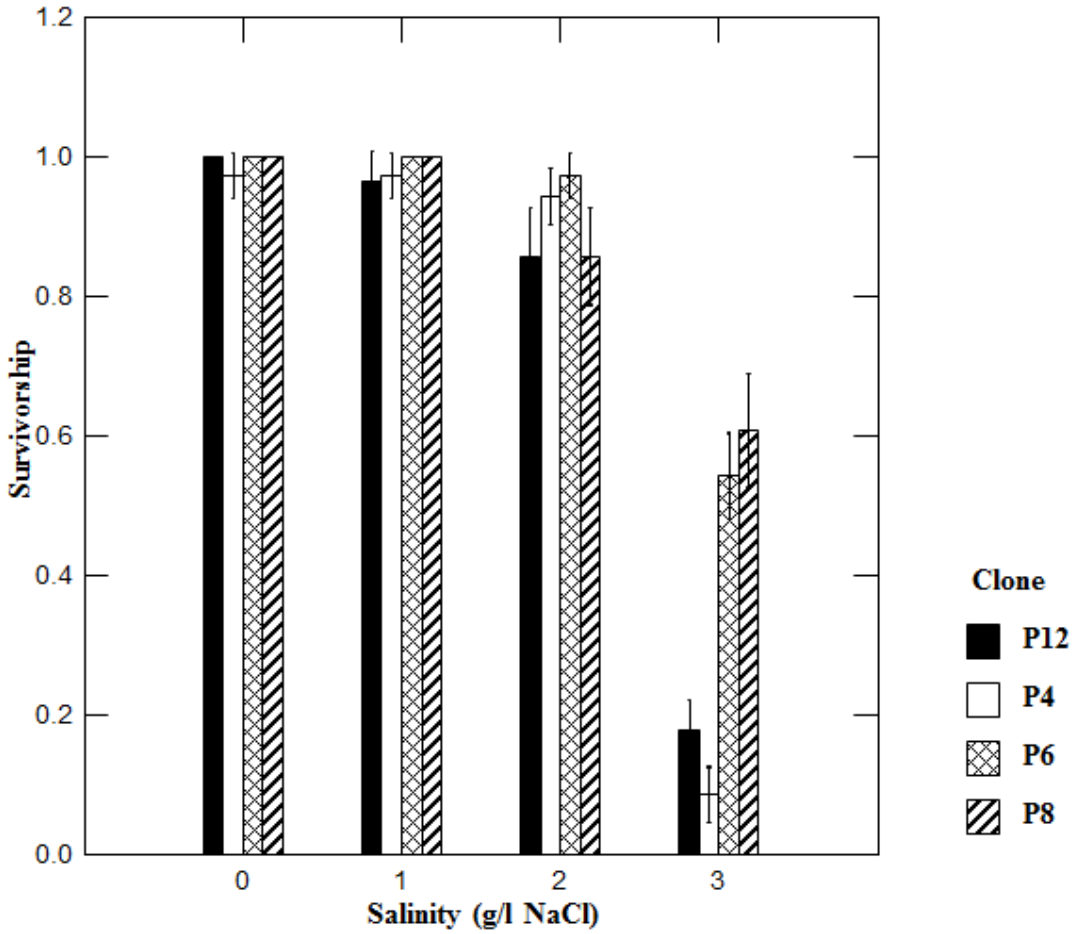

Figure 12: Survivorship of *D. pulex* from four ponds (P12, P4, P6, and P8) in Southeast Michigan after being exposed to different salinities for 48 hours

Daphnia pulex relative abundance appeared to be related to pond depths, with the deepest ponds in category 3 having no *D. pulex* (Fig. 13). However, depth effect on *D. pulex* relative abundance was not significant ($p = 0.15$, ANOVA). Partial least squares regression of residuals from the depth effect ANOVA of *D. pulex* relative abundances produced a first component axis that accounted for 35% of the variation in residuals. The second axis only explained an additional 14% of the variation. Thus, I focused on axis 1. When examining axis loadings of the predictors,

salinity, temperature and conductivity were most strongly and negatively related to axis 1 (Table 2). Thus, residual variation in *D. pulex* relative abundances increased with decreasing salinity, temperature and conductivity (Fig. 14) after accounting for depth category effects.

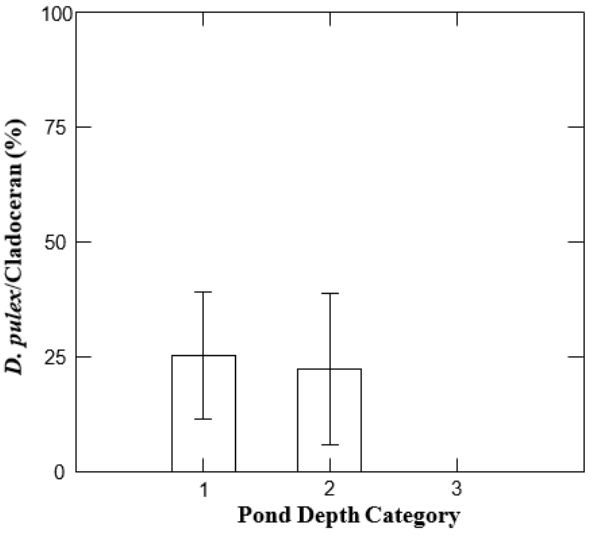

Figure 13: The average *D. pulex* relative abundance to Cladoceran in the ponds from each depth category

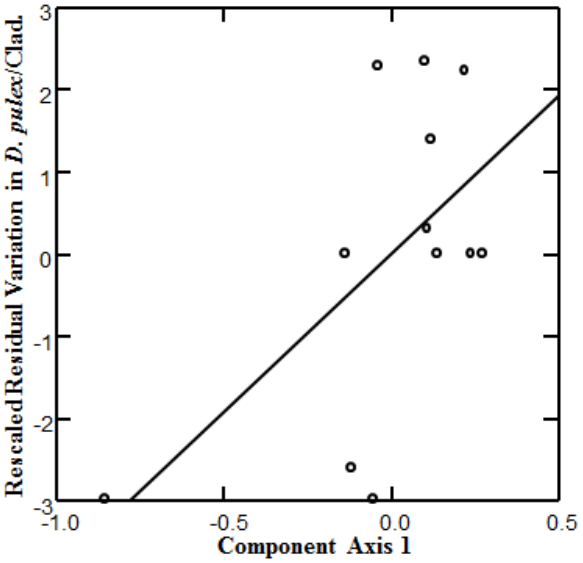

Figure 14: Relationship between the component axis 1 and rescaled residual variation in *D. pulex* relative abundance after removing depth category effects

Predictors	Salinity (g/l NaCl)	pH	Cond. (ms/cm)	Temp. (°C)	Total Chl. a (µg/l)	< 30 µm Chl. a (µg/l)	TP (mg/l)	lg (surface area) (m^2)
Axis loadings	-3.1	0.708	-3.056	-2.561	1.11	0.955	0.495	1.62

Table 2: Axis loadings of each predictor in the component axis 1 explaining residual variation of *D. pulex* relative abundance after removing the effects of depth category in PLSR. Cond.: conductivity; Temp.: Temperature; Chl. a: Chlorophyll a; TP: total phosphorus.

Discussion

In contrast to predictions, salinities of the surveyed ponds did not show a significant correlation with the distances of the ponds from major roads. Four ponds along I-75 (pond #16, #17, #18, and #20) with salinities greater than 2.0 g/l NaCl were possibly storm water retention

ponds (personal observation) that directly collect runoff from roads and thus may be more susceptible to road salt intrusion. However, no significant correlation (rho = -0.02, p = 0.93, Spearman Rank Correlation) was found between pond salinity and distance to roads after removing these four ponds in the analysis. Important trends in the data were still evident; ponds greater than 50m from their nearest major road had relatively lower levels of salinity. In contrast, ponds less than 50m showed a wide range of variation in salinity. This could be due to multiple reasons. Road salt usage might differ on different roads with freeways usually receiving more salt input than other road categories due to their heavier traffic usage. Road salt application may also vary on roads of the same hierarchy in different counties and cities, which might result in higher salinities in the ponds along I-75 in Oakland County, Michigan than those in the ponds along I-94 and M-14 in Washtenaw County, Michigan.

In addition, buffer zones between pollution sources and waterways play important roles in mitigating pollution pressure (Hoffmann and Willi, 2008). Infiltration capability differs in buffer zones with different vegetation composition (Polyakov et al., 2005). For example, Bharati et al. (2002) found that the infiltration rates of multi-species buffers in Midwestern regions were five times greater than buffers with cultivated fields and pasture. Infiltration rates have also been found to be highly dependent on soil properties, land management, and topography (Herron and Hairsine, 1998). The buffer zones of the ponds I surveyed had a high diversity in terms of vegetation composition, land use, and topography (personal observation). These factors may have contributed to differences in salinities among the study sites. Slope of the buffer zone can also be used as a predictor for trapping efficiency of buffers (Jin and Romkens, 2001). Steeper slopes may assist road salt flow into waterbodies due to gravity. Therefore, distances to road salt pollution sources cannot be used as the only predictor of pond salinities. More comprehensive studies including

more factors described above have to be conducted to explain freshwater salinization from road salt pollution.

In keeping with my general predictions, I found significant variation in salinity tolerance among the clones isolated from my study sites. More importantly, relative differences in salinity tolerance among the clones appeared to be associated with salinity levels in their source ponds. Clones from the ponds with higher salinity levels (P6 and P8) displayed stronger salinity tolerance than the ones from the ponds with lower salinities (P12 and P4; Table 1 and Fig. 12). Assuming that the salinity concentrations I measured were representative of past spring levels, the capacities of clones from P6 and P8 to tolerate elevated salinity levels may be due to past selection pressure and evolutionary adaptation to road salt intrusion. This is consistent with prior studies that have that suggested that evolutionary responses by *D. pulex* are common when facing elevated salinity stress and ion cytotoxicity (Latta et al., 2012; Zhu, 2002). The capacity of species, especially keystone taxa, to adapt to anthropogenic stressors has important implications for predicting community responses to environmental change. High adaptive capacity could contribute to the stability and resilience of populations and communities facing persistent environmental change and novel stressors. There are important caveats to consider, however. First, *D. pulex* was found in only a minority of the surveyed ponds. More importantly, they were all found in ponds with relatively low NaCl concentrations (all less than 0.6 g/L). This suggests that *D. pulex* may have a limited ability to adapt to the direct negative effects of increasing salinity stress or increasing indirect effects via interactions with other abiotic stressors (e.g. increasing temperatures; see below) or biotic interactions (e.g. predation and competition).

Daphnia pulex relative abundances appeared to be associated with pond depth, with the deepest category 3 ponds having no *D. pulex*. A likely explanation is the presence of planktivorous

fish. Deeper aquatic systems are generally associated with residency of fish (Kushlan, 1976; Snodgrass et al., 1996) and many planktivorous fish are known to selectively feed on large-bodied zooplankton such *Daphnia pulex* (Galbraith Jr., 1967; Macchiusi and Baker, 1991; Zaret and Kerfoot; 1975). *Daphnia pulex* relative abundance (once depth effects were removed) was negatively associated with increasing temperature, salinity and conductivity. This finding is consistent with prior studies of cladoceran community responses to environmental stressors. For example, Thompson and Shurin (2012) demonstrated a stronger negative association of *Daphnia* with increasing salinity compared to other cladoceran genera, suggesting that osmotic stress tolerance of *D. pulex* is lower than other pond taxa.

Temperature increases in freshwater systems, especially small and shallow ponds, can also significantly alter community composition due to differences in thermal tolerances of different species (Hogg and Williams, 1996). Thompson and Shurin (2012) found that small-bodied cladoceran taxa (including *Scapholeberis, Ceriodaphnia, Bosmina, Diaphanosoma, Polyphemus* and *Chydorus*) benefited from higher temperatures while *Daphnia* abundances were negatively associated with increasing temperature. This supports my finding that *Daphnia pulex* relative abundances were lower in warmer ponds. Therefore, my field survey provides implications for how salinization and temperature increase can jointly alter freshwater community structure.

There are important limitations to this study that are worth considering. First, the survey was only one snapshot of the characteristics of the ponds and communities in them. Even though the timing of sampling was selected at mid-April to capture possible maximum salt input through run-off and initial population growth of *D. pulex* populations, the survey may not be representative of conditions that the ponds and their communities experienced later in the growing season. It is possible that salinity levels fluctuate through time due to different input rates, retention, and release

of salt (Kelly et al., 2008; Kincaid and Findlay, 2009). Aquatic community structure is also known to change seasonally because of various factors such as temperature and species interactions (Hawkins and Sedell, 1981; Pires et al., 1999; Daufresne et al., 2004). Repeated sampling efforts over the growing season could reveal more detailed community fluctuations and their association with salinity stress. Second, many different kinds of pollutants such as gasoline and heavy metals can be jointly emitted into roadside freshwater systems through runoff (Bäckström et al., 2003; Mangani et al., 2005; Drapper et al., 2000). The effects of these pollutants may be confound with perceived road salt impacts and should be considered when interpreting my findings. Third, predation is widely considered to be a primary driver of zooplankton population dynamics and community structure (Hansson et al., 2007; Luecke et al., 1990; Gliwicz and Pijanowska; 1989). Predators of *D. pulex* may include both invertebrate and fish predators in pond systems. Although, I attempted to control for potential fish predation effects by removing the effects of pond depth, this does not negate the possibility that fish may have been present in some of the shallow systems. Furthermore, I did not sample invertebrate predator assemblages in the ponds. These unknown factors may have driven some of the unexplained variation in *D. pulex* relative abundance that was not accounted for by environmental factors in the PLSR analysis, which was substantial. Lastly, I assessed salinity tolerance of the *D. pulex* populations using one *D. pulex* clone from each population. It is possible that populations were composed of multiple genotypes during the sampling period. Thus, one individual may not be representative for all the possible genotypes and corresponding salinity tolerances. A more rigorous approach would be to assay numerous clones from each pond and from different times of the year.

In conclusion, my field survey and laboratory experiments suggested that salinity tolerance of *D. pulex* is positively associated with salt exposure in their source ponds, indicating possible

evolutionary adaptation to elevated salinity stress. However, the limited range of pond salinities that *D. pulex* was found in, further suggest a limited capacity of this species to adapt to high levels of salt intrusion. *D. pulex* relative abundance was negatively associated with increasing salinity, conductivity, and temperature in their habitats, suggesting potential impacts on their population from global climate change and freshwater salinization. Salinity levels in the roadside ponds did not have any significant relationship with road adjacency, based on the parameters I investigated. Understanding freshwater salinization from road salt pollution may require examination of more factors including buffer zone properties.

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ABSTRACT

EVOLUTIONARY ECOTOXICOLOGY OF SALINITY TOLERANCE IN *DAPHNIA PULEX*: INTERACTIVE EFFECTS OF CLONAL VARIATION, SALINITY STRESS, AND PREDATION

by

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Evolutionary ecotoxicology addresses effects of toxic chemicals in an ecological context and considers the potential evolutionary responses of organisms following exposure to toxins. Despite decades of research, the effects of salinity stress in freshwater systems, partly from road salt pollution, on a keystone species, *Daphnia pulex,* in its interaction with predators have received very limited attention. In this study, I quantified *D. pulex* clonal variation in response to salinity stress and the lethal and non-lethal effects of *Chaoborus* (a dominant planktivore in fishless ponds). Behavioral, morphological, and life history responses of two *D. pulex* clones, known to differ in salinity tolerance, were quantified in the presence/absence of *Chaoborus* chemical cues (kairomone) under different salinity levels. I predicted that kairomone would induce both clones to develop neckteeth structure, mature later at larger sizes, and give birth to fewer but larger neonates. Also, I predicted that the clone with stronger salinity tolerance would show stronger responses for these traits as well as higher escape efficiency from *Chaoborus* predation. I found some support for my predictions. Elevated salinity generally weakened some responses of both

clones. However, clonal variation in salinity tolerance did not significantly mediate the effects of salinity on the responses. Additionally, I found that both clones reproduced more but smaller neonates, in contrast to my predictions. In an April field survey of 21 roadside ponds in Southeast Michigan, USA, *D. pulex* relative abundance was found to be negatively associated with increasing salinity, suggesting that community structure and *Daphnia* persistence may be impacted by freshwater salinization. Laboratory assays of clones collected from a subset of the surveyed ponds, provided evidence of local adaptation of *D. pulex* to pond salinity levels. Clones collected from ponds with higher salinities exhibited stronger salinity tolerance. Overall, my study revealed the negative impacts of salinity stress on *D. pulex* populations and its responses to *Chaoborus*. My work also suggests that intraspecific variation and the evolution of salinity tolerance may mediate some of the impacts of salinity stress on *Daphnia* populations. Keywords: *Daphnia pulex*, *Chaoborus*, zooplankton, predator-prey interactions, non-lethal predator effects, escape behavior, life history, clonal variation, phenotypic plasticity, salinity stress, and road salt

AUTOBIOGRAPHICAL STATEMENT

My strong interest in and connection with nature have been established since my childhood in countryside China. Additionally, a bike packing trip to Tibet triggered my interest in outdoors. A perfect way to integrate my passion in nature and outdoor work is ecological study. This drove me to pursue a graduate degree in Aquatic Ecology at Wayne State University. I have enjoyed every moment in my graduate study in this field despite some frustrations from the hard work. Now, with a Master's degree, I am more prepared to pursue my passion. Protecting our beautiful nature using my knowledge and skills is the next challenge that I will focus on.