

THE EFFECT OF SALINITY AND TEMPERATURE ON SURVIVAL
OF THE ORANGE-BLACK HAWAIIAN DAMSELFLY, *Megalagrion xanthomelas*

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By
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ABSTRACT

Decreased ground-water flux associated with urbanization has the potential to increase the salinity of anchialine ponds at Hawai‘i’s Kaloko-Honokōhau National Historical Park. These unique coastal aquatic ecosystems possess an endemic invertebrate fauna including candidate endangered crustaceans and aquatic insects. To assess the effects of rising salinities on anchialine pond biota, our objective was to determine the effect of salinity and temperature on egg hatch and naiad (larval) survival of the endemic orange-black Hawaiian damselfly (*Megalagrion xanthomelas*). The study used a 5 x 3 factorial experimental design consisting of constant salinities of 0, 5, 10, 15, and 20 ppt and constant temperatures of 20, 24, and 28°C. Results revealed that eggs and naiads were sensitive to increased salinity and temperature, although different salinity-temperature combinations yield differing effects on hatching success and naiad survival. Generally, egg hatch increased with increased temperature and decreased salinity, whereas, naiad survival increased with decreased temperature and increased at intermediate salinity. Naiads also displayed a threshold response to salinity above 15 ppt with no naiads surviving at 20 ppt despite successful hatch observed at this salinity. Given the limited information on the effects of increased salinity on anchialine pond biota, the results of this study has significant management implications and raises awareness on the importance of water quality and quantity for organisms in these rare environments.

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INTRODUCTION

Anchialine ponds are unique coastal features found in low-lying areas throughout the tropics and subtropics (Maciolek 1983). Such brackish-water ecosystems are limited to porous substrates that allow the mixture of seawater and fresh groundwater through permeable aquifers (Oki et al. 1999). Anchialine ponds are often defined as exposed portions of the groundwater table that lack a direct surface connection with the sea, yet experience dampened tidal fluctuations in water level and contain measurable salinity (Holthuis 1973). Salinity may be seasonally and spatially variable and can be influenced by changes in sea level, freshwater recharge (groundwater and precipitation) and evaporation (Chai et al. 1989). Anchialine ponds are considered to be particularly vulnerable to increased groundwater withdrawals (Ridgley & Chai 1990, Stephens 2006), since the extraction of groundwater associated with urbanization can exacerbate saltwater intrusion into aquifers, thereby raising pond salinity. In Hawai‘i, for example, there has been a documented decline in the water quality and biological integrity of anchialine ponds adjacent to developments along the western coast of Hawai‘i Island [hereafter ‘West Hawai‘i’] (Brock et al. 1987).

Of the 600 estimated anchialine ponds located throughout Hawai‘i, more than 70% are found within a few hundred meters along a 55 km continuous section of the West Hawai‘i coastline (Brock 1977). In this area, seaward flowing fresh groundwater mixes with underlying saltwater forming a brackish water transition zone (Oki et al. 1999). Approximately 170 anchialine ponds at Kaloko-Honokōhau National Historical Park (NHP) are situated directly above this brackish-water table. The primary freshwater

component of the brackish water flowing through the Park originates from inland subsurface flow and ultimately discharges into the ponds or directly into the ocean (Oki et al. 1999). Results of a non-calibrated hydrological model of the Park's groundwater resources indicated that groundwater withdrawals would reduce the rate of freshwater discharge within the Park, thereby increasing salinity in anchialine ponds by an unquantified amount (Oki et al. 1999). Currently there is no evidence suggesting that increased anchialine pond salinity negatively impacts anchialine habitats, however this should be examined as development increases along the West Hawai'i coast.

Hawaiian anchialine pond ecosystems are dominated by a characteristic assemblage of organisms including crustaceans, fishes, mollusks, a hydroid, sponges, polychaetes, tunicates, aquatic insects, algae and aquatic macrophytes (Brock & Kam 1997). Because of the geographic isolation of the Hawaiian Islands, a greater percentage of anchialine species are endemic with pond habitats displaying high biological diversity (Maciolek 1986). Anchialine pond habitats sustain biological communities representing both hypogeal (utilizing subterranean interstitial waters) and epigeal (restricted to surface waters) species. Salinity is believed to be among the most important factors affecting the distribution of anchialine species (TNCH 1987). For example, hypogeal organisms are frequently found in waters exceeding 12 ppt, whereas epigeal organisms are often found in lower salinity waters (Maciolek 1983, Maciolek 1986). Anchialine pond surveys in National Parks throughout Hawai'i suggest that the least documented epigeal organism is the endemic orange-black Hawaiian damselfly (*Megalagrion xanthomelas*) (Odonata: Coenagrionidae) (Foote et al. in review). Once widespread, *M. xanthomelas* has experienced population reductions in the past century and is currently listed as a

candidate for inclusion on the List of Endangered and Threatened Wildlife in the Federal Register (USFWS 2009).

M. xanthomelas completes its life cycle in coastal aquatic waters with salinities less than 8 ppt (Polhemus 1996, Foote et al. in review). The genus *Megalagrion* is one of the few diversified groups of Hawaiian aquatic insects that are derived from freshwater ancestors (Polhemus 1997). The majority of freshwater odonates occupying brackish waters do not exist where salinities exceed about 10‰ seawater (Corbet 1999).

Anchialine ponds, for example, provide breeding habitat for *M. xanthomelas* only if sufficient freshwater inflow is present (Polhemus & Asquith 1996). Consequently, *M. xanthomelas* may be among the most sensitive anchialine organisms to increasing pond salinity.

Like most odonates (dragonflies and damselflies), *M. xanthomelas* undergoes incomplete metamorphosis occurring in four stages: egg, pre-larva, larva (naiad) and adult (imago). Egg and naiad stages are exclusively aquatic. Female *M. xanthomelas* often insert their eggs endophytically (within plant tissue) into submerged or partially submerged aquatic plants (Evenhuis et al. 1995). Eggs are surrounded by a chorion (outer shell) containing structures that allow oxygen intake while simultaneously preventing the osmotic penetration of water (Wichard et al. 2002). Eggs hatch after 21 days (Evenhuis et al. 1995) and the pro-larvae (first naiad stage) emerges. Developing *M. xanthomelas* naiads go through 11 to 17 larval stages (instars) lasting from 103 to 111 days (Evenhuis et al. 1995). In brackish waters (anchialine ponds), naiads are likely osmoregulators maintaining (or regulating) their internal fluids within a narrow range even when the osmolality (salinity) of the surrounding water fluctuates (Bayly 1972).

However, with the increased osmolality of the surrounding water beyond the limits of osmoregulation (increasing anchialine pond salinity, for example), naiads may experience lethal and sub-lethal stress (changes in growth, development, feeding and osmoregulation without mortality) (Hassel et al. 2006). *M. xanthomelas* adults can escape local habitat disturbances and recolonize sites upon the return of suitable conditions (Pangelinan 1997). Consequently, conservation strategies for *M. xanthomelas* should target the egg and naiad phase, which combined spans approximately 64% of their lifespan (Pangelinan 1997).

The purpose of our study was to determine the effects of rising anchialine pond salinity and temperature on *M. xanthomelas* egg hatch, naiad survival and naiad growth. In anchialine ponds, salinity and temperature are often coupled (inputs from colder freshwater and warmer saltwater) and both are two essential limiting factors governing populations of anchialine organisms (Maciolek 1983), so a comprehensive study on the effect of salinity should be done in the context of temperature. Experimental survival studies recording egg hatch success, naiad survival, and naiad growth were conducted to evaluate the effect of elevated salinity on the biology and life history of *M. xanthomelas* in anchialine ponds. We test the hypothesis that the increased salinity results in the reduced egg hatch, naiad survival and naiad growth of *M. xanthomelas*.

METHODS

Sampling Area and Collection Procedures

Copulating tandem adult *Megalagrion xanthomelas* were collected from anchialine ponds at Lokoaka Wilderness Park, Keaukaha, eastern coast of Hawai'i Island (19°43'49"N, 155°2'47"W). Previous *M. xanthomelas* collections from this region suggest a longstanding population in the area (Polhemus 1996) and surveys conducted in 2004 and 2005 indicated that this area supports a relatively large and accessible population (D. Foote unpubl. data). The collection site is located approximately 5 km east of Hilo Bay between Carlsmith Beach Park to the west and Leleiwi Beach Park to the east (Figure 1). Mean annual rainfall in the Keaukaha region is approximately 300 cm (Giambelluca et al. 1986) and climate is classified as tropical continuously wet (Köppen classification; Juvik et al. 1978). The high regional rainfall contributes to rapid groundwater recharge, circulation, and discharge within the anchialine ponds (Stearns & MacDonald 1946, Chai 1993).

M. xanthomelas specimens used in experiments were collected with sweep nets at low tide along the margins of two adjacent anchialine ponds ≤ 10 m from the coastline. Collections occurred at three sampling intervals: (1) 8 August 2008 to 18 August 2008; (2) 21 January 2009 to 24 January 2009; and (3) 27 October 2009 to 30 October 2009. At each sampling interval, at least three tandem adults were collected. Measurements of water temperature ($^{\circ}\text{C}$), specific conductivity (mS cm^{-1}), conductivity (mS cm^{-1}), and salinity (ppt) were taken at each collection site at a depth of 10 cm with a YSI model 30 meter (YSI Inc., Yellow Springs, Ohio, USA). Mean water temperature in ponds at low

tide ranged from 19.83 to 22.63°C and mean salinity ranged from 5.97 to 7.43 ppt (Table 1). Following collection, mated pairs were held in one liter, mesh-sealed, plastic holding containers containing one bamboo skewer (to serve as a perch) and one 9.0 cm filter paper disc (Whatman qualitative no. 5 cat. no. 1005-090) moistened with pond water (to serve as an oviposition site). Adult mortality prior to oviposition was high for all sampling intervals with an average of 1 in 4 females successfully ovipositing in holding containers. Each successful female oviposited approximately 350 eggs.

Experimental Design

A total of ten isofemale lines (progeny of a single wild-caught female) were examined in this study: four during the first sampling interval and three during each of the second and third sampling intervals. Eggs and naiads were divided among fifteen treatment combinations from a 5 x 3 factorial design using constant salinities of 0, 5, 10, 15 and 20 ppt and constant temperatures of 20, 24 and 28°C. Salinities and temperatures selected were intended to resemble current and slightly elevated surface conditions for anchialine ponds at Kaloko-Honokōhau NHP (mean $\pm$ SE: salinity = 9.73 ± 0.15 ppt; temperature = $22.84 \pm 0.18^\circ\text{C}$) (Foote et al. in review). Freshwater used in the study was obtained from the University of Hawai'i at Hilo College of Agriculture Farm in Panaewa (source = 76 m+ deep well). Saltwater was obtained from the University of Hawai'i at Hilo Pacific Aquaculture and Coastal Resources Center in Keaukaha (source = 106 m+ deep well). Both fresh and saltwater were aerated through air stones for a minimum of 24 hours prior to use. Intermediate salinities were obtained via dilution mixtures of fresh and saltwater and monitored via YSI model 30 meter. Temperatures for all experiments

were maintained through the use of Percival-Scientific Reach-In biological incubators (Model E-30B) set to a 12-hour light-dark cycle.

For the hatching success experiment, ten eggs from each female were assigned to one of the fifteen salinity-temperature combinations ($n = 1500$ total eggs). The filter paper to which the eggs were oviposited was cut so that ten eggs could be allocated to each treatment combination. Eggs were kept embedded within the filter paper to resemble endophytic oviposition conditions. Filter paper containing eggs were placed in 30 ml plastic holding cups (Solo P100 1 oz. translucent plastic soufflé cups) containing approximately 25 mL of the treatment salinity water. Water was replaced every five to seven days and topped up in a similar fashion between replacements. Eggs were monitored daily over 28 days for hatch. The 28-day study period, selected to allow for hatch at the range of salinities and temperatures tested, was based on our pilot studies and the previously observed 21 day duration from egg laying to hatching for *M. xanthomelas* (Evenhuis et al. 1995).

The remaining eggs not used in the egg hatch experiment were reared to naiads at 0 ppt and 28°C for use in the survival experiment. During preliminary studies approximately 99% of eggs ($n = 714$) held at this treatment combination hatched in 14 days. At least ten naiads from each female were divided among the same fifteen salinity-temperature combinations as the eggs ($n = 2,036$). Naiads were acclimatized to treatment salinities by means of gradual exposure to increased salinity at a rate of 5 ppt per hour. Following acclimatization, naiads were kept individually in 30 ml plastic cups containing approximately 25 ml of the treatment salinity water and maintained as described for eggs. Naiads were fed laboratory-reared brine shrimp (*Artemia nauplii*, from Great Salt Lake

Artemia Cysts, Artemia-International) hatchlings every two days. Uneaten prey, fecal pellets, and molts were removed from cups prior to feeding. Naiads were monitored daily over 84 days for survival. The 84-day study period intended to encompass the early-naiad stage, which may be more sensitive to salinity than older stages (Kefford et al. 2004a), for the range of salinities and temperatures tested and was selected based on our pilot studies and the previously observed 103 to 111 day duration from hatch to eclosion for *M. xanthomelas* (Evenhuis et al. 1995).

Naiads used in this study fed solely on *Artemia nauplii* hatchlings, yet our observations suggested that survival of *A. nauplii* differed between salinity treatments with *A. nauplii* hatchling survival increasing with increased salinity. *M. xanthomelas* naiads are passive predators that typically feed on live prey that move within reach of their protrusible labium (Evenhuis et al. 1995). Because of the difficulty in identification of a readily available prey item that would survive indefinitely in the range of salinities tested, we conducted a feeding experiment involving *A. nauplii* hatchlings, early instar *Culex quinquefasciatus* larvae and a combination of both. The results demonstrated that consumption was not significantly different among prey types. This suggested that the lack of a continuous supply of live prey for an extended period of time at different salinities did not have direct adverse effects on naiads. We also compared feeding at 0 and 15 ppt (representing salinities at the thresholds for survival and growth) and found no difference in the amount of prey consumed between the two salinities.

The head-widths of a subset of naiads from the survival experiment were measured at hatch. All head-width measurements were made with an eyepiece micrometer using a Leica MS5 dissection microscope. Naiad head-width measured as

the widest width of the head (including the eyes) is a standard assay used to assess growth (Kefford et al. 2006). Head-width measurements at hatch showed little variation (mean $\pm$ SE = 0.34 ± 0.0005 mm). Instead head-widths of naiads that survived to 84 days ($n = 1070$) were used as an index of growth for the purposes of this experiment. Larger head-widths often correspond to greater body mass, so we assumed that higher growth indicates greater fitness (see Kefford et al. 2006).

Statistical Analyses

The responses from each subject (egg or naiad) were used to construct a single number that summarized the hatching success or naiad survival at different salinity-temperature combinations. This particular approach is useful for repeated-measures data (Reiczigel 1999) and can elucidate scientific questions of interest and simplify analyses (K. Gerow, pers. comm.). Summarized data were graphed prior to analyses, since graphical exploration is a good first step of any analysis, with statistics often playing a secondary, confirmatory role (Murtaugh 2007).

Summarized egg hatch and naiad survival data were analyzed separately by logistic regression. Logistic regressions are well suited for describing and testing hypotheses about relationships between a binary response variable and one or more categorical or continuous predictor variables (Hosmer & Lemeshow 1989). Since the objective of this study was to determine the effect of salinity and temperature on *M. xanthomelas* egg hatch and naiad survival, the outcome or response variables were simply egg hatch: yes/no and naiad survival: yes/no and the continuous explanatory predictor variables were temperature and salinity. A reliable goodness-of-fit test was not

possible for logistic regressions, as continuous covariates (temperature and salinity) were present (Hosmer et al. 1997).

Residual plots were used to detect outliers and nonlinearity between response variables and predictors (Quinn and Keough 2002). Naiad survival plots revealed two unusual observations from different isofemale lines within the same sampling interval at the 20°C–20 ppt treatment combination. The unusual observations indicated that two naiads survived the 84-day study, however, excluding these observations (which were likely attributed to observer error), no naiads survived at 20 ppt. Accordingly, the two observations were too small to expect any decent model fitting and the differences in the output including versus excluding the observations were minimal, so they were removed from analyses, as they were probably not representative of the population.

For all models, isofemale line was regarded as a fixed blocking factor. When the experimental units in blocks are more similar to each other than to other experimental units in different blocks, the blocking factor may explain some of the residual variation resulting in a smaller error term and a more powerful test for treatments (Quinn & Keough 2002). For example, each isofemale line represented a distinct genetic sample from their natural population. We assumed eggs and naiads from the same isofemale line at the same treatment are more likely to have similar responses to treatments than eggs and naiads from another isofemale line at the same treatment.

The resulting logistic regression equation for egg hatch and naiad survival was:

$$\text{Logit} = \beta_0 + \beta_T X_T + \beta_S X_S + \beta_{TS} X_{TS}$$

where Logit is the natural logarithm (ln) of the odds of response (egg hatch or naiad survival) given a particular level of a predictor, β_0 is a constant, β_T is the slope coefficient

of the predictor variable X_T (temperature), β_S is the slope coefficient of the predictor variable X_S (salinity), and $\beta_{T \times S}$ is the slope coefficient of the interaction term $X_{T \times S}$ (temperature x salinity). The slope coefficients (β) of the variables included in the model are best explained when expressed in terms of the odds ratio (e^β). For scaled continuous predictor variables (temperature and salinity), e^β is a measure of how much more likely (or unlikely if the odds ratio is <1) it is for the outcome to occur when the variable is increased by 1 unit (Bartol et al. 2002). Furthermore, the estimate of the predicted probability (p) of egg hatch or naiad survival is given by:

$$p = \exp(\beta_0 + \beta_T X_T + \beta_S X_S + \beta_{T \times S} X_{T \times S}) / (1 + \exp(\beta_0 + \beta_T X_T + \beta_S X_S + \beta_{T \times S} X_{T \times S}))$$

where p is used to predict the probability of egg hatch or naiad survival that would be observed with the explanatory variables set at specific values.

Time to egg hatch, naiad survival over time, and naiad head-width measured at 84 days were analyzed separately by Linear Regression Model. These are useful analyses for a single continuous response variable and any combination of categorical and continuous explanatory variables (Dobson 2002) and have been used in related studies to determine the effect of different temperatures and salinities on growth and survival (Kumlu et al. 2000, Jackson and Burford 2003).

Linear regression models were equivalent to logistic regression models, with egg hatch time, naiad survival time or naiad growth as the response variables, temperature and salinity treated as continuous covariates, and the interaction of temperature and salinity, blocked by isofemale line. The resulting regression equations for egg hatch time and naiad survival was:

$$Y = \beta_0 + \beta_T X_T + \beta_S X_S + \beta_{T \times S} X_{T \times S}$$

where Y = egg hatch time or naiad survival time, β_0 is a constant, β_S is the slope coefficient of the independent variable X_S (salinity), β_T is the slope coefficient of the independent variable X_T (temperature) and $\beta_{T \times S}$ is the slope coefficient of the interaction term $X_{T \times S}$ (temperature x salinity). Curvature for the response variable naiad growth was detected by residual plots and was reflected in the model. The resulting regression equation was:

$$Y_{\text{Naiad growth}} = \beta_0 + \beta_T X_T + \beta_S X_S + \beta_{S^2} X_{S^2} + \beta_{T \times S} X_{T \times S} + \beta_{T \times S^2} X_{T \times S^2}$$

where $Y_{\text{Naiad growth}}$ = naiad growth, β_{S^2} is the slope coefficient of the squared term X_{S^2} (salinity²) and $\beta_{T \times S^2}$ is the slope coefficient of the interaction term $X_{T \times S^2}$ (temperature x salinity²).

A significance level of $\alpha \leq 0.05$ was used for all statistical analyses. Data are presented as mean $\pm$ SE mean. All statistical analyses were completed in Minitab® 15 statistical software (© 2007 Minitab, Inc.). Figures were prepared in SigmaPlot for Windows Version 10.0 (© 2006 Systat Software, Inc.).

RESULTS

Hatching Success

Successful *Megalagrion xanthomelas* egg hatch exceeded 50% for 13 of the 15 salinity-temperature combinations examined in this study (Figure 2). Hatching success was highest and greater than 80% for the treatment combinations at 0, 5, 10 and 15 ppt and 24 and 28°C, but was reduced below 80% at the 28°C–20 ppt and 24°C–20 ppt combinations (69% and 56%, respectively). Hatching success at 20°C resembled the higher salinity-temperature combinations and was greater than 60% at the three lower salinities (0, 5 and 10 ppt) but was reduced to 14% at the 20°C–15 ppt combination and was not observed at the 20°C–20 ppt combination. These observations suggested that hatching success increased with temperature and decreased with salinity.

Logistic regression results indicated that salinity had a strong influence on egg hatch probability ($p < 0.001$) but the effect was confounded by significant temperature $\times$ salinity interaction ($p < 0.001$) (Table 2). The logistic regression model was highly significant: log-likelihood = -671.118; goodness-of-fit statistic, $G = 481.805$, $df = 12$, $p < 0.001$, goodness-of-fit (Hosmer-Lemeshow method) = 59.267; $df = 8$; $p < 0.001$. Salinity had a greater impact on hatch probability at 20°C where the predicted probability of hatch decreased by 20% for every 1 ppt increase in salinity ($\beta = -0.23 \pm 0.02$, odds ratio = 0.80). Temperature had a larger influence on egg hatch probability at 20 ppt where the predicted probability of hatch increased by 61% for every 1°C increase in temperature ($\beta = 0.48 \pm 0.04$, odds ratio = 1.61). Combined, these results suggested that high temperature and low salinity had less of impact on egg hatch considering hatch

probability was high regardless of salinity at 28°C ($\beta = -0.04 \pm 0.02$, odds ratio = 0.96) and temperature at 0 ppt ($\beta = 0.00 \pm 0.04$, odds ratio = 1.00). Accordingly, mean percent egg hatch was highest at 28°C ($82.6 \pm 3.15\%$, $n = 413$) and 0 ppt ($85.3 \pm 3.13\%$, $n = 256$), supporting the observation that egg hatch generally increased with increased temperature and decreased salinity.

The time to successful egg hatch was influenced by both temperature and salinity. At 28°C, hatch was observed 10 days following oviposition for the three lower salinities (0, 5 and 10 ppt) but was not observed until day 22 for the same salinities at 20°C (Figure 3). Hatching success was also high for these salinities, exceeding 75% by day 16 at 28°C but not reaching 75% success until day 28 at 20°C. This suggested that the reduced hatching success across all salinities at 20°C could be attributed to increased time to hatch. Furthermore, at the 28°C–20 ppt combination, hatch was not observed until day 14. By this time more than half of the eggs at the three lower salinities had successfully hatched. The apparent lag in time to hatch at 28°C–20 ppt could explain for the lack of hatch at the 20°C–20 ppt combination within the 28-day study. Together, these observations indicated that higher salinity and lower temperatures contributed to delayed egg hatch.

Linear regression analysis results revealed that temperature ($F = 2331.77$, $p < 0.001$) and salinity ($F = 30.96$, $p < 0.001$) had strong effects on days to hatch, although the effects were confounded by significant temperature $\times$ salinity interaction ($F = 74.55$, $p < 0.001$). The effect of salinity on time to successful egg hatch was greatest at 28°C ($\beta = 0.36 \pm 0.03$). At this temperature, eggs hatched in fewer days compared to eggs at this salinity and lower temperatures. Combined with hatching success, these results indicated

that hatch was high and fastest at 28°C (15.4 ± 0.15 days, $n = 413$) and 0 ppt (18.4 ± 0.33 days, $n = 256$), supporting observations that time to hatch generally decreased with decreased salinity and increased temperature.

Naiad Survival

Megalagrion xanthomelas naiad survival was greater than 50% for 10 of the 15 salinity-temperature combinations examined in this study (Figure 4). Excluding two unusual observations at the 20°C–20 ppt combination, which were removed from analyses, no naiads survived at 20 ppt. Naiad survival was highest, exceeding 80%, at the 20°C–5 ppt and 20°C–10 ppt combinations compared to survival at the 20°C–0 ppt ($74.86\% \pm 7.14$, $n = 110$) and 20°C–15 ppt ($58.73\% \pm 11.73$, $n = 79$) combinations. Survival at 24 and 28°C was considerably lower and did not exceed 80% at any of the salinity-temperature combinations, yet survival trends were similar to 20°C, suggesting survival optima around 5 and 10 ppt across all temperatures. These observations suggested that naiad survival increased with decreased temperature and increased at intermediate salinity levels.

Logistic regression results for naiad survival indicated that temperature ($p < 0.001$) and salinity ($p < 0.001$) had strong impacts on survival probability, with significant temperature $\times$ salinity interaction ($p < 0.001$) (Table 3). The logistic regression model was highly significant: log-likelihood = -1066.990; goodness-of-fit statistic, $G = 683.404$, $df = 12$, $p < 0.001$, goodness-of-fit (Hosmer-Lemeshow method) = 136.255; $df = 8$; $p < 0.001$. Salinity had a greater impact on survival probability at 20°C where the predicted probability of survival decreased by 18% for every 1 ppt increase in salinity ($\beta = -0.19 \pm 0.01$, odds ratio = 0.82). Temperature had a larger influence on

survival probability at 0 ppt where the predicted probability of survival decreased by 28% for every 1°C increase in temperature ($\beta = -0.33 \pm 0.03$, odds ratio = 0.77). Together, these results suggested that salinity had less impact on survival probability at 28°C given the low survival probability among salinity levels ($\beta = -0.11 \pm 0.01$, odds ratio = 0.90), whereas, temperature had less impact at 20 ppt given the high survival probability among temperature levels ($\beta = -0.06 \pm 0.03$, odds ratio = 0.94). However, the predicted probabilities at 20 ppt were not biologically relevant due to an apparent threshold response to salinity above 15 ppt (Figure 4). This rendered the predicted probabilities irrelevant near the threshold boundaries since the model was predicative only within the biologically functional range where naiads survived. A reduced logistic regression model excluding 20 ppt and including a polynomial covariate for salinity reflecting the curvilinear response to naiad survival indicated that temperature ($\beta = -0.23 \pm 0.03$, $p < 0.001$) and the polynomial covariate for salinity, salinity^2 ($\beta = -0.04 \pm 0.02$, $p = 0.036$), significantly influenced naiad survival probability (Table 4). The logistic regression model was highly significant: log-likelihood = -877.679; goodness-of-fit statistic, $G = 445.138.404$, $df = 14$, $p < 0.001$, goodness-of-fit (Hosmer-Lemeshow method) = 59.061; $df = 8$; $p < 0.001$. These results suggested that the predicted probability of survival decreased by 21% for every 1°C increase in temperature (odds ratio = 0.79), and that the relationship between survival probability and salinity was not linear, but rather reflected the survival optima at intermediate salinity, therefore the predicted probability of survival would differ depending on salinity. The results of the reduced model also supported the observation that naiad survival increased with decreased temperature and increased at intermediate salinity.

Naiad survival rates varied greatly among the salinity and temperature combinations. At 20 ppt, survival declined faster compared to lower salinities and was not observed beyond day 44 for all three temperatures (Figure 5). At the four lower salinities, naiad survival decreased slightly by no more than 30%, but appeared to stabilize by day 26 with naiad survival remaining above 60% at the lower temperatures (20 and 24°C) for the duration of the study. At 28°C, a similar naiad survival plateau beyond day 26 was observed at 5 and 10 ppt, whereas, naiad survival continued to decline at 0 and 15 ppt. Combined, these observations suggested that naiad survival over time was inversely related to temperature and salinity.

Linear regression results revealed that temperature ($F = 23.67$, $p < 0.001$) and salinity ($F = 61.96$, $p < 0.001$) had strong temporal effects on naiad survival, although the effects were confounded by significant temperature $\times$ salinity interaction ($F = 33.34$, $p < 0.001$). The effect of salinity was greatest at 28°C ($\beta = -0.88 \pm 0.37$), with less than 60% of naiads surviving beyond day 26 (Figure 5). The effect of temperature was greatest at 20 ppt ($\beta = 0.86 \pm 0.61$), with less than 40% of naiad surviving beyond day 14. Combined with mean percent naiad survival, these results indicated that naiads lived longer at low temperature and intermediate salinity, supporting the observation that naiad survival was reduced with increased temperature and increased salinity.

Naiad Growth

Naiad head-width was approximately 4.5 times larger at day 84 compared to size at hatch across all salinity-temperature combinations. Average head-widths values were largest, above 2 mm, at the 24°C–5 ppt, 28°C–5 ppt, 24°C–10 ppt and 28°C–10 ppt combinations and were considerably larger compared to head-width values for the 0 and

15 ppt salinity-temperature combinations. Average head-width values at the 20°C were smaller in comparison to higher temperatures but followed the same trends, suggesting that head-widths measured at 84 days reflected survival optima around 5 and 10 ppt across all temperatures. These observations indicated that naiad head-width increased with increased temperature and increased at intermediate salinity.

Linear regression results indicated that temperature ($F = 30.19$, $p < 0.001$), salinity ($F = 35.93$, $p < 0.001$) and the polynomial covariate for salinity, salinity² ($F = 35.93$, $p < 0.001$) had strong effects on head-width measured at 84 days, with significant temperature $\times$ salinity ($F = 5.29$, $p < 0.001$) and temperature $\times$ salinity² interaction ($F = 8.70$, $p = 0.003$). These results suggested that the effect of salinity on head-width was greatest at 20°C ($\beta = 0.12 \pm 0.61$), the level at which head-widths values were smaller compared to naiads at the same salinity and higher temperatures. Therefore, the relationship between head-width and salinity was not linear, but rather was greatest at intermediate salinity as observed for naiad survival. Both results support the observation that naiad head-width increased with increased temperature and increased at intermediate salinity.

DISCUSSION

Decreased ground-water flux associated with urbanization has the potential to increase the salinity of anchialine ponds at Hawai‘i’s Kaloko-Honokōhau National Historical Park (Oki et al. 1999). Although rigorous evidence is lacking, there are no studies suggesting that increased anchialine pond salinity impacts anchialine habitats. Therefore, the response of anchialine organisms to elevated salinity remains largely uncharacterized. Our results are the first to demonstrate that increasing salinity negatively affects the endemic (candidate endangered) orange-black Hawaiian damselfly (*Megalagrion xanthomelas*), a species that completes its life cycle in anchialine ponds. We found that *Megalagrion xanthomelas* were particularly sensitive to increased salinity, although differentially affected by various salinity-temperature combinations. Generally, egg hatch increased with increased temperature and decreased salinity, whereas, naiad survival increased with decreased temperature and was greatest at intermediate salinity (5 and 10 ppt), and naiad growth increased with increased temperature at intermediate salinity. The survival and growth optimum for *M. xanthomelas* naiads at intermediate salinity was unexpected and may have important implications for environmental change in anchialine pond environments. Additionally, *M. xanthomelas* naiads displayed threshold responses to salinity above 15 ppt with no naiads surviving at 20 ppt despite successful hatch observed at this salinity. This suggested that eggs were less sensitive to the salinity-temperature combinations used in this study compared to naiads.

The observed differences in salinity tolerance between *M. xanthomelas* eggs and naiads are particularly interesting when considered in context with the dynamic character

of anchialine pond environments. Eggs are stationary and remain within the medium to which they were oviposited until hatch. Because eggs are immobile, they must be able to tolerate large fluctuations in salinity. The reduced sensitivity of eggs to higher salinity could be attributed to osmotic protection supplied by the surrounding chorion (Wichard et al. 2002) and the additional buffer provided by the endophytic nature in which the eggs were deposited. These salinity buffers (with filter paper providing the experimental endophytic properties) may explain the observed high egg hatch success at 20 ppt at the two greatest temperature levels in the study (24 and 28°C), whereas, naiad survival was not observed beyond 15 ppt. However, egg hatch success, was substantially reduced at the 20°C–15 ppt combination and was not observed at the 20°C–20 ppt combination. We do not suspect that these combinations represent the salinity-temperature thresholds for hatching, but the low hatch success at these combinations is possibly a reflection of the study duration selected. At 20°C egg hatch success was delayed at all salinities compared to results at higher temperatures. Therefore, the 28-day study duration was likely not long enough to observe hatch at the 20°C–15 ppt and 20°C–20 ppt combinations. Additionally, eggs did not exhibit threshold responses to salinity similar to naiads, although successful hatch was not observed within 28 days at 32 ppt during our pilot studies suggesting the hatching threshold for *M. xanthomelas* is between 20 and 32 ppt.

In contrast to eggs, *M. xanthomelas* naiads have the ability to respond behaviorally and seek out lower salinity waters and are frequently observed free swimming near the surface of small stream pools (Englund 1999). Such behavioral adaptations are especially significant in anchialine pond habitats where salinity is often

vertically stratified. Salinity in west coast anchialine ponds of Hawai'i Island differ by an average of 12.6 ppt between subsurface and bottom waters (Maciolek & Brock 1974) creating a substantial salinity gradient (halocline) in ponds. Naiads appeared to exhibit a threshold response to salinity above 15 ppt suggesting that if baseline anchialine pond salinity were to exceed 15 ppt naiad mortality would ensue. With the impending threat of increased groundwater withdrawals and salinization of anchialine ponds, the salinity threshold for *M. xanthomelas* naiad survival may be surpassed resulting in large mortality and potential extirpation.

The apparent threshold to salinity for naiads beyond 15 ppt may be an underestimate and could differ among the populations and locations. Hawai'i Island populations of *M. xanthomelas* show a great deal of genetic diversity (Jordan et al. 2005), however, we cannot assume that the effects of salinity are similar for populations around the island. Eggs and naiads used in this study were collected from a single population from anchialine ponds on the eastern coast of Hawai'i Island. Mean annual rainfall is approximately 300 cm in this area, compared to 60 cm in West Hawai'i. Differences in regional rainfall may also account for differences in anchialine pond salinity; average salinity is 3.5 ppt along the eastern coast (Chai 1993) and 7.0 ppt along the western coast (Maciolek & Brock 1974). Therefore, it is possible that populations of *M. xanthomelas* may have adapted to differences in salinity, with western coast populations potentially less sensitive to higher salinity than eastern coast populations. If so, our results provide a conservative estimate for the impact of salinity on *M. xanthomelas* survival. Kefford et al. (2004b) demonstrated that laboratory salinity tolerance could reflect the maximum salinity that macroinvertebrates inhabit in the field. Therefore, comparing the field

distribution for *M. xanthomelas* with our laboratory observations may reveal analogous results.

While the physiological mechanisms responsible for the survival and growth optima have not been investigated, several mechanisms may contribute. Diet can have physiological effects on life history characteristics among various environmental conditions (Kefford et al. 2006). Although our naiad feeding experiment did not demonstrate differential feeding of prey used in the experiment, differential effects may occur in anchialine ponds. While feeding appears to be similar at the opposite ends of the salinity range tested in this experiment for *M. xanthomelas*, we cannot entirely rule out salinity and its effects on feeding because we did not examine feeding rates within the salinity optima. However, because feeding was similar between *A. nauplii* hatchings and early instar *C. quinquefasciatus* larvae, we can speculate that differences in survival and growth may be due to direct effects of salinity and not indirect effects of prey availability.

Respiration rate may also contribute to the survival and growth optima. Respiration was not examined in this study. However, respiration and the concentration of dissolved oxygen in water are known to affect the distribution and survival of damselflies (Corbet 1999). Therefore, respiration rates near the salinity optimum for *M. xanthomelas* could be reduced to allow for increased survival and growth. Kefford et al. (2006) found that respiration rates for the damselfly *Ischnura heterosticta* were similar across a large salinity range. Like *M. xanthomelas*, naiad survival and growth for *I. heterosticta* were higher and faster in intermediate salinity. During this study, naiads were kept individually in 30 mL plastic cups containing approximately 25 mL of the treatment salinity water. Although water was replaced every five to seven days, oxygen

concentration was not monitored and the effect of oxygen utilization was not evaluated. We can only speculate that dissolved oxygen was higher in salinities less than 10 ppt, allowing naiads to be more efficient at breathing which subsequently resulted in increased survival and growth under these conditions.

Related to respiration rates and oxygen consumption, osmoregulation could be another physiological mechanism contributing to the survival and growth optima at intermediate salinity. Under optimum conditions, it is hypothesized that damselflies spend less energy on osmoregulation to allow for significant whole organism responses, such as growth (Kefford et al. 2006). Since the osmoregulatory abilities of *M. xanthomelas* have not been studied and remain unknown, it would be difficult to infer the relationship between osmoregulation and salinity preference. Osmoregulation could also have important implications for acclimation and long-term survival. For example, the time necessary for naiads to reach their isosmotic point (where internal and external salinity are equal) may be critical under conditions where salinity can change rapidly (e.g., within an anchialine pond).

The results of this investigation indicated that naiads are well adapted to anchialine pond environments, with optima for naiad survival and growth at intermediate salinities. This is especially noteworthy, given there are few salt-tolerant species that reportedly prefer, rather than merely tolerate, brackish waters (Corbet 1999). Rather, most damselflies found in brackish waters are primarily freshwater species that are, to varying degrees, tolerant of salinity (Bayly 1972, Corbet 1999). It is unknown whether other species within the genus *Megalagrion* are adapted to brackish waters, but the

apparent adaption of *M. xanthomelas* to brackish waters may have important evolutionary implications for this predominately freshwater genus.

Future studies on *M. xanthomelas* in anchialine ponds should examine the salinity and temperature thresholds especially for the naiad stage. In addition the salinity (and temperature) tolerance for populations around the island should be studied to determine whether different populations are adapted to local conditions. Comparative studies investigating younger and older life stages' response to salinity could also provide interesting information for the long-term success of the species within anchialine ponds. Behavioral studies could also reveal how naiads respond to salinity gradients and how salinity affects the distribution of *M. xanthomelas* naiads within anchialine ponds. It would also be interesting to examine the osmoregulatory and respiratory capabilities for *M. xanthomelas*. Both measures could provide further insight to the mechanisms responsible for lethal and sub-lethal effects.

Damselflies are exceptionally vulnerable to habitat alteration and degradation associated with urbanization (Corbet 1980). Habitat degradation exerts especially great pressure on damselflies confined to small areas (Clausnitzer et al. 2009). We have demonstrated that *Megalagrion xanthomelas* in anchialine ponds are particularly sensitive to increased salinity and may become extirpated from pond habitats if salinities rise beyond 15 ppt. Therefore, it is important that anchialine pond water quality and quantity are maintained in order to provide sufficient breeding habitat for *M. xanthomelas* and the integrity of anchialine pond communities.

Table 1. Summary of water quality data collected in anchialine ponds at Lokoaka Wilderness Park during *Megalagrion xanthomelas* collection, Aug 2008-Oct 2009. Parameters included water temperature (Temp), conductivity (Cond), specific conductivity (SpC) and salinity (Salin). Data for each parameter are mean $\pm$ SE; n = 4 for sampling interval I; n = 3 for sampling intervals II and III.

Sampling interval	Date	Temp ($^{\circ}\text{C}$)	Cond (mS cm^{-1})	SpC (mS cm^{-1})	Salin (ppt)
I	August 2008	22.63 ± 0.66	12.96 ± 0.72	12.41 ± 0.63	7.43 ± 0.45
II	January 2009	19.83 ± 0.13	11.41 ± 0.13	10.30 ± 0.05	6.53 ± 0.07
III	October 2009	22.07 ± 0.32	10.57 ± 0.79	9.90 ± 0.75	5.97 ± 0.47

Table 2. Results of logistic regression analysis for the *Megalagrion xanthomelas* hatching success experiment. Output presented for influences of temperature, salinity, and the interaction (temperature x salinity) include slope coefficient (β), standard error of the slope coefficient (SE β), z-value, p-value, and odds ratio (e^β). The interaction term was highly significant ($p < 0.001$) and accounted for at each level of temperature and salinity.

Predictor	β	SE β	z-value	p-value	Odds ratio (e^β)
Constant	2.24	1.06	2.12	0.03	NA
Temperature	0.00	0.04	-0.04	0.97	1.00
Salinity	-0.71	0.08	-8.43	< 0.001	0.49
Temperature x Salinity	0.02	0.00	6.90	< 0.001	1.02
20°C	-0.23	0.02			0.80
24°C	-0.13	0.01			0.90
28°C	-0.04	0.02			0.96
0 ppt	0.00	0.04			1.00
5 ppt	0.12	0.03			1.13
10 ppt	0.24	0.02			1.27
15 ppt	0.36	0.03			1.43
20 ppt	0.48	0.04			1.61

Table 3. Results of logistic regression analysis for the *Megalagrion xanthomelas* naiad survival experiment. Output presented for influences of temperature, salinity, and the interaction (temperature x salinity) include slope coefficient (β), standard error of the slope coefficient (SE β), z-value, p-value, and odds ratio (e^β). The interaction term was highly significant ($p < 0.001$) and accounted for at each level of temperature and salinity.

Predictor	β	SE β	z-value	p-value	Odds ratio (e^β)
Constant	7.57	0.81	9.36	< 0.001	NA
Temperature	-0.31	0.32	-9.85	< 0.001	0.73
Salinity	-0.42	0.06	-6.45	< 0.001	0.66
Temperature x Salinity	0.01	0.00	4.29	< 0.001	1.01
20°C	-0.19	0.01			0.82
24°C	-0.15	0.01			0.86
28°C	-0.11	0.01			0.90
0 ppt	-0.33	0.03			0.72
5 ppt	-0.26	0.04			0.77
10 ppt	-0.20	0.06			0.82
15 ppt	-0.25	0.07			0.86
20 ppt	-0.06	0.03			0.94

Table 4. Results of logistic regression analysis for *Megalagrion xanthomelas* naiad survival using a reduced model excluding 20 ppt and including the polynomial covariate for salinity (salinity²). Output presented include slope coefficient (β), standard error of the slope coefficient (SE β), z-value, p-value, and odds ratio (e^β).

Predictor	β	SE β	z-value	p-value	Odds ratio (e^β)
Constant	4.55	0.87	5.25	<0.001	NA
Temperature	-0.24	0.03	-6.92	<0.001	0.79
Salinity	0.52	0.29	1.78	0.075	1.69
Salinity ²	-0.04	0.02	-2.29	0.022	0.96
Temperature $\times$ Salinity	-0.01	0.01	-0.79	0.428	0.99
Temperature $\times$ Salinity ²	0.00	0.00	1.09	0.274	1.00

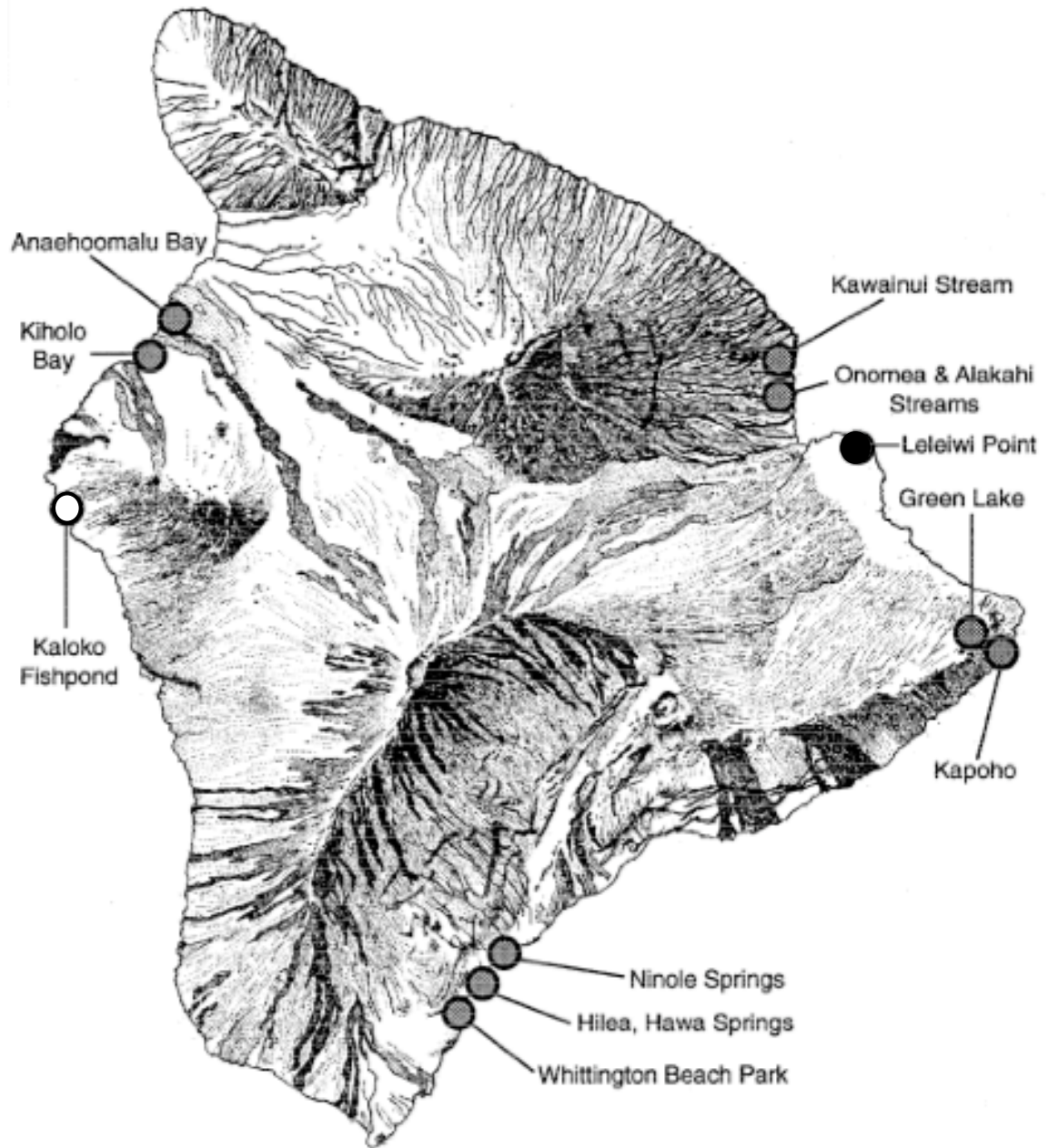


Figure 1. Hawai'i Island map showing known populations of *Megalagrion xanthomelas* (Polhemus 1996). Anchialine ponds at Lokoaka Wilderness Park collection site are denoted by solid circle, (●) and anchialine ponds at Kaloko-Honokōhau National Historical Park are denoted by open circle (○).

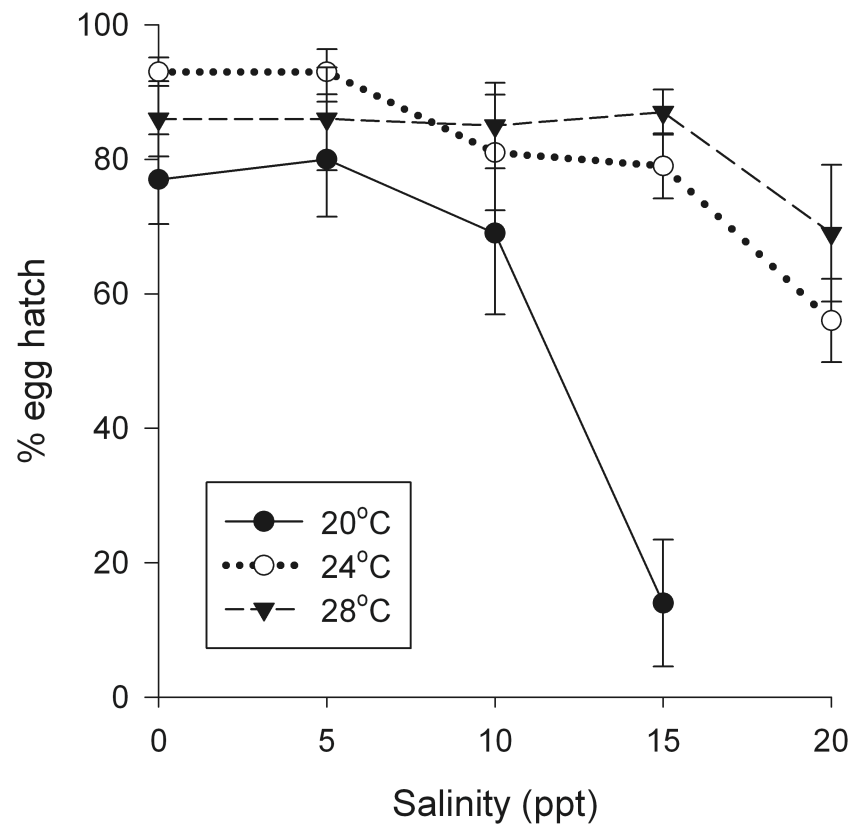


Figure 2. Percent egg hatch (mean $\pm$ SE) for *Megalagrion xanthomelas* among three temperature levels (20°, 24° and 28°C) plotted against salinity. Egg hatch was not observed at 20°C–20 ppt combination during the 28 day study.

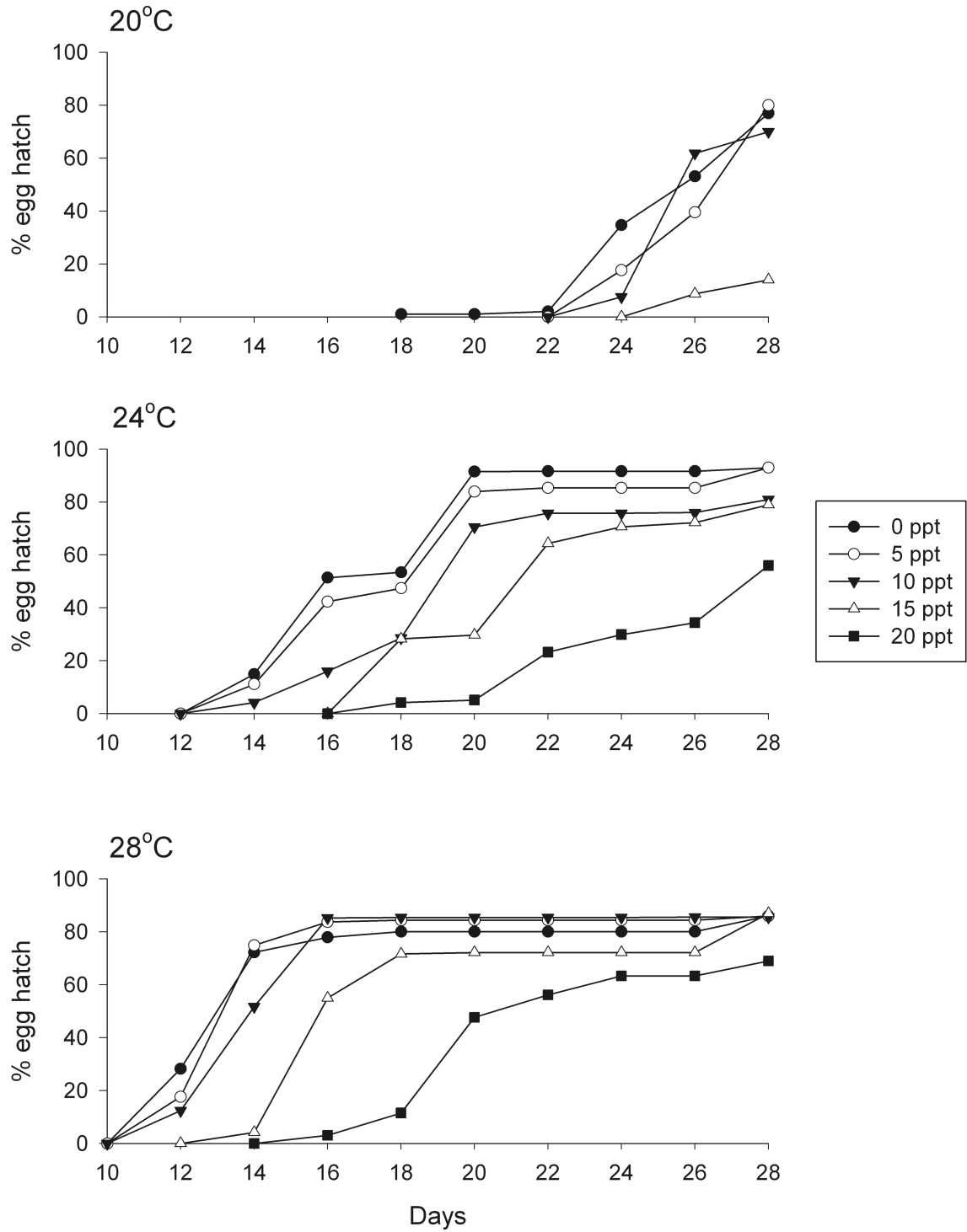


Figure 3. Percent egg hatch for *Megalagrion xanthomelas* among three temperature levels (20°, 24° and 28°C) and five salinity levels (0, 5, 10, 15 or 20 ppt) over 28 days.

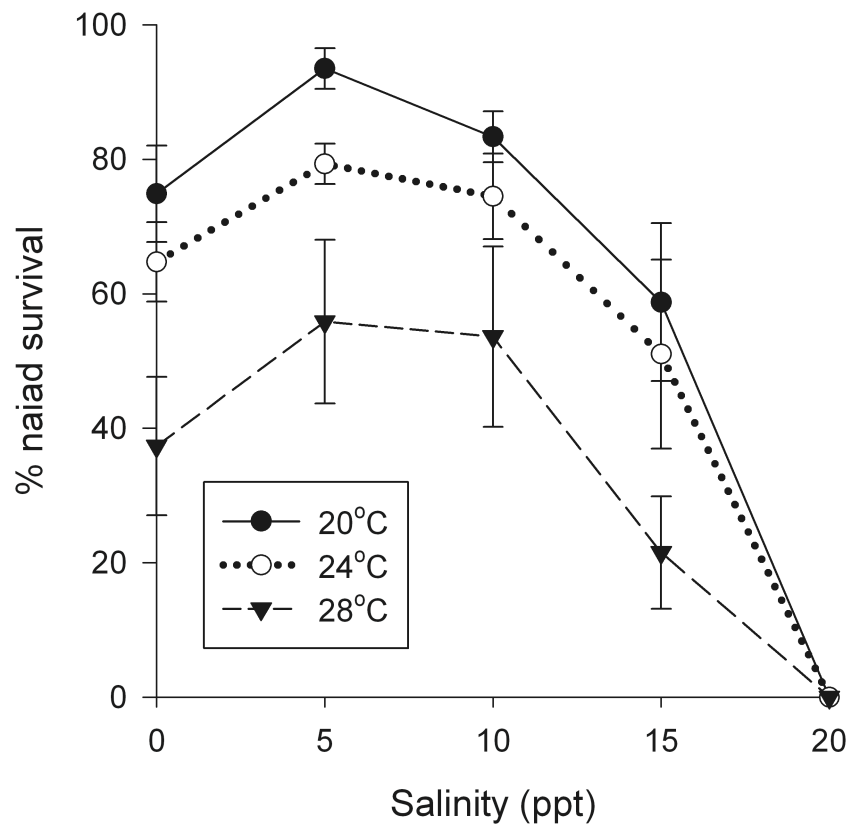


Figure 4. Percent naiad survival (mean $\pm$ SE) for *Megalagrion xanthomelas* among three temperature levels (20°, 24° and 28°C) plotted against salinity. Excluding two outliers (at 20°C–20 ppt combination) that were removed from analyses, no naiads survived at 20 ppt during the 84-day study.

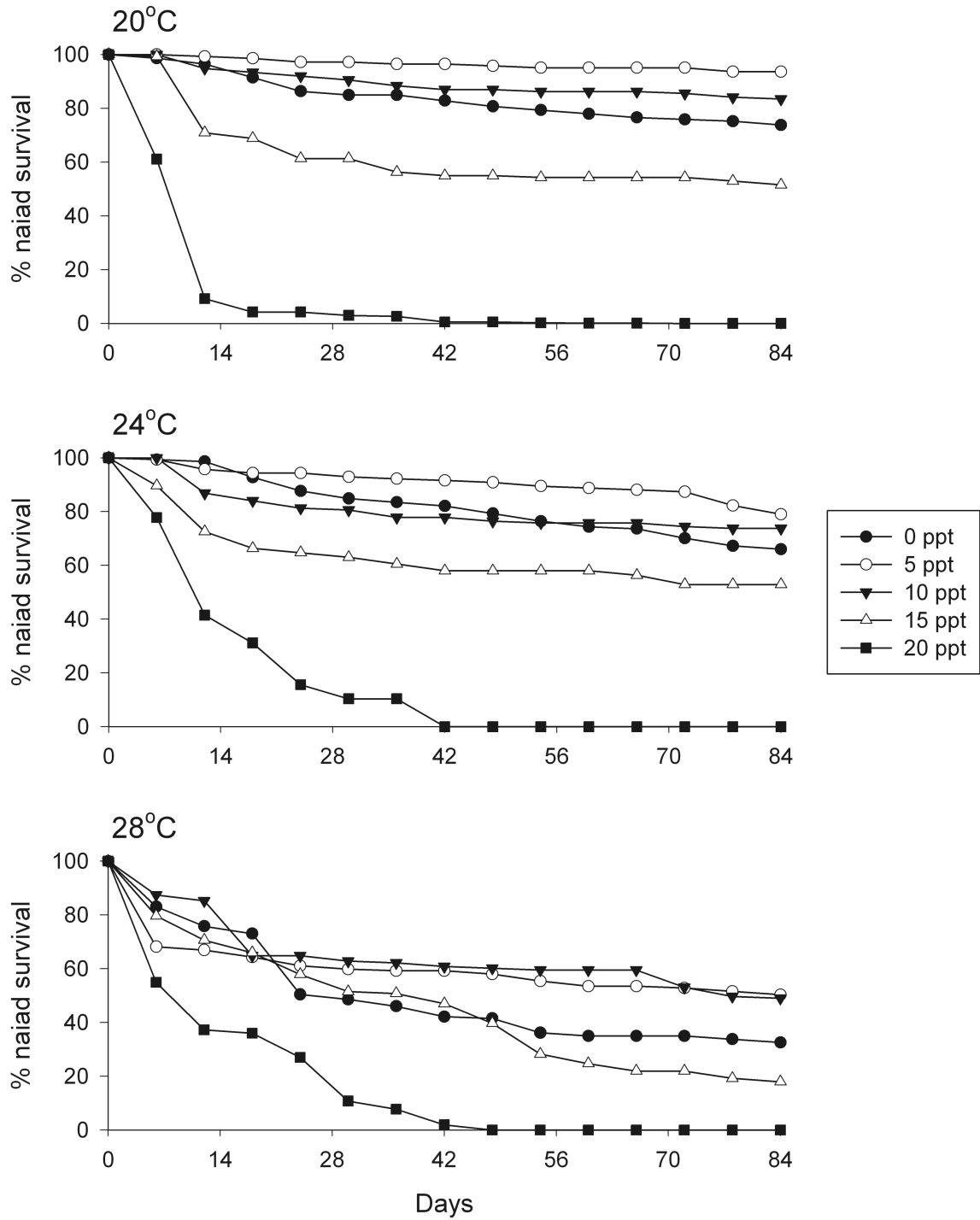


Figure 5. Percent naiaid survival for *Megalagrion xanthomelas* among three temperature levels (20°, 24° and 28°C) and five salinity levels (0, 5, 10, 15 or 20 ppt) over 84 days.

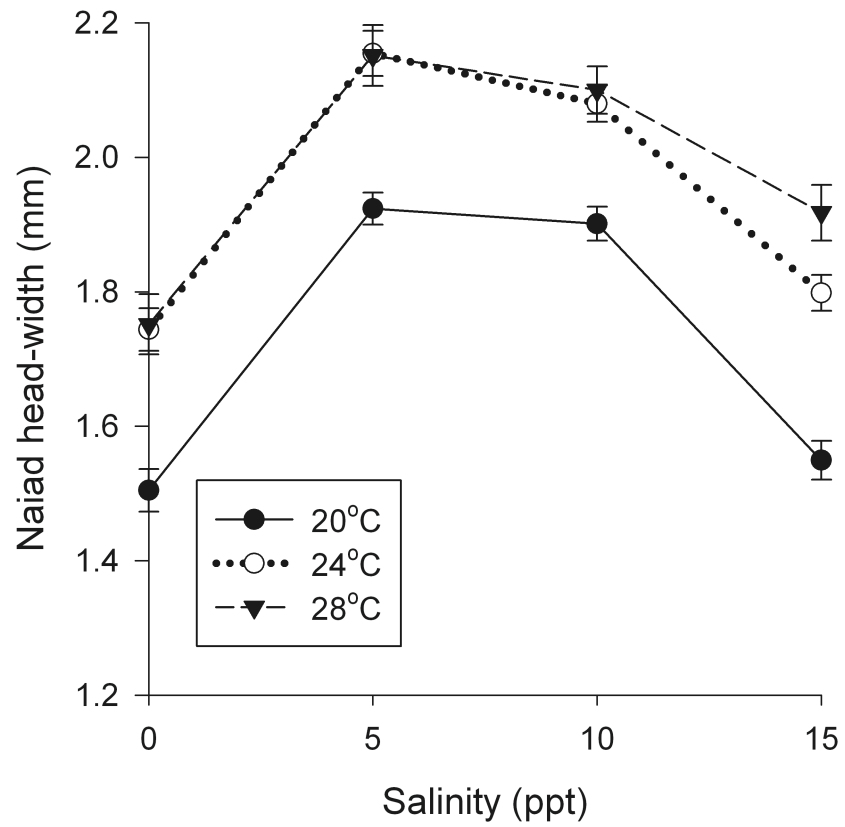


Figure 6. Naiad head-width growth (mean $\pm$ SE) for *Megalagrion xanthomelas* measured at 84 days among three temperature levels (20°, 24° and 28°C) plotted against salinity.

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