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Within the past century, the copepod *Eurytemora affinis* have invaded freshwater from saline sources throughout the Northern Hemisphere. Two genetically divergent clades (Atlantic and North Atlantic) of *E. affinis* coexist in the St. Lawrence estuary, but only the Atlantic clade was able to invade into freshwater ecosystem. A common-garden reaction norm experiment was performed on populations from these two clades to determine their differences in survival and development time across three food levels (high, medium and low) in fresh water. The populations were fed with the freshwater alga *Rhodomonas minuta*. The developmental progress and survival of the populations were monitored throughout the experiment. The results indicated that the population from the invasive Atlantic clade is more starvation resistant and has faster development time to adulthood. The differences between the populations might be due to their different osmoregulatory mechanisms and energy usages. These different physiological responses to starvation are likely to have functional consequences for the populations' ability to expand their ranges into novel environments.

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Introduction

More and more species are being introduced into novel environment due to the increase in global trade and shipping traffic (Perrings et al. 2005). These invasive species pose threats to biodiversity, ecosystem integrity, agriculture, fisheries and public health (Lee, 2002). It is estimated that in the United States, the economic cost due to invasive species such as weeds, agricultural pests, zebra mussels, and plant pathogens is $137 billion per year (Pimentel et al. 2000). Therefore, it is important to understand the mechanisms by which organisms invade. Understanding the mechanism of invasion would allow us to predict which populations may invade and their potential range of expansion. We could then apply preventive measures to populations that pose the greatest threats (C. E. Lee, proposal).

Extrinsic factors in the environment impose challenges and opportunities on invading populations. These extrinsic factors include the availability of food and other resources, the presence of predators and competitors, and abiotic factors such as salinity and temperature. The invading populations respond to these extrinsic factors through mechanisms of phenotypic adaptation and evolutionary adaptation. Phenotypic plasticity is the capacity of a genotype to develop different phenotypes under different environmental conditions (Lee, 2002). It could allow an organism to invade novel habitats without the act of selection (Baker, 1965; Agrawal, 2001; Yeh and Price, 2004). On the other hand, evolutionary adaptation is an organism's adaptation to environmental conditions as a result of natural selection. It determines the organism's ability to persist in a novel environment (Reznick et al., 1997; Huey et al., 2000; Reznick and Ghalambor, 2001; Lee, 2002; Donohue et al., 2005; Lee et al., 2007).

The copepod species complex *Eurytemora affinis* are crustaceans that are ubiquitous in estuaries and salt marshes throughout the Northern Hemisphere. It offers an excellent model for studying the mechanisms of aquatic invasions. *E. affinis* populations have invaded freshwater ecosystem from saline sources multiple times independently within the past century (Lee, 1999). The species consists of both invasive and noninvasive clades, and these clades often have overlapping distributions (Lee, 1999; Lee, 2000). This allows us to directly compare the characteristics of successful and unsuccessful invaders. Moreover, the organisms are small and easy to culture in large numbers under laboratory conditions. They have a short reproductive cycle with a generation time of 10-20 days. Powerful genetic tools, such as cDNA libraries, cDNA microarrays and molecular markers are available for genetic and molecular study (C. E. Lee, proposal). The results obtained from the study of *E. affinis* could possibly be extended to other freshwater invaders, because it is likely that similar mechanisms govern the expansion of other freshwater invaders.

The populations that we used in this experiment came from the St. Lawrence drainage system (Fig. 1). Two genetically divergent clades, the Atlantic clade and the North Atlantic clade, have overlapping distributions in this drainage system. However, only populations from the Atlantic clade were able to invade into freshwater reservoirs along the St. Lawrence River and into the Great Lakes following the opening of the St. Lawrence Seaway circa 1959 (Willey, 1923; Anderson and Clayton, 1959; Engel, 1962; Faber et al., 1966; Patalas, 1972; Gannon, 1974; Lee, 1999; Lee, 2000). The damming of reservoirs and construction of canals along the St. Lawrence river led to an increase in shipping and dumping of ballast water into the Great Lakes, which facilitated invasions. However, populations from the North Atlantic clade have not been able to invade freshwater habitats (Lee, 1999).

Food is an important variable that limits the expansion of freshwater invaders. Many freshwater invaders are inefficient osmoregulators compared to native freshwater species (Dietz et al. 1996; Taylor and Harris 1986). They must expend large amounts of energy, and hence require high nutritional intake, in order to maintain osmotic balance in a freshwater habitat. *E. affinis* are more likely to invade reservoirs and altered lake (Lee, 1999) where phytoplankton is more abundant due to cultural eutrophication (Davis 1964; Hasler 1947).

When compared to saline habitats, freshwater habitats have less food available for *E. affinis*. In saline habitats, *E. affini*s can survive on a wide range of diets (e.g. *Ankistrodesmus, Chlamydomonas, Isochrysis, Rhodomonas, Scenedesmus, Spirulina,* or *Tetraselmis sp.*) (C. E. Lee, unpub. data). However, they are picky eaters in freshwater and only feed on the freshwater algae *Rhodomonas minuta* (C. E. Lee and G. W. Gelembiuk, unpub. data). Comparing the R. minuta levels in the Great Lakes to the total algae levels in the St. Lawrence showed that the food available in saline habitats is two orders of magnitude greater than that in freshwater habitats (Makarewicz 1993; Makarewicz et al. 1994; Reuter 1979; Barnard et al. 2003; Lovejoy et al. 1993; Martineau et al. 2004; Vincent et al. 1994).

In this study, we investigated the physiological responses of two *E. affinis* populations, Montmagny and St. Jean Port Joli, to food quantity. The Montmagny population belongs to the invasive Atlantic clade, whereas St. Jean Port Joli belongs to the noninvasive North Atlantic clade. Percentage survival and development time of both populations were examined across a range of food concentrations. Given that *E. affinis* needs more energy to maintain osmotic balance in freshwater than in saltwater environments, and there may be less food available to them in freshwater habitats, we hypothesized that the population from Montmagny (invasive Atlantic clade) would be more starvation resistant than the population from St. Jean Port Joli (noninvasive North Atlantic clade).

Methods and Materials

Population sampling

Populations from the St. Lawrence drainage system, sampled at Montmagny and St. Jean Port Joli (Fig.1), were used in this experiment. These populations were chosen because of their close proximity to each other in their native range (35 km apart) and to the freshwater habitats they invaded (Great Lakes) (Lee, 1999). The populations were collected using a 63 µm plankton net in June 2004 from the surface layer (1-2 m deep) of the St. Lawrence River at Montmagny and St. Jean Port Joli. The populations were reared at a common salinity of 5 PSU in the laboratory. They were maintained with 20mg/L Primaxin (to prevent bacterial infections) at 12^{o}C on a 15L:9D photoperiod. Both cultures were fed with a mixture of freshwater *Rhodomonas minuta* and saline *R. salina* in excess three times weekly. At the time when this experiment started (October 2006), the populations had been cultured in the laboratory for about 28 months (~40 generations).

Experimental design

A common-garden reaction norm experiment was performed on the two populations to determine differences in survival and development time across three different food levels (high, medium and low) at 0 PSU. A reaction norm is the set of phenotypes produced by a genotype exposed to different environmental conditions, and is the result of phenotypic plasticity (Schlichting and Pigliucci 1998).

To perform the experiment, eight full-sibling egg sacs were taken from females from each population and the eggs were split into three food concentration treatments: high, medium (1/5 of high), and low (1/20 of low). From each full-sib clutch, five eggs were placed at each food concentration treatment. Each full-sib clutch arose from a different male/female pairing, and thus represented a distinct genotype. Eggs were placed in 20-mL scintillation vials filled with 15 mL fresh water (0 PSU) from Lake Michigan (Racine, WI). The water was sterilized by filtering through a 0.22 μm mesh. To prevent bacterial growth, vials were given 20 mg/L Primaxin. Vials were kept at 12° on a 15L:9D photoperiod, and were placed randomly in two 3 x 8 racks rotated daily to compensate for differences in the incubator's microenvironment. The copepods were fed freshwater *Rhodomonas minuta* daily. The high food treatment was prepared by centrifuging 50 ml *R. minuta* from algal cultures, removing 30 μl of the spun down algal pellet using a micropipette, and resuspending it in 10 ml fresh water. The medium and low food concentrations were 1/5 and 1/20 of the high food concentration respectively, and were made from dilutions of the high food treatment. Since this experiment was an investigation of the effect of food levels on survival, it was important to feed copepods within each treatment identical amounts of food. Thus, the volume of water in the vials and the amount of food fed were adjusted according to the number of individuals inside each vial. For vials with 4-5 individuals, the water volume was kept at 15 ml and 400 μl of food was fed. For vials with 2-3 individuals, the water volume was 9 ml and 240 μl of food was fed. For vials with only 1 individual, the water was reduced to 3 ml and 80 μl of food was fed. Within each food treatment, this ensured the approximate consistency of: (1) the amount of algae per copepod fed to each vial, and (2) the algal cell concentration in each vial after feeding.

Experimental Observations

Vials were visually observed every two days. The number of nauplii, juveniles and adults in each vial were counted, and sexes of newly developed adults were recorded. Individuals were classified as juveniles when they developed into the copepodid I stage. Individuals were classified as adults (C6 stage) when males developed geniculate right antennules, and when females developed wing-like processes on the posterior end of their body. For both populations, the average development time (in days) and average percentage survival to metamorphosis and adulthood were measured across the three food treatments.

Cell Count

The number of *R. minuta* cells/mL in the high food treatment was counted eight times during the course of the experiment using a hemocytometer. The cells were counted using methods suggested by Hansen (2000). At each of the eight counting events, three samples were counted, with the hemacytometer washed with ethanol in between countings. The average of the eight counting measurements was used as an approximation of the algal cell concentration in the high food treatment. The cell concentration in the medium and low food treatment were 1/5 and 1/20 of the high food treatment, respectively. By assuming the cell shape to be a prolate spheroid, the cell volume of *R. minuta* was calculatd by averaging the measurements of twenty cells. Biomass of *R. minuta* for each treatment level was then estimated by converting biovolume (mm^3/l) to biomass (mg/m^3) assuming a specific gravity of 1.0 (Willén 1959).

Haplotype sequencing

Haplotype sequencing using mitochondrial cytochrome oxidase subunit I (COI, 652 base pairs) was performed to verify the identity of the populations used in this experiment. For each population, the COI sequences of the maternal parents of five clutches were obtained using the methods outlined in Lee (2000).

Statistical Analysis

The ordinal probit model was employed to analyze differences in survival (Albert and Chib 2001) between the populations. This model is appropriate because of the discrete nature of survival data. Survival data are discrete in that the individuals are either dead or alive and there are no intermediates. An observation was coded as 0 (if not hatched), 1 (if hatched), 2 (if survived to metamorphosis) and 3 (if survived to adulthood). The detailed statistics were performed according to previous methods mentioned in Lee et. al. (2007).

T-test was used to analyze differences in development time between the two populations. The t-test is suitable for continuous data such as development time. The development time to metamorphosis and adulthood was being compared between the two populations. Separate t-tests were conducted at each food level. The difference is significant when the p-value < 0.05. The t-tests were performed using the "t.test function" in the statistical software "R".

Results

Percentage survival

The two populations showed similar survival rates in high food treatment, but showed significant differences in survival to metamorphosis (under low food conditions) and to adulthood (under medium and low food conditions), with the Montmagny population (invasive Atlantic Clade) being more resistant to starvation (Fig. 2, 3). Under the high food treatment, the Montmagny population had a slightly higher survival percentage to both metamorphosis and adulthood than the St. Jean Port Joli population (noninvasive North Atlantic Clade). However, the difference is not significant (ordinal probit model; $P > 0.05$). Under medium food treatment, the Montmagny population had significant higher survival to adulthood than the population from St. Jean Port Joli (*T* = 2.13, df = 47, *P* = 0.0384). Under low food treatment, the Montmagny population had significantly higher survival to both adulthood and metamorphosis than the population from St. Jean Port Joli (ordinal probit model; survival to metamorphosis: *T* = 2.46, df = 47, *P* = 0.0176; to adulthood: *T* = 2.18, df = 47, *P* = 0.0345). No individuals from the St. Jean Port Joli population were able to survive to adulthood in the low food treatment (Fig. 3).

Development time

The two populations had similar development times to metamorphosis, but the Montmagny population had a significantly faster development time to adulthood. The populations did not show significant differences in development time to metamorphosis across all three food treatments (Fig. 4) (T-test; high food*:* *T* = 0.1166, *P* = 0.9104; medium food: *T* = -0.6818, *P* = 0.5166; low food: T = 0.8177, P = 0.4402). Montmagny had a significantly faster development time to adulthood under both the high and medium food treatment (Fig. 5) (T-test; high food: T = -4.6692 P = 0.001300; medium food: T = -5.1452, P = 0.000555). No individuals from St. Jean Port Joli were able to survive to adulthood under the low food treatment, so no comparison was possible.

Cell concentration

The cell concentration in the high food treatment was found to be approximately 14,000 cells/ml, and the corresponding biomass was calculated to be approximately 0.410g/m^3. The medium food treatment was a 1/5 dilution of the high food treatment, and its cell concentration and biomass were calculated to be 2800 cells/ml and 0.082g/m^3 respectively. The low food treatment was a 1/20 dilution of the high food treatment, and thus its cell concentration and biomass were 700 cells/ml and 0.021g/m^3 respectively.

Discussion

The food levels used in our experiment are relevant to the levels of preferred food sources in the wild. *R. minuta* is the most abundant phytoplankton species in the Great Lakes (Makarewicz 1993; Munawar and Munawar 1975; Reuter 1979), and is probably the primary food source for *E. affinis* in the wild. Other algae in the Great Lakes (e.g. *Isochrysis* and *Scenedesmus sp.*) are not able to support the growth and reproduction of *E. affinis* in the laboratory (C. E. Lee and G. W. Gelembiuk, unpub. data). *R. minuta* is needed for the growth and reproduction of freshwater populations of *E. affinis* in the laboratory, probably because it contains high levels of polyunsaturated fatty acids (Ahlgren et al. 1992; Park et al. 2002). Since the mid 1970s, the concentration of *R. minuta* in the Great Lakes has been between the medium (2800 cell/ml; 0.082 g/m^3) and low (700 cells/ml; 0.021 g/m^3) food levels in this experiment, or lower (Makarewicz 1993; Makarewicz et al. 1994; Reuter 1979). Before the passage of the measures to reduce phosphorus loading in the early 1970s, the *R. minuta* levels in the Great Lakes were higher. However, the level only approached the high food treatment (14000 cells/ml; 0.410 g/m^3) in Lake Erie, the most eutrophic lake among the Great Lakes (Munawar and Munawar 1975).

In contrast to freshwater habitats, saline habitats have a wider variety of food sources (e.g. *Ankistrodesmus, Chlamydomonas, Isochrysis, Rhodomonas, Scenedesmus, Spirulina,* or *Tetraselmis sp.*) available to *E. affinis* (C. E. Lee, unpub. data). The total phytoplankton level is thus a better measure of the food quantity available to *E. affinis* in saline environments. The phytoplankton biomass near the regions where the populations of this experiment were collected, approximated (Moal et al. 1987; Montagnes et al. 1994) from chlorophyll a measurements (Barnard et al. 2003; Lovejoy et al. 1993; Martineau et al. 2004; Vincent et al. 1994), ranges from 0.3 g/m^3 to over 6.0 g/m^3. These values are similar to or exceed food levels in the high food treatment, and are two orders of magnitude greater than the *R. minuta* levels in the Great Lakes. Therefore, the food available to *E. affinis* in freshwater habitats is less than that in saline habitats, and food might be an important extrinsic factor that limits the range expansion of some populations into fresh water.

Given the limitation of food in freshwater environment, the ability to resist starvation might be an important asset to successful invasion. This experiment demonstrated that the population from the invasive Atlantic clade (Montmagny) is more resistant to starvation than the population from the noninvasive North Atlantic clade (St. Jean Port Joli). The differences in starvation resistance may be due to the populations' differences in (1) mechanisms of osmoregulation and (2) energy usages.

Starvation may lead to problems in osmoregulation. For some populations, dietary salt intake is an important ion source for maintaining the osmotic balance in freshwater (Aladin and Potts 1995). Starvation could lead to poor development of osmoregulatory structures. For example, selenium deficiency leads to cuticular deterioration in *Daphnia pulex* and *D. magna* (Keating and Dagbusan 1984). Osmoregulatory mechanisms in copepods are not well known, but St. Jean Port Joli might have a relatively inefficient osmoregulatory system. This may lead to their lower survival in fresh water when food is limited. Osmoregulation is metabolically costly in crustaceans (Anger 2003; Pequeux 1995) and poor osmoregulatory mechanisms would require high energy expenditure to maintaining osmotic balance. The more energy a copepod uses for osmoregulation, the less energy will be available for growth and reproduction (Dybdahl 1995). The effect is particularly detrimental in freshwater environment where the food is scarce. Moreover, populations from the noninvasive North Atlantic clade are more active than populations from the invasive Atlantic clade. Swimming is a metabolically expensive activity (Morris et al. 1985; Svetlichny and Hubareva 2005) and might not be favorable in freshwater habitats where food is limited.

The noninvasive North Atlantic clade had a slower development time to adulthood than the invasive Atlantic clade. This may be due to tradeoffs between osmotic tolerance and development rate. Since osmoregulation is energetically expensive (Anger 2003; Pequeux 1995), a large proportion of energetic resources might be allocated to maintaining osmotic balance in freshwater. With less energy available for development, there could be retardation in development time (Hart, 1998). Both populations had similar development time to metamorphosis. The accelerated development of the Atlantic clade at later life history stages might be a means to achieve reproductive maturity faster in order to escape the high parasite loads in fresh water (Lee et. al., 2003).

Conclusions and future directions

The population from Montmagny (invasive Atlantic clade) showed greater starvation resistance and faster development time to adulthood than St. Jean Port Joli (noninvasive North Atlantic clade) in freshwater environment where food is scarce. This might be due to inefficient osmoregulatory mechanisms or higher energy expenditures of the population from St. Jean Port Joli. To verify that these results generalize to the clade level, it would be useful to repeat the study on other populations from these two clades.

In the future, this study could also be extended to other clades within *E. affinis* and to other freshwater invading species. It is possible that other freshwater invaders also use similar mechanisms to invade, and it would be interesting to see if they also show similar responses to starvation. To fully understand the mechanisms of freshwater invasion, it would also be useful to examine the effects of other conditions, such as temperature and salinity, on the survival and development time of freshwater invaders.

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Figures and Graphs

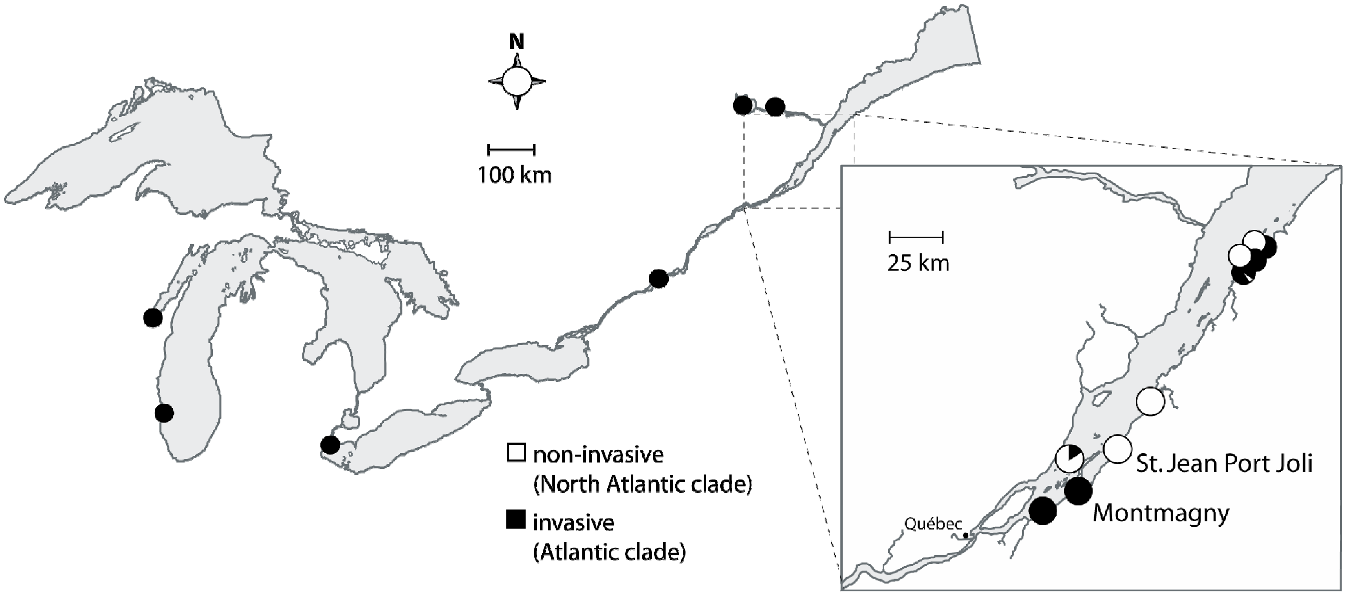

Fig. 1: A map showing the geographical distribution of the Atlantic and North Atlantic clades in the St. Lawrence River system and the Great Lakes. Patterned dots indicate locations of populations of *E. affinis* (black = invasive Atlantic clade, white = non-invasive N. Atlantic clade). Proportions of each circle represent proportion of animals from each clade. The inset is an amplification of the St. Lawrence estuarine transition zone and downstream regions of the lower estuary.

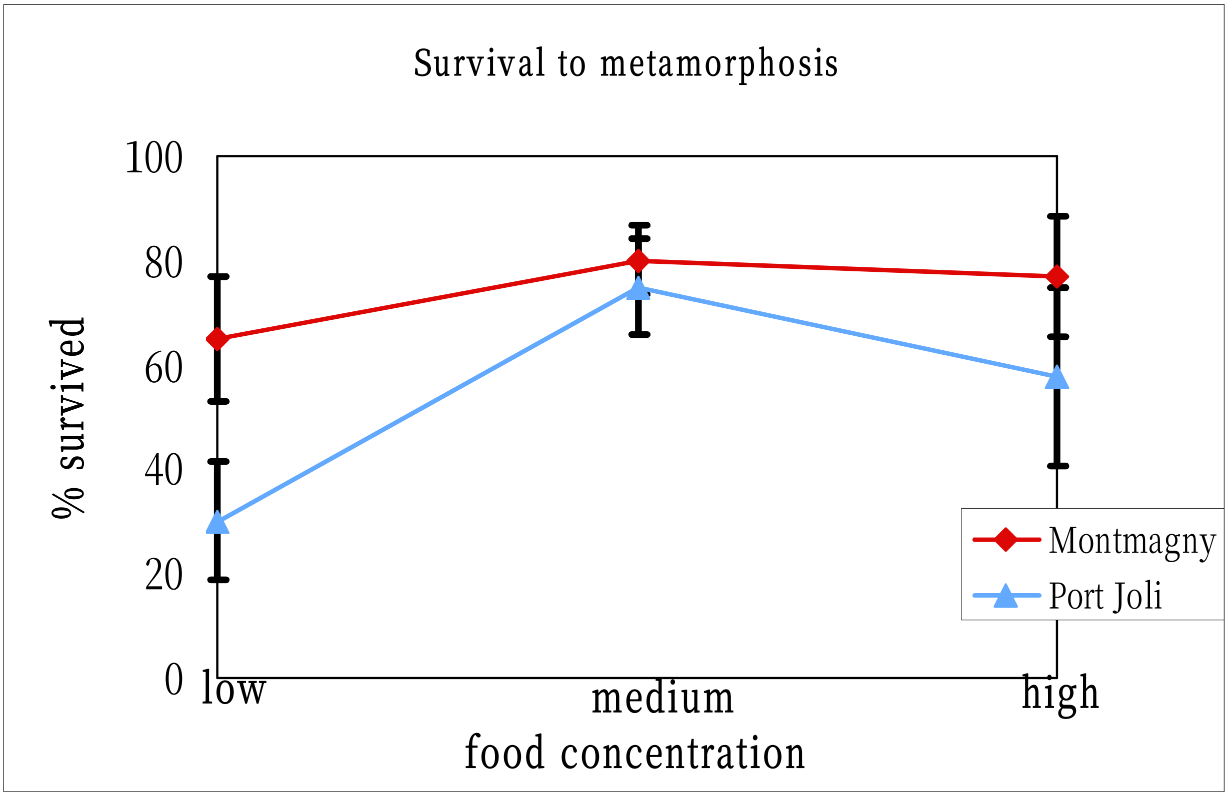

Fig. 2: A graph showing the mean percentage survival ± SE of Montmagny (Atlantic clade) and St. Jean Port Joli (North Atlantic clade) to metamorphosis under the three food treatments: high (14000 cells/ml; 0.410 g/m^3), medium (2800 cell/ml; 0.082 g/m^3) and low (700 cells/ml; 0.021 g/m^3).

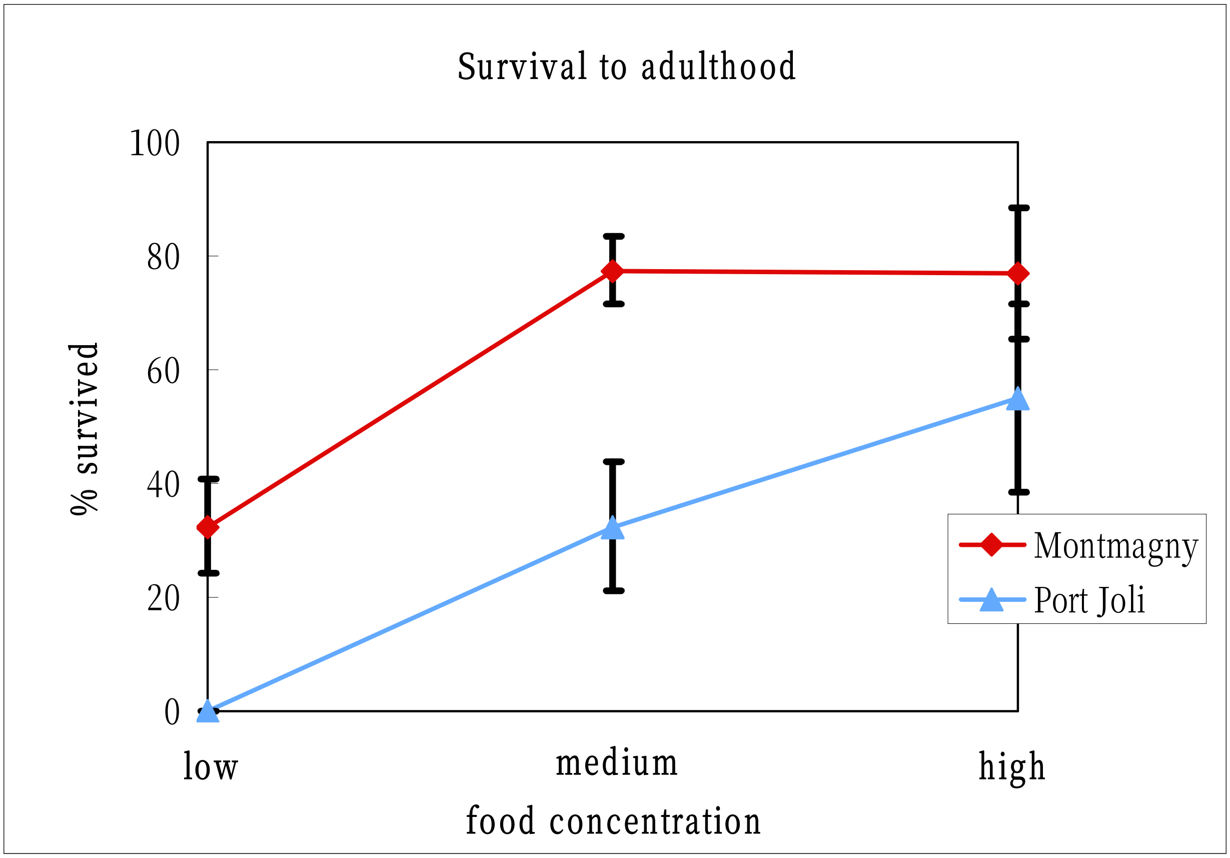

Fig. 3: A graph showing the mean percentage survival ± SE of Montmagny (Atlantic clade) and St. Jean Port Joli (North Atlantic clade) to adulthood under the three food treatments: high (14000 cells/ml; 0.410 g/m^3), medium (2800 cell/ml; 0.082 g/m^3) and low (700 cells/ml; 0.021 g/m^3).

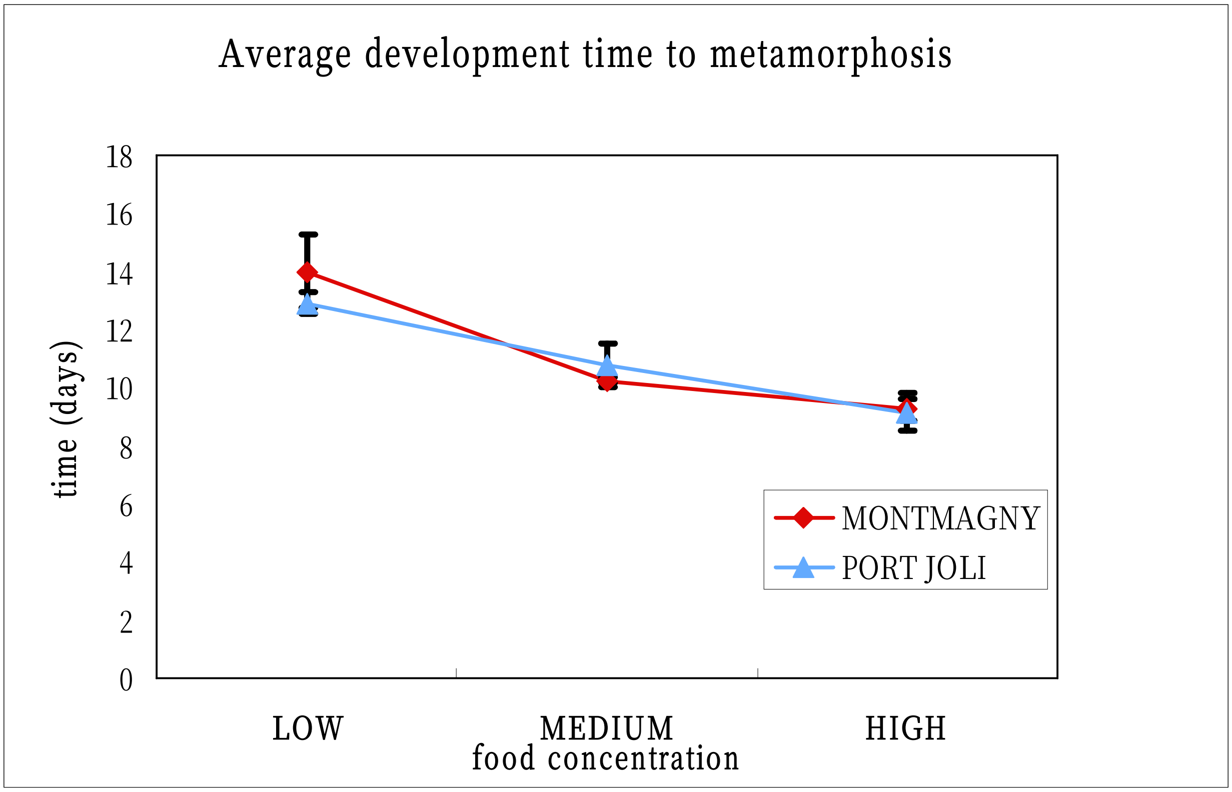

Fig. 4: A graph showing the mean development time ± SE of Montmagny (Atlantic clade) and St. Jean Port Joli (North Atlantic clade) to metamorphosis under the three food treatments: high (14000 cells/ml; 0.410 g/m^3), medium (2800 cell/ml; 0.082 g/m^3) and low (700 cells/ml; 0.021 g/m^3).

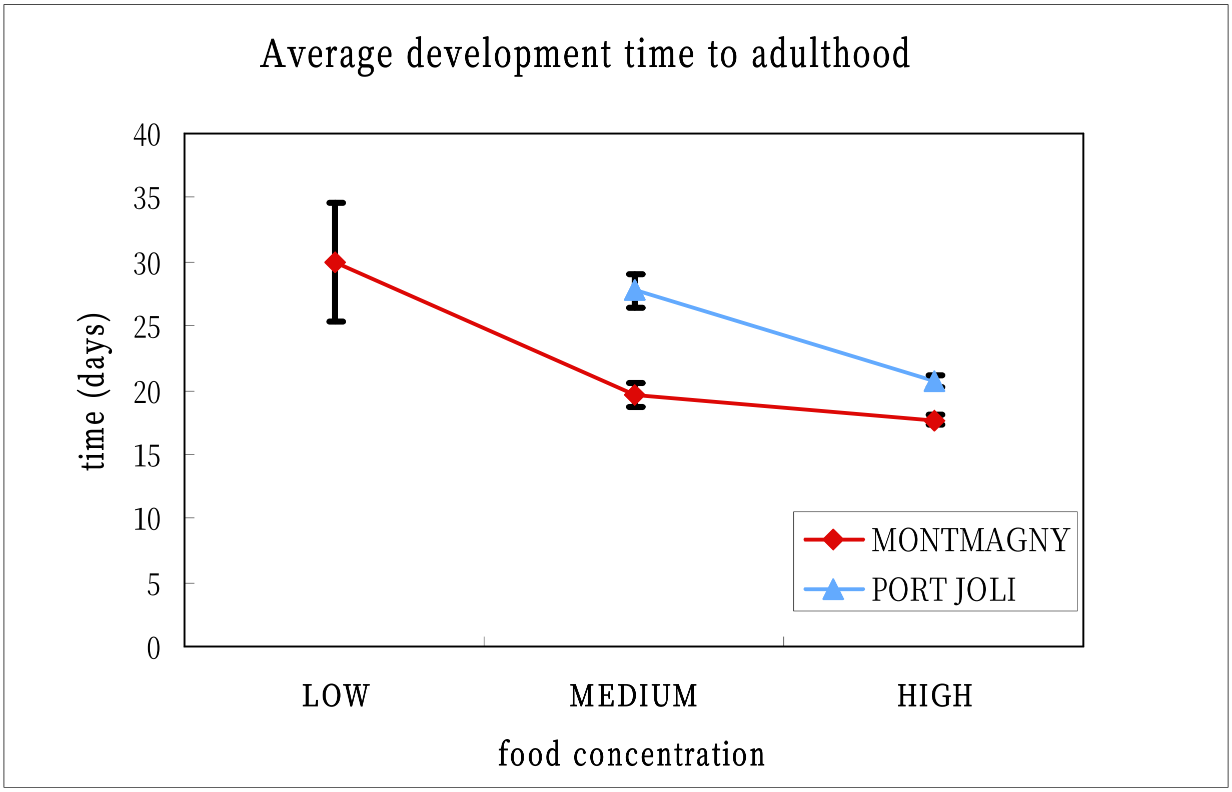

Fig. 5: A graph showing the mean development time ± SE of Montmagny (Atlantic clade) and St. Jean Port Joli (North Atlantic clade) to adulthood under the three food treatments: high (14000 cells/ml; 0.410 g/m^3), medium (2800 cell/ml; 0.082 g/m^3) and low (700 cells/ml; 0.021 g/m^3).

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