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Bet hedging and cold-temperature termination of diapause in the life history of the Atlantic salmon ectoparasite *Argulus canadensis*

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Research Article

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Abstract

Argulus canadensis is a crustacean ectoparasite observed increasingly on wild migrating adult Atlantic salmon. We investigated temperature and salinity tolerance regarding development, survival and hatch of *A. canadensis* eggs to help understand spatiotemporal features of transmission. *Argulus canadensis* eggs differentiate to pharate embryos by 35 days buttheir hatch is protracted to ~7 months. Cold treatment ⩾75 days mimics overwintering and terminates egg diapause, with 84.6% (72.1–100%) metanauplius hatch induced ⩾13 °C and synchronized to 3–4 weeks. Inter- and intra-clutch variability and protracted hatch in the absence of cold-temperature termination of diapause is compatible with bet hedging. Whereas diapause likely promotes phenological synchrony for host colocalization, bet hedging could afford temporal plasticity to promote host encounter during environmental change. Our egg storage and hatch induction/synchronization methodologies can be exploited for empirical investigations. Salinity tolerance reveals both significantly higher embryonic development (94.4 ± 3.5% *vs* 61.7 ± 24.6%) and metanauplius hatch (53.3 ± 7.5% *vs* 10.1 ± 8.2%) for eggs in freshwater than at 17 ppt. Unhatched embryos were alive in freshwater by the end of the trial (213 days) but were dead/dying at 17 ppt. Eggs did not develop at 34 ppt. Salinity tolerance of *A. canadensis* eggs supports riverine transmission to adult Atlantic salmon during return to freshwater for mating each year.

Introduction

Atlantic salmon (*Salmo salar*) are anadromous fish that live in salinities ranging from ~0 ppt in freshwater to 33–35 ppt in the Northwest Atlantic Ocean (Reddin, 2006). Ectoparasites are considered a major threat for wild Atlantic salmon in the Northwest Atlantic region (Clarke *et al*., 2014). Species of ectoparasites observed commonly on Atlantic salmon in some home ranges include *Lepeophtheirus salmonis*, *Caligus elongatus* and *Argulus canadensis*. These ectoparasites are observed in freshwater on returning wild Atlantic salmon during passage at hydroelectric dams ~50–150 km from the Atlantic Ocean (Powell *et al*., 1999; Clarke *et al*., 2014). *Lepeophtheirus salmonis* and *C. elongatus* are referred to commonly as sea lice (Copley *et al*., 2005), they occur predominantly in marine environments and usually die within a week following the return of their salmon host to freshwater (McLean *et al*., 1990).

Unlike *L. salmonis* and *C. elongatus* that are copepods, *Argulus* spp. are branchiurans. *Argulus* spp. are distributed broadly and infest fish from equatorial to temperate regions in both hemispheres (Neethling and Avenant-Oldewage, 2016). The genus *Argulus* includes >120 valid species, with some adapted to marine (Devaraj and Ameer Hamsa, 1977) or estuarine conditions (Natarajan, 1982) and nearly two-thirds occurring primarily in freshwater (Neethling and Avenant-Oldewage, 2016). Many *Argulus* spp. are stenohaline, infesting fishes from either freshwater or marine environments, whereas others are euryhaline (Neethling and Avenant-Oldewage, 2016). However, general salinity tolerance of *Argulus* spp. (i.e. Neethling and Avenant-Oldewage, 2016) is based primarily on field observations of parasites encountered on wild fishes and not on empirical studies. *Argulus* spp. are obligate and cosmopolitan parasites of fishes, infesting a wide variety of warm-water species (Carvalho *et al*., 2004) and cold-water salmonids (Powell *et al*., 1999). Their global distribution in aquatic environments and parasitism of a broad variety of animals suggests high adaptability of *Argulus* spp. in diverse environments. Their occurrence on an increasing proportion of wild Atlantic salmon is of concern (Clarke *et al*., 2014) and raises questions about phenology. Specifically, unknown are sites of parasite acquisition during migration and how spatiotemporal co-localization of these parasites is achieved in the face of depressed Atlantic salmon populations (Chaput, 2012).

Reports of *A. canadensis* on wild migrating adult Atlantic salmon occur exclusively during upstream passage at hydroelectric dams (Powell *et al*., 1999; Clarke *et al*., 2014), rather unique sites for population-level assessments and monitoring. These dams occur ~50–150 km from the Atlantic Ocean (Powell *et al*., 1999; Clarke *et al*., 2014) and ~20–80 km upstream of saltwater influence. Adult salmon are observed rarely and in comparatively small numbers at other locations and times and so many spatiotemporal aspects of *A. canadensis* biology are unknown. Despite predominant observations of *A. canadensis* on migratory diadromous fishes

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in downstream reaches of freshwater systems (Davis, 1956; Powell *et al.*, 1999; Clarke *et al.*, 2014), neither sites of *A. canadensis* acquisition by their preferred Atlantic salmon host nor parasite salinity tolerance have been investigated. Initial attempts to hatch metanauplii for empirical investigations using conditions established for many congeneric species (Shafir and Van As, 1986) were unsuccessful. This promoted our investigation of salinity tolerance of *A. canadensis* eggs and effects of temperature on embryonic development, survival and hatch to help understand spatiotemporal features of transmission to wild migrating Atlantic salmon and to elucidate life history features that can be exploited to support empirical investigations.

Materials and methods

Ectoparasitic crustaceans and egg collection

Adult *A. canadensis* were collected from wild Outer Bay of Fundy Atlantic salmon (*Salmo salar*) handled by Department of Fisheries and Oceans (DFO) Canada personnel at the Mactaquac Biodiversity Facility on the St. John River, New Brunswick. Live female *A. canadensis* were collected by gentle removal from the fish epidermis using a dull knife. Parasites were transferred to 10-gallon recirculation aquariums (Top Fin®, Pet Smart, Fredericton, Canada) to promote egg laying for up to 48 h at room temperature (~21 °C) in dechlorinated tap water.

Embryonic development, larval hatch and fate of eggs at room temperature

Egg clusters laid by *A. canadensis* (June 28–July 3) were maintained in dechlorinated tap water at room temperature. Egg clusters were chosen randomly and removed from aquarium glass and silicone using a scalpel blade and individual clusters were contained within 70-μm sieves (Fisherbrand™ Cell Strainers, Fisher Scientific, Ottawa, Canada). Eggs were observed daily for 35 days at ~21 °C to monitor embryonic development.

An aquarium containing 138 adherent egg clusters was maintained at room temperature and monitored daily for ~230 days (229–234 days) to determine number and timing of metanauplius hatch. After ~230 days, 20 egg clusters were selected randomly and examined to determine the fate of embryos in individual eggs contained within each cluster (i.e. dead, live, hatched, unfertilized).

Influence of cold-temperature treatment on hatch

14-day and 28-day cold treatment

Metanauplii hatched prior to the beginning of this experiment (230 days maintenance at room temperature) were counted. Eggs were assessed microscopically to determine whether they were empty (i.e. hatched), not fertilized, contained embryos that were dead, or contained embryos that were live. From these eggs, 10 clusters with at least 60 live unhatched *A. canadensis* embryos were used to assess the influence of a cold-temperature treatment on their hatch. Each of 10 clusters was cut roughly in half to ensure a minimum of 30 eggs with developed embryos were present in each sibling group. The number of unhatched eggs containing live embryos in each paired group was 30–106 (50.5 ± 19.9). Each sibling group was placed individually into a 70-μm sieve floating in a 100 × 80-mm^2 Pyrex dish (No. 3250), with each of the 20 dishes containing 300 mL of dechlorinated tap water. One half of each sibling group was allocated randomly to incubation at either room temperature (~21 °C; control) or 7 °C for cold treatment (Fig. 1). Five of 10 cold treatment clusters were assigned randomly to a 14-day cold treatment and the other five received a 28-day cold treatment. Replacement water was aerated and adjusted to treatment temperature (~21 °C or 7 °C) for 24 h prior to exchange of 100 mL water volume each week. Hatched metanauplii were removed from each sieve daily and were enumerated. Pyrex dishes containing the cold-treatment half of sibling groups were returned to room temperature following 14- or 28-day cold treatments (Fig. 1). Larval hatch was monitored daily in all eggs for another 30 days.

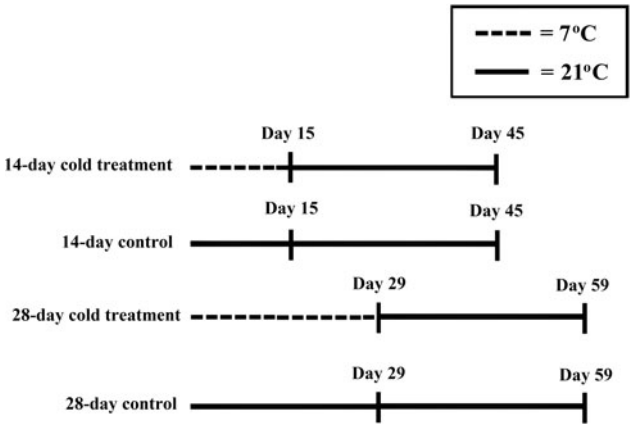

Fig. 1. Schematic of experiments assessing 'influence of cold-temperature treatment on hatch'. Ten egg clusters were cut to yield two siblings groups per cluster. One half of each sibling group (i.e. 10 controls) was maintained at 21 °C with the other half at 7 °C for cold treatment. Ten cold treatment sibling groups were assigned randomly to 14-day ($n = 5$) or 28-day ($n = 5$) cold treatment. The other half of each sibling group was maintained at 21 °C. Cold treatment was terminated after 14 days (Day 15) or 28 days (Day 29) with return to 21 °C. Egg hatch was monitored in all sibling egg clusters until experiments were terminated 30 days after return of cold treatments groups to 21 °C.

The effect of cold dormancy on *A. canadensis* metanauplius hatch was analysed using a linear mixed-effects model using Treatment, Time and their interactions as fixed effects, with Cluster (nested in Treatment) treated as the random effect to test the H_o that there was no statistical difference in hatching induction or synchrony between cold treated eggs and their corresponding room temperature controls over time. Fixed-effect correlations were examined and were not highly correlated for any test, suggesting that collinearity was not an issue.

For statistical analyses, 14- and 28-day cold-treated eggs and their associated controls were assessed separately. For the 14-day group, post-cold treatment hatch was compared to its control for the duration of the experiment (days 1–45), to test *A. canadensis* hatch induction following cold treatment. Additionally, the 30-day period post-cold treatment exposure (days 15–45) was analysed to test *A. canadensis* hatch synchronization following cold treatment. Similarly, for the 28-day group, post-cold treatment hatch was compared to its control for the duration of the experiment (days 1–59) to assess hatch induction, and hatch synchronization was assessed for the 30-day period post-cold treatment exposure (days 29–59). A *t*-test was conducted to test the H_o of no significant difference in the overall hatch of *A. canadensis* metanauplii between cold temperature treated eggs and their associated controls (i.e. cumulative hatch for days 1–45 days for 14-day cold-treated eggs *vs* controls, and for days 1–59 days for 28-day cold-treated eggs *vs* controls).

75-day cold treatment

Metanauplii hatched prior to the beginning of this experiment (150 days maintenance at room temperature) were counted. Three egg clusters were subjected to cold treatment (7 °C) for 75 days, after which daily hatches were monitored at room temperature as described above. The average number of eggs in

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each cluster was 91.3 (84–111). There was no room temperature control for this observation.

Long-term egg viability

Egg clusters containing fully developed embryos were subjected to uninterrupted cold treatment (⩽7 °C) for 2.5 years prior to their return to favourable temperatures (~21 °C). Long-term embryo survival was assessed based on their ability to hatch.

Influence of post-diapause water temperature on hatch

A 10-gallon recirculation aquarium containing dechlorinated tap water and egg clusters laid by *A. canadensis* over a 22-day period (July 11–August 1) was maintained at room temperature for ~150 days (134–155 days) after egg laying. The aquarium was subjected to cold treatment at 7 °C for 108 days. Six egg clusters were selected randomly and assessed to determine the number of empty eggs prior to initiating the experiment. Egg clusters were cut roughly into thirds to yield three sibling groups per cluster. Each sibling group was placed individually into a 70-μm sieve floating in a 100 × 80-mm^2 Pyrex dish (No. 3250), with each of the 18 dishes containing 300 mL of dechlorinated tap water. The average number of eggs in each sibling group was 69.4 (41–102). Each sibling group was assigned randomly to one of three incubation temperatures (9 °C, 13 °C, 17 °C) to assess larval hatch. Treatment temperature and photoperiod were controlled using a Conviron CMP 3246 growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba). Pyrex dishes were secondarily contained and submerged 2/3 depth within a water bath to promote constant water temperature during daily observations. Incandescent light [Sylvania 6000-h bulbs (60 w; 120–125 v)] with 14.5-h light and 9.5-h dark cycle was used in chambers to mimic the light cycle at the estimated time of hatch in the wild (i.e. early May). Replacement water was aerated and adjusted to treatment temperature (9 °C, 13 °C, 17 °C) for 24 h prior to exchange of 100 mL water volume each week. Hatched metanauplii were enumerated and removed daily from each sieve.

The effect of incubation temperature on *A. canadensis* metanauplii hatch from eggs following cold temperature treatment was analysed using a linear mixed-effects model. Temperature, Time and their interactions were treated as fixed effects, with Cluster (nested in Temperature) treated as the random effect to test the H_o that there was no statistical difference in *A. canadensis* hatch at different temperatures over time. Fixed-effect correlations were examined for all tests and were not highly correlated, suggesting that collinearity was not an issue. A one-way ANOVA was conducted to test the H_o of no significant difference in overall hatch success of *A. canadensis* metanauplii at different temperatures at the end of the experiment (i.e. cumulative hatch over the 21 days of monitoring following cold treatment).

Salinity tolerance of A. canadensis eggs

Egg clusters were collected and maintained at room temperature as described above. Nine egg clusters were selected randomly 1–3 days after laying and were cut from the aquarium using a scalpel blade. Clusters had an average of 111 (76–178) eggs. Clusters were contained individually in 70-μm sieves and were allocated randomly to one-gallon aquariums (Top Fin®, PetSmart) containing one of three water preparations: dechlorinated tap water (0 ppt; Freshwater (FW)); Bay of Fundy seawater diluted 1:1 with dechlorinated tap water (17 ppt; Estuarine water (EST)); and, undiluted Bay of Fundy seawater (34 ppt; Seawater (SW)). All water was filter-sterilized (0.2 μm) prior to egg incubation. Egg development was monitored daily for the first 45 days and periodically for another ~150 days. All three aquariums were then subjected to cold treatment (7 °C) for 33 days. Following cold treatment, all three aquariums were maintained at room temperature and hatched metanauplii were removed daily from each sieve and enumerated.

Statistical analyses

Analyses were completed using the *lme4* package (Bates *et al.*, 2015; version 1.1-21) in R (version 3.6.1, R Core Team, 2019) within RStudio (version 1.2.1335, RStudio Team, 2019). Data were visualized using the *ggplot2* package (Wickham, 2016; version 3.2.1).

Results

Embryonic development, larval hatch, and fate of eggs at room temperature

Eggs of *A. canadensis* were observed daily to monitor embryonic development in freshwater. Eyes began to develop in embryos by day 17, development of the median 'nauplius eye' was complete by day 29 and no further changes to embryo morphology were observed after day 35 (Fig. 2). Hatch of a single *A. canadensis* metanauplius was observed 31 days after egg-laying.

Egg clusters were randomly chosen and removed from an aquarium after ~150 days ($n = 9$ clusters) and after ~230 days ($n = 10$ clusters) at room temperature. The fate of eggs from these clusters was assessed regarding whether they: contained embryos that were dead (Fig. 3A); contained embryos that were live (Fig. 3A–C); were empty because metanauplii hatched (Fig. 3B); or, were unfertilized when laid (Fig. 3C). Nine egg clusters removed ~150 days after laying revealed 3.2 ± 3.6% (0–9.1%) of metanauplii hatched. Six of these clusters showed relatively low hatch 0.8 ± 0.8% (0–1.9%; Fig. 4A) whereas the other 3 clusters showed comparatively high hatch 7.9 ± 0.7% (6.7–9.1%; Fig. 4A). Ten egg clusters removed ~230 days after laying revealed 18.6 ± 15.7% (1–45.8%) of metanauplii hatched. Seven of these clusters showed relatively low hatch 8.9 ± 4.5% (1.0–14.9%; Fig. 4A) whereas the other 3 clusters showed comparatively high hatch 41.1 ± 5.5% (35.1–45.8%; Fig. 4A). These results support large variation in per cent metanauplius hatch between different egg clusters in the absence of environmental hatching cues and prompted the examination of a higher number of egg clusters. Data from another 138 egg clusters removed ~230 days after laying were split evenly regarding hatch percentage with 69 clusters showing relatively low hatch 9.8 ± 5.2% (0–17.3%; Fig. 4A) and 69 clusters showing relatively high hatch 34.4 ± 14.5% (18.3–69.2%; Fig. 4A). These 138 clusters had 15–163 eggs per cluster (7241 eggs total) and it was observed that 1.1% (0–19%) of embryos in each cluster died ($n = 76$), 71.2% (19.0–100%) in each cluster were live within their egg chorion ($n = 5158$), 22.6% (0–69%) in each cluster hatched ($n = 1641$) and 5.1% (0–60%) in each cluster were unfertilized ($n = 366$; Fig. 4B).

Influence of cold-temperature treatment:

14-day cold treatment

A total of 52 *A. canadensis* metanauplii hatched from control egg groups at room temperature (26.0 ± 20.0%; 5.0–53.3%; Fig. 5), with a maximum hatch of 2 metanauplii per day over the duration of the experiment (days 1–45; Fig. 6). This includes 33 *A. canadensis* metanauplii hatched from control egg groups from days 15 to 45 (15.4 ± 11.7%; 5.0–33.3%), with a maximum hatch of 2 metanauplii per day (Fig. 6). No hatches were recorded for any sibling group during cold temperature treatment (days 1–14). For days following cold temperature treatment and after transfer

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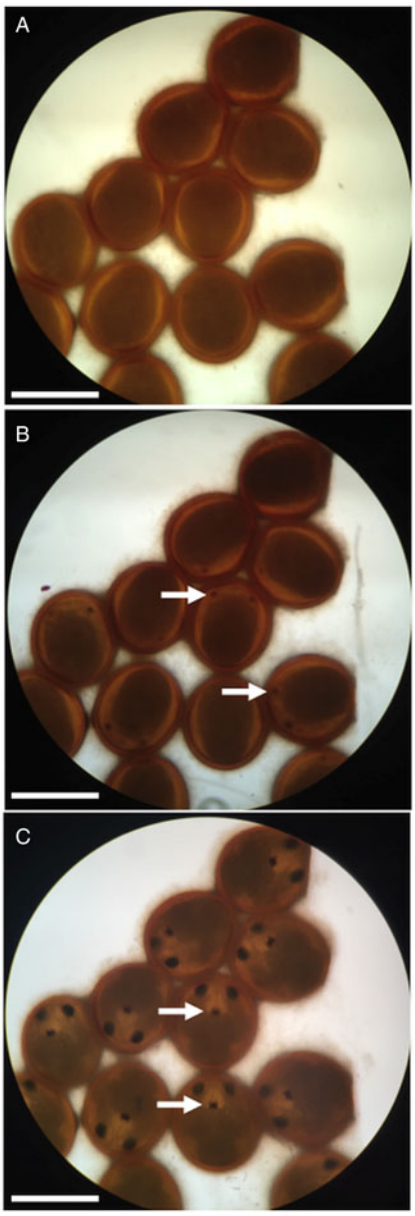

Fig. 2. Embryonic development within *Argulus canadensis* eggs maintained in freshwater at ~21 °C show: the developing embryo contracted away from the egg chorion (13 days after egg laying, A); initiation of eye formation (17 days after egg laying, B; arrows); and, the median 'nauplius eye' in each fully developed embryo (35 days after egg laying, C; arrows). White lines in the lower left of each image represent scale bars of 0.4 mm.

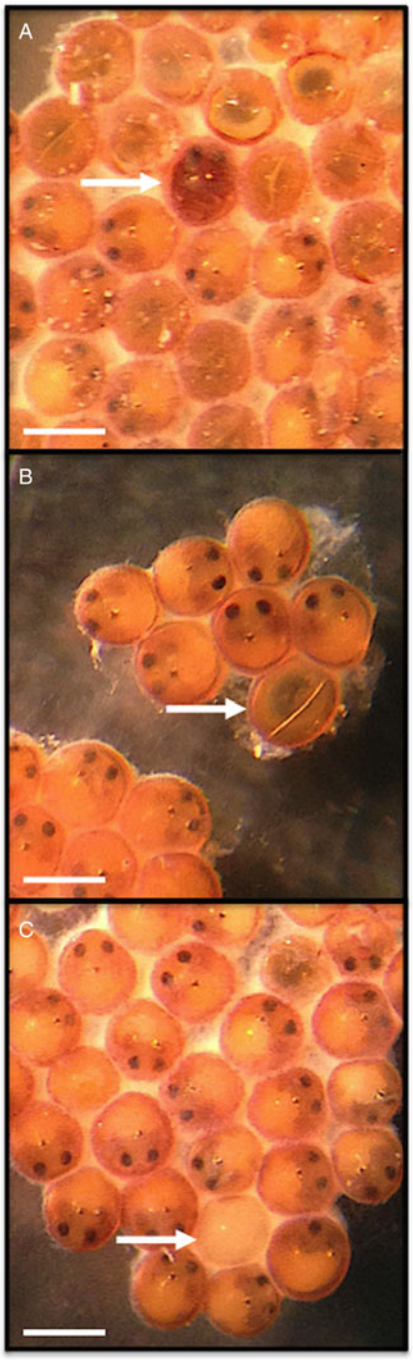

Fig. 3. *Argulus canadensis* eggs achieve different developmental outcomes in clusters maintained in freshwater at ~21 °C for ~230 days. Eggs with brown colouration contain a dead or dying embryo (A; arrow). Eggs are live if they contain an intact and non-discoloured eyed embryo (visible in all images). An empty egg with a cleavage furrow signifies metanauplius hatch (B; arrow). Eggs with persistent white colouration and lacking an identifiable embryo are unfertilized (C; arrow). White lines in the lower left of each image represent scale bars of 0.4 mm.

to room temperature (days 15–45), 77 *A. canadensis* hatched from cold temperature treated egg groups (35.3 ± 15.6%; 20.8–55.1%; Fig. 5), with a maximum hatch of 5 metanauplii per day (Fig. 6). Peak hatch of metanauplii occurred 8 days after return to room temperature (Fig. 6). More metanauplii hatched from cold-treated than control eggs, but the difference was not significant (Welch's two-sample *t*-test: $t = -0.82$, d.f. = 7.6, $P = 0.44$).

For these data, the interaction term in the linear mixed-effects model is the difference in the slope of the relationship for the cold temperature treatment relative to its room temperature control. For days 1–45, the interaction between *A. canadensis* metanauplii *Hatch* and *Time* did not differ ($t = 1.512$, $P = 0.131$), but slightly higher hatch occurred from room temperature controls ($\beta = 0.007$). For days 15–45, the interaction between *A. canadensis* metanauplii *Hatch* and *Time* was found to be statistically significant as compared to the room temperature control ($t = -3.032$; $P = 0.003$), with slightly higher hatch occurring in the cold temperature treatment ($\beta = -0.003$). The random effect of replicates (nested in treatment) for days 1–45 and days 15–45 explained 2.82% and 2.72%, with 95.0% and 93.7% residual variance, respectively.

28-day cold treatment

A total of 58 *A. canadensis* hatched from room temperature control egg groups (22.7 ± 13.7%; 9.4–43.5%; Fig. 5), with a maximum hatch of 3 metanauplii per day over the duration of the experiment (days 1–59; Fig. 6). This includes 31 *A. canadensis* metanauplii hatched from control egg groups (days 29–59: 12.0 ± 6.5%, 5.7–

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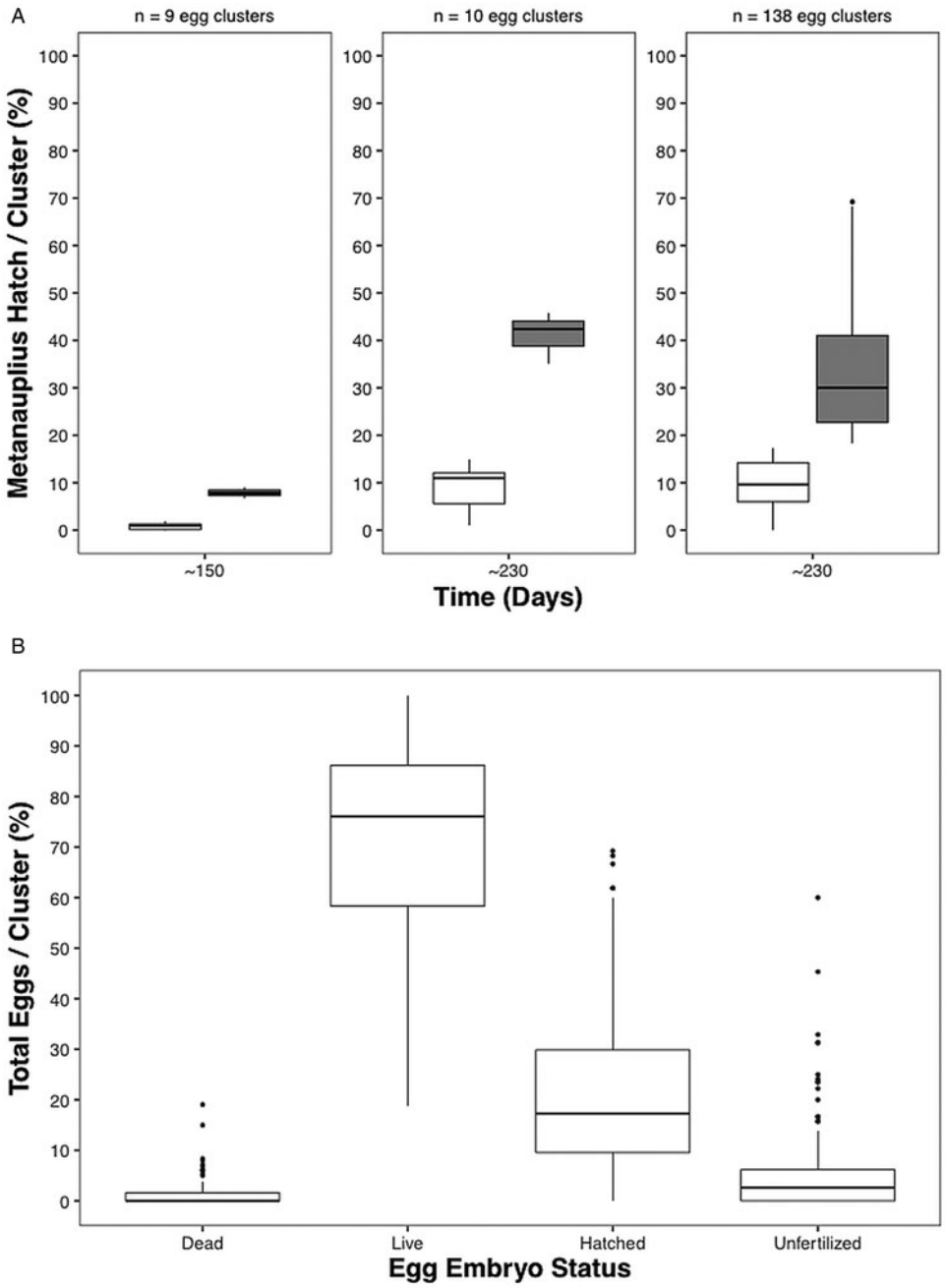

Fig. 4. Box and whisker plots show differential per cent hatch of metanauplii from representative egg clusters maintained in freshwater at ~21 °C (A). White boxplots show egg cluster subpopulations demonstrating relatively low per cent metanauplius hatch at ~150 or ~230 days after laying. Gray boxplots show egg cluster subpopulations demonstrating relatively high per cent metanauplius hatch at ~150 or ~230 days after laying. Box and whisker plots show the developmental outcomes for 7241 *A. canadensis* eggs from 138 clusters (⩾15 eggs per cluster) maintained in freshwater at ~21 °C for ~230 days (B). Eggs with internal brown colouration that contain degrading embryos are considered dead ($n = 76$). Eggs that contain a fully-developed embryo are considered live ($n = 5158$). Empty eggs with a cleavage furrow indicate that metanauplii hatched successfully ($n = 1641$). Eggs with persistent white colouration are unfertilized ($n = 366$). Horizontal black lines in boxplots represent the median per cent hatch, and boxes showing the lower 25th and upper 75th quartiles of the data. Vertical black lines represent differences in quartile distribution to the minimum and maximum bounded data values, and outliers are shown as dots.

21.7%), with a maximum hatch of 3 metanauplii per day (Fig. 6). No hatches were recorded for any egg groups during cold temperature treatment (days 1–28). For days following cold temperature treatment and after transfer to room temperature (day 29–59), 107 *A. canadensis* hatched from cold treatment egg groups (39.8 ± 10.6%; 31.6–56.1%; Fig. 5), with a maximum hatch of 9 metanauplii per day (Fig. 6). Peak hatch of metanauplii occurred 9 days after return to room temperature following cold temperature treatment (Fig. 6). Differences in per cent egg hatch for 28-day control and 28-day cold temperature treatments (days 1–59) approached significance (Welch's two-sample *t*-test: $t = -2.2$, d.f. = 7.5, $P = 0.06$), with more eggs hatching from cold-treated eggs.

For these data, the interaction term in the linear mixed-effects model is the difference in the slope of the relationship for the cold temperature treatment relative to its room temperature control. For days 1–59, the interaction between *Argulus* metanauplii *Hatch* and *Time* was statistically significant as compared to the cold temperature treatment ($t = 2.856$, $P = 0.004$), with slightly higher hatch occurring in the room temperature control over time ($\beta = 0.001$). For days 29–59 the interaction between *Argulus* metanauplii *Hatch* and *Time* was statistically significant as compared to the room temperature control ($t = -2.390$, $P = 0.0175$), with higher hatch occurring in the cold temperature treatment over time (β −0.316). The random effect of replicates

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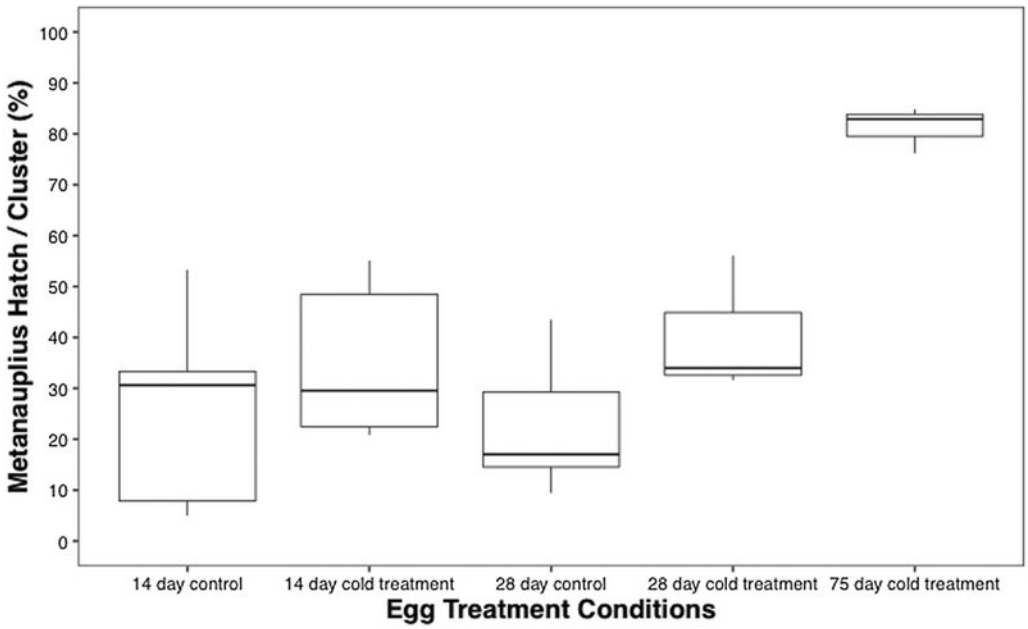

Fig. 5. Box and whisker plots show that hatch of *Argulus canadensis* metanauplii is induced by, and per cent hatch increases with, increasing the duration of cold temperature treatment. Control groups (14 and 28 day) were maintained at ~21 °C for the duration of the experiment. Cold temperature treatment groups were maintained for 14-day, 28-day and 75-day cold treatments, respectively, and then returned to ~21 °C for the duration of the experiment. Metanauplius hatch is cumulative over the 30 days at ~21 °C following treatment. The 75-day cold treatment lacks true control. Horizontal black lines in boxplots represent the median per cent hatch and boxes showing the lower 25th and upper 75th quartiles of the data. Vertical black lines represent differences in quartile distribution to the minimum and maximum bounded data values.

(nested in treatment) for days 1–59 and days 29–59 explained 0.605% and 1.25%, with 98.7% and 94.1% residual variance, respectively.

75-day cold treatment

Following a 75-day cold temperature treatment and return to room temperature, 223 *A. canadensis* metanauplii hatched from the three replicate treatments over the next 30 days (81.3 ± 4.5%; 76.2–84.8%; Fig. 5), with a maximum hatch of 16 metanauplii per day (Fig. 6). No hatches were recorded during the 75-day cold temperature treatment for any egg cluster. Peak hatch of metanauplii occurred 9 days after return to room temperature following cold temperature treatment (Fig. 6).

Long-term egg viability

Eggs returned to favourable temperatures (~21 °C) after 2.5 years at ⩽7 °C showed successful induction of live metanauplius hatches.

Influence of post-diapause water temperature on hatch

The increasing water temperature had a positive influence on inducing *A. canadensis* metanauplius hatch. Overall hatches were lowest in the 9 °C treatment (Fig. 7A) but daily hatches trended upwards over the 21-day experiment (Fig. 7B). From the 9 °C treatment, 105 *A. canadensis* metanauplii hatched (23.4 ± 15.3%; 5.1–42.4%), with a maximum hatch of 7 metanauplii per day from a single cluster (Fig. 7A) and 10 per day for the six clusters combined. Overall hatches for the 13 °C treatment were substantially higher than for the 9 °C treatment (Fig. 7A) and daily hatches trended downwards over the experiment as hatching progressed in each egg cluster (Fig. 7B). From the 13 °C treatment, 318 *A. canadensis* metanauplii hatched (82.6 ± 12.6%; 72.1–100%), with a maximum hatch of 11 metanauplii per day from a single cluster (Fig. 7A) and 33 per day for the six clusters combined. Overall hatches for the 17 °C treatment were substantially higher than for the 9 °C treatment and slightly higher than the 13 °C treatment (Fig. 7B). *Argulus canadensis* metanauplius hatch was highest initially in the 17 °C treatment (Fig. 7A), then trended downwards with the steepest slope as hatching progressed in each egg cluster (Fig. 7B). From the 17 °C treatment, 360 *A. canadensis* hatched (86.5 ± 7.1%; 79.4–98.4%), with a maximum hatch of 20 metanauplii per day for a single cluster (Fig. 7A) and 49 per day for the six clusters combined.

Of the remaining unhatched eggs containing embryos following this experiment, all were dead in the 17 °C (11% of total; 7.4–21.1%) and 13 °C (16.2% of total; 6.9–23.1%) treatments, and were either dead or dying in the 9 °C (66.0% of total; 50.6–82.1%) treatment at 270–291 days after they were laid (July 11–Aug. 1).

For experimental analysis, replicate clusters were nested within each temperature, with the 9 °C treatment assigned as the baseline temperature for comparison. The models interaction term is the difference in the slope of the relationship for the 13 °C and 17 °C treatments relative to 9 °C. The interaction between *Argulus* metanauplius *Hatch* and *Time* was statistically significant when comparing the 17 °C and 13 °C to the 9 °C baseline ($t = -7.52$, $P < 0.001$; $t = -3.247$, $P = 0.001$), with higher hatch occurring in both the 17 °C and 13 °C treatments, over time ($\beta = -0.389$; $\beta = -0.168$). None of the random effect variances was attributed to differences among replicates (nested in temperature). No significant difference was observed in overall per cent hatch between the 17 °C (87.3%) and 13 °C (84.0%) treatments ($P = 0.986$). A significant difference was observed in overall per cent hatch for the 9 °C treatment (23.3%) as compared to the 17 °C ($P < 0.001$) and 13 °C ($P < 0.001$) treatments by an ANOVA.

Salinity tolerance of A. canadensis eggs

Increasing water salinity had a negative effect on both development of embryos and their eventual hatch to metanauplii.

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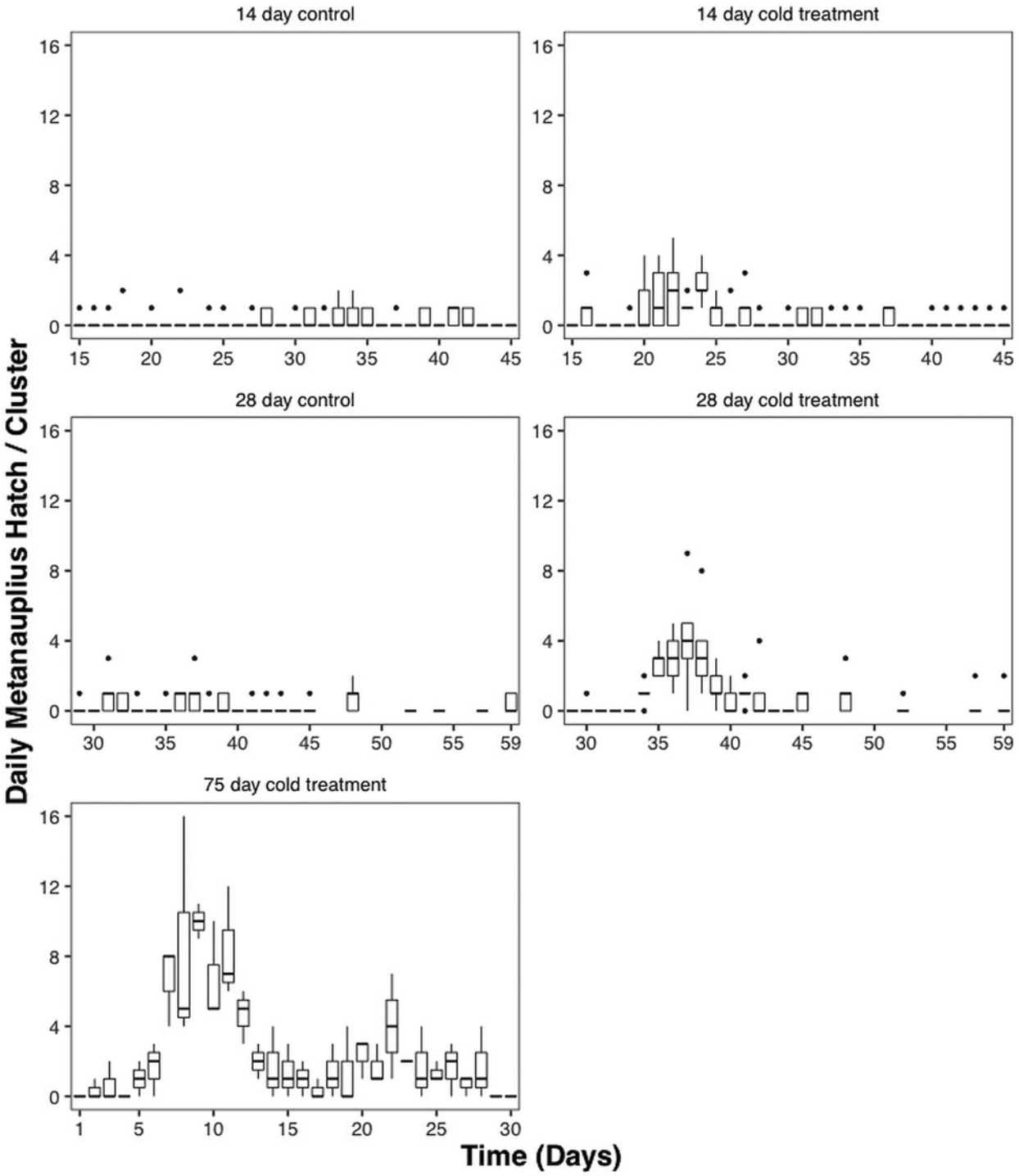

Fig. 6. Box and whisker plots show that hatch of *Argulus canadensis* metanauplii is both induced and synchronized by increasing durations of cold temperature treatment. Egg clusters were paired, with one half kept at ~21 °C while the other half received cold temperature treatment for 14 days or 28 days. Additional egg clusters lacking room temperature controls received cold temperature treatment for 75 days. Horizontal black lines in boxplots represent the median per cent hatch and boxes showing the lower 25th and upper 75th quartiles of the data. Vertical black lines represent differences in quartile distribution to the minimum and maximum bounded data values and outliers are shown as dots.

Embryo contraction from the chorion was initiated day 1 (±2 days) for all eggs that eventually developed to the eyed stage for FW and EST treatments, respectively. Eye formation was initiated on days 18 and 20 and was completed for all eggs that eventually developed to the eyed stage by days 23 and 27 for FW and EST treatments, respectively. Nauplius eye formation was initiated on days 22 and 24 and was completed by days 26 and 40 for FW and EST treatments, respectively. An average of 94.4% (90.4–96.6%; Fig. 8A) and 61.7% (36.9–86.1%; Fig. 8C) of eggs completed nauplius eye formation and became fully developed embryos in FW and EST treatments, respectively. Eggs maintained in SW did not develop into discernable embryos (data not shown).

Following the completion of larval development of *A. canadensis* to eyed embryos, eggs from FW and EST treatments (Fig. 8A and C) were maintained and monitored at room temperature for an additional 102 days. In total, 10.1% (4.8–19.6%) of *A. canadensis* metanauplii hatched from eggs in EST treatments but no hatches were recorded from eggs in FW treatment during this time. However, 53.3% (32.4–77.4%) of embryos

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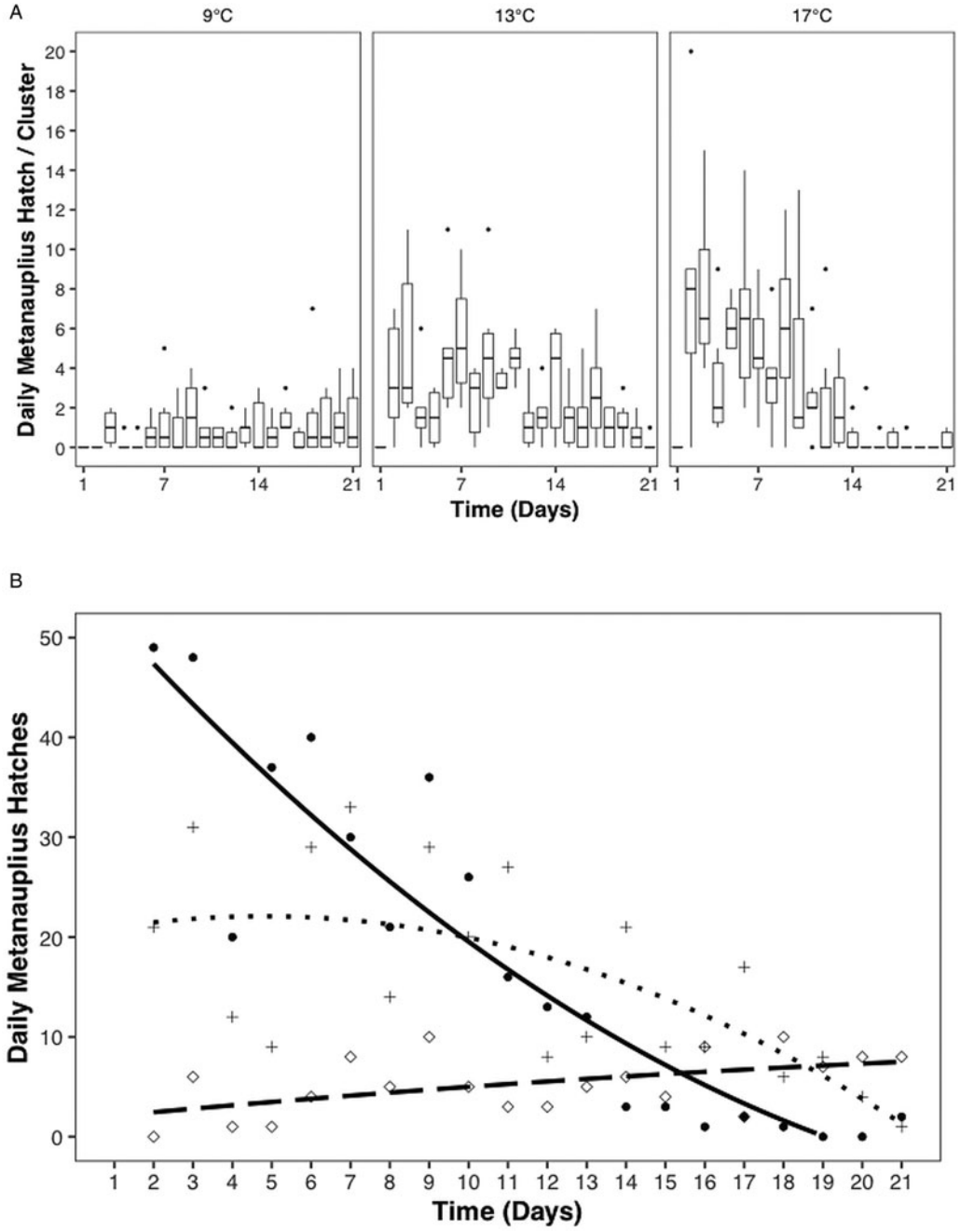

Fig. 7. Box and whisker plots show that higher water temperature serves to both induce higher per cent hatch and to synchronize hatch of *Argulus canadensis* metanauplii to a shorter timeframe (A). Horizontal black lines in boxplots represent the median per cent hatch and boxes showing the lower 25th and upper 75th quartiles of the data. Vertical black lines represent differences in quartile distribution to the minimum and maximum bounded data values and outliers are shown as dots. Scatter plot shows cumulative hatch of *A. canadensis* metanauplii throughout the duration of the experiment for each treatment (B). Each data point represents the total number of hatched metanauplii for a given day and treatment. The open diamonds, crosses and solid squares represent the 9 °C, 13 °C and 17 °C treatments, respectively. The dashed, dotted and solid lines represent the regression plot for each of the 9 °C, 13 °C and 17 °C treatments, respectively.

remaining in EST treatment eggs were dead or dying by day 102 but no such change was observed for embryos from FW treatment eggs. Following maintenance at room temperature, *A. canadensis* eggs were exposed to 33 days of cold temperature treatment at 7 °C. Following re-acclimation to room temperature, 55.8% (50–64.3%) of FW treatment eggs hatched live metanauplii (Fig. 8B), with peak hatch occurring 7 days after cold treatment. The remaining 44.2% (35.7–50%) of eggs in freshwater appeared live (Fig. 8B) whereas all embryos remaining in eggs in EST treatment water were dead or dying (Fig. 8D). No successful hatches of metanauplii were recorded from EST treatment eggs following cold temperature treatment.

Discussion

Our initial attempts to hatch metanauplii from *A. canadensis* eggs were unsuccessful. In spite of embryonic development to pharate embryos by 35 days after laying (21 °C), hatch was highly protracted with only ~3% hatch by 150 days and ~23% by 230 days after laying. This is incompatible with many reports of hatch times for *Argulus* spp. but was compatible with observations of the highly protracted hatch for congeneric species in temperate regions (Pasternak *et al.*, 2000; Hakalahti *et al.*, 2004*a*). Increasing daily photoperiod and rising water temperatures signify annual completion of winter and emergence of spring, thus cueing migrations of anadromous fishes like Atlantic salmon

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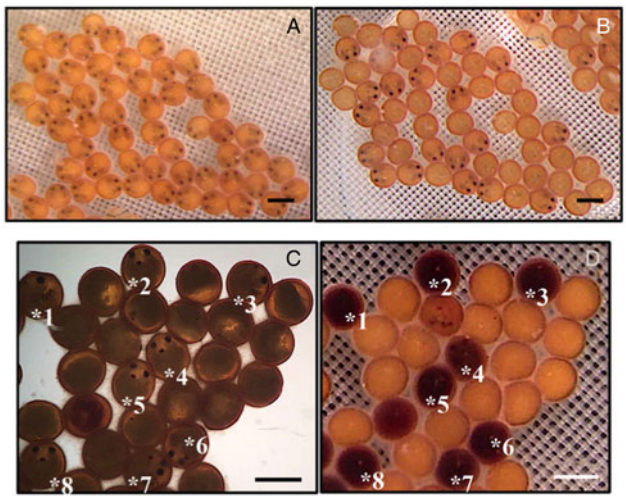

Fig. 8. Images show embryonic development of *Argulus canadensis* in eggs maintained in 0 ppt water (FW) and 17 ppt diluted seawater (EST). A high proportion of embryos developed successfully at 0 ppt (A) whereas development to eyed embryos occurred in a lower proportion of eggs in water at 17 ppt (C). Metanauplius survival at day 213 after egg-laying, following a 33-day cold diapause treatment (7 °C), was ~100% in FW (B). However, all embryos were dead or dying by this same time in eggs maintained in water at 17 ppt (D). Development was greatly restricted in eggs maintained in full-strength seawater (34 ppt) with no evidence of discernable embryo development in any egg (data not shown). Lines in the lower right corner of each image represent scale bars of 0.4 mm.

(Migaud *et al.*, 2010) and hatch of some crustaceans (Gyllstrom and Hansson, 2004). This includes congeneric *Argulus* species following cold temperature dormancy (Hakalahti *et al.*, 2004*a*). Hatch of *A. canadensis* eggs was similarly induced and synchronized following cold-treatment and return to favourable temperatures. High-efficiency hatch of *A. canadensis* occurs only following termination of diapause by extended cold-temperature exposure (⩾75 days). Such evolutionarily-derived reproductive strategies in parasites often determine their potential for spatio-temporal interactions with a host (Wimberly *et al.*, 2008; Loot *et al.*, 2011).

For many parasites, host availability and encounter are considered somewhat random, but essential for survival (Hakalahti *et al.*, 2004*a*, 2004*b*). Newly hatched *Argulus* metanauplii must attach to a host quickly as they survive free swimming for only 2–5 days (Pasternak *et al.*, 2000; Hakalahti *et al.*, 2005; Mikheev *et al.*, 2007). Fecundity can be selected upon to improve odds for host encounter (Poulin, 1995) but synchrony in host-parasite life cycles can promote encounters in space and time for successful infection/infestation (Calero-Torralbo and Valera, 2008). In spite of egg hatch occurring at fixed times following development for numerous species (Stewart *et al.*, 2017), hatch by some parasites relies upon direct (Warkentin, 2011) or indirect cues that reflect host availability (Calero-Torralbo and Valera, 2008; Warkentin, 2011). Egg hatch stimulated by abiotic conditions or biotic cues that favour larval survival is referred to as environmentally-cued hatching (Warkentin, 2011). In absence of appropriate environmental hatching cues, embryos that hatch early could die before suitable hosts are available, whereas those that delay hatch too long can die after exhaustion of energy reserves (Warkentin, 2011; Wei *et al.*, 2014). Mechanisms for maintenance of egg dormancy are crucial to fitness with selection favouring individuals that emerge coincident with resource/host availability.

Quiescence represents physiological conformity to unfavourable environmental conditions and includes developmental processes restricted by cold temperatures. Conversely, diapause occurs when growth, development, or hatch do not occur at times when suitable conditions (i.e. temperature) support these processes. Diapause is a cessation of physiological processes, seemingly in anticipation of upcoming unfavourable conditions (Hand *et al.*, 2016). For some temperate invertebrates, cold temperatures terminate diapause during winter, larval reactivation is positively correlated with increasing spring temperatures, and hatch generally is synchronized with food/host availability (see Calero-Torralbo and Valera, 2008; Hodkinson, 2009; Wei *et al.* 2014). Diapause is controlled by a combination of genetic and environmental factors, often viewed as a fitness advantage to avoid harsh environmental conditions (Gyllstrom and Hansson, 2004). The adaptive value of diapause includes synchrony of reactivation with the return of favourable conditions (Saunders, 2020), whereby abiotic factors indirectly signal food availability. Similar egg diapause is reported for *Argulus* species in temperate regions (Meehean, 1940; Shafir and Van As, 1986; Pasternak *et al.*, 2000; Mikheev *et al.*, 2001; Hakalahti *et al.*, 2004*a*). Overwintering eggs are the only surviving members in these parasite populations (Shimura, 1983; Mikheev *et al.*, 2001; Hakalahti and Valtonen, 2003) and larval hatch occurs once water temperatures warm in spring (Taylor *et al.*, 2009; Mikheev *et al.*, 2015). Our observations are compatible with these temperate *Argulus* species where eggs overwinter for extended periods and show synchronized hatch upon return to favourable temperatures (Mikheev *et al.*, 2001; Hakalahti *et al.*, 2004*a*). Cold temperature diapause termination presumably requires sufficient duration to ensure that only a small fraction of the population emerge if conditions become temporarily 'suitable' due to random environmental events, rather than a true change in season that cues resource availability.

Hatches of *A. canadensis* were best synchronized by cold treatments that mimic overwintering (i.e. ⩾75 days), with hatches synchronized to ~3–4 weeks following acclimation to suitable temperatures. Overwintering congeneric *Argulus* species show most hatches in spring (Taylor *et al.*, 2009; Mikheev *et al.*, 2015) but hatch of some metanauplii extends into summer (Shimura, 1983; Mikheev *et al.*, 2001; Hakalahti and Valtonen, 2003; Hakalahti *et al.*, 2004*a*). It was observed that *A. canadensis* metanauplius hatches were first initiated ⩾9 °C and with hatch induction and synchrony positively correlated with increasing temperature. Cold-temperature treatment terminates diapause and return of favourable temperatures induces hatch of ~86% (72–100%) of eggs. Remarkably similar to *A. canadensis* is egg diapause demonstrated by terrestrial insects that parasitize one life stage of a migratory bird host, exclusively within their breeding range (Calero-Torralbo and Valera, 2008). These arthropods also show relatively short larval development periods, they enter diapause as pharate embryos, cold exposure terminates diapause, increasing temperature induces physiological activation and hatch is synchronized with host availability (see Calero-Torralbo and Valera, 2008; Hodkinson, 2009; Wei *et al.* 2014).

Argulus canadensis metanauplius hatch is induced/synchronized by extended cold treatment and return of favourable temperatures but protracted hatch of ~75% of eggs eventually occurs at constant favourable temperatures. This phenomenon is similarly documented for congeneric species in temperate regions (Pasternak *et al.*, 2000; Hakalahti and Valtonen, 2003; Hakalahti *et al.*, 2004*a*). Most eggs are refractory to hatch for 3–5 months in spite of favourable temperatures (Pasternak *et al.*, 2000; Hakalahti *et al.*, 2004*a*; and, the current study). The linear hatch of >70% of diapausing eggs then occurs over the subsequent 5–6 months even without cold temperature diapause termination (Pasternak *et al.*, 2000; Hakalahti *et al.*, 2004*a*). The requirement for cold temperature to terminate diapause is accordingly time-limited. Diversified diapause length among dormant eggs that promotes extended hatching to ensure the survival of at least some in the cohort is known as bet hedging (Slusarczyk *et al.*, 2019).

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Delayed hatch by some individuals in congeneric *Argulus* populations is bet hedging to increase odds of successful infestation during periods of environmental stochasticity or suboptimal host availability (Pasternak *et al.*, 2000; Mikheev *et al.*, 2001). We provide evidence supporting both inter- and intra-cluster bet hedging. Protracted hatch by *A. canadensis* was most commonly observed in eggs lacking cold temperature termination of diapause but further illustrates bet hedging to increase odds for host encounter in the absence of environmental cues.

Diapause was once considered obligate for gypsy moths (Leonard, 1968) but subsequent studies support substantial plasticity across their range (Keena, 2016). Some diapausing insects are univoltine in cold parts of their range but multivoltine elsewhere (see Hodkinson, 2009). Similar plasticity likely pertains for biological rhythms in host/parasite relationships. The capacity for bet hedging by *Argulus* spp. supports tremendous plasticity following introduction to non-endemic regions (Avenant-Oldewage, 2001) and during an environmental change in endemic regions (Hakalahti *et al.*, 2006). Environmental change and plasticity in biological rhythms can alter relationships such that host/parasite interactions are promoted/inhibited for a given species (Kharouba *et al.*, 2018). Diapause is not observed in temperate *Argulus* species introduced to warmer climes (Shafir and Van As, 1986). Bet hedging by eggs of *A. canadensis* and congeneric species held at constant suitable temperatures support their establishment in warmer environments where suitable hosts exist. Diapause is not displayed by tropical *Argulus* species in their native range but the potential is demonstrated for use diapause in temperate conditions (Shafir and Van As, 1986; Sahoo *et al.*, 2012). Observations from gypsy moths support a genetic basis underlying bet hedging and diapause with variation in regional hatch phenotypes and rapid selection following environmental change (Keena, 2016).

Physiological mechanisms for bet hedging in invertebrates remain unknown but sequential degradation of a differentially allocated, maternally-derived, molecule is proposed (Slusarczyk *et al.*, 2019). This is compatible with protracted but linear hatch by *Argulus* spp. in absence of overwintering (Pasternak *et al.*, 2000; Hakalahti *et al.*, 2004*a*; current study). Gray *et al.* (1991) describe dynamic thermal responses with young insect eggs less sensitive to isolated warming events, presumably to prevent hatch prior to true seasonal warming that promotes survival. Conversely, older, more physiologically advanced eggs, become more responsive to temperature cues over time and are induced to hatch by these same temperatures once spring arrives (Gray *et al.*, 1995; Gray, 2009). Such age-dependent differences are compatible with observations of temperate *Argulus* spp. (Pasternak *et al.*, 2000; Hakalahti *et al.*, 2004*a*; current study).

Empirical investigations on parasites often require large numbers produced over a short time. Gravid parasites are observed on migrating wild salmon predominantly over a 6-week period from mid-June through early August each year (unpublished data). Water temperatures in the St. John River support egg development to hatch-ready embryos by early September. Failure of eggs to hatch under apparently favourable temperatures in the laboratory prompted investigations of diapause. Previous studies of congeneric *Argulus* species show hatch is prevented below 10 °C (Shimura, 1983), others report hatching at 8–10 °C (Pasternak *et al.*, 2000; Mikheev *et al.*, 2001) and we observed 23% egg hatch at 9 °C. We exposed *A. canadensis* eggs containing pharate hatch-ready embryos to cold temperature conditions (<7 °C) to terminate diapause and then manipulated temperatures to both induce and synchronize larval hatch to acquire sufficient parasite numbers in support of empirical investigations. Our maintenance of hatch-ready embryos ⩽7 °C permits storage and extends egg viability to 2.5 years with the induction of metanauplius hatches following return to favourable temperatures. Egg viability might persist longer but was not assessed. Whereas cold storage of eggs at earlier developmental stages could promote longer survival, early gypsy moth embryos subjected to cold temperatures can show impaired viability (Bell, 1996). Diapausing pharate embryos of *Argulus* species likely possess low respiration rates, similar to insects (Gray *et al.*, 1991), to promote such extended survival.

Abiotic factors influence animal life history with physiological adaptations determining spatiotemporal distribution in the environment. Whereas temperatures vary widely in temperate regions, salinity is relatively constant over time but with spatial variability. For host/parasite interactions, additional complexity exists when parasites have restricted environmental tolerance (i.e. salinity) and their host is both migratory and in specific locations for only short periods. Eggs of *A. canadensis* fail to initiate development in full strength seawater but the majority developed successfully in estuarine conditions. Embryos of stenohaline freshwater crustaceans typically die before hatch at 7 ppt (Holdich *et al.*, 1997; Susanto and Charmantier, 2001). In total, 11% of *A. canadensis* eggs hatched live metanauplii at 17 ppt but remaining *A. canadensis* embryos were unable to hatch and/or died, presumably following depletion of resources. A rapid decrease in the osmolality of periembryonic fluid (Susanto and Charmantier, 2001) occurs due to a change in the inner egg membrane of freshwater crustaceans, including *Argulus* sp. (Davis, 1966), immediately prior to hatch (Davis, 1959). A hyposaline freshwater environment promotes water influx and swelling of freshwater crustacean eggs late in embryonic development to induce hatch (Susanto and Charmantier, 2001). Hatch often cannot occur in hyperosmotic solutions due to the absence of an osmotic differential that promotes rapid water influx (Davis, 1959; Katre and Pandian, 1972). Eggs containing hatch-ready embryos at ⩾17 ppt failed to hatch following cold exposure, despite the return to favourable temperatures. Impaired egg development and hatch support *A. canadensis* as predominantly freshwater parasites with Atlantic salmon likely becoming infested upstream of saltwater influence during return to freshwater systems for spawning. To our knowledge, this is the first assessment of the effect of salinity on *Argulus* eggs.

Conclusion

Argulus canadensis eggs develop to pharate hatch-ready embryos and likely enter diapause to forego costs of winter survival and limited host availability. Cold winter temperatures terminate diapause to facilitate ectoparasite hatch with increasing water temperatures, hatches are synchronized to ~3–4 weeks following return of favourable conditions and some individuals within an egg cluster are less reliant on the need for cold to terminate diapause (i.e. bet hedging). Phenological synchrony of the *A. canadensis* life cycle likely promotes spatiotemporal colocalization of metanauplii with their preferred Atlantic salmon definitive host in the St. John River. Similar diapause in temperate congeneric *Argulus* spp. (Pasternak *et al.*, 2000; Mikheev *et al.*, 2001; Hakalahti *et al.*, 2004*a*) and in some temperate insects (see Calero-Torralbo and Valera, 2008; Hodkinson, 2009; Wei *et al.*, 2014) also afford phenological synchrony with their preferred food source/host. Variation in hatch time of individual eggs (inter- and intra-cluster) is likely a mechanism for bet hedging in the event that environmental change impacts phenology. Whereas examples of this egg diapause phenomenon are rare in other crustacean ectoparasites, it is of obvious importance for short-lived parasites such as *Argulus*, and especially so for univoltine species that occur in temperate regions. Observations of phenology for *A. canadensis* might be best viewed as one moment

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in time but with plasticity persisting to accommodate environmental stochasticity.

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Conflict of interest. The authors declare there are no conflicts of interest.

Ethical standards. Not applicable.

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