Voltinism-associated differences in winter survival across latitudes: integrating growth, physiology and food intake

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Abstract

Species that span large latitudinal gradients face strong differences in voltinism and in winter conditions within their range. Latitudinal gradients in winter survival and especially their underlying mechanisms and association with voltinism patterns are poorly studied. We tested in the damselfly *Enallagma cyathigerum* whether high-latitude populations were better in dealing with the longer winters compared to central- and low-latitude populations and whether this was associated with changes in voltinism. We thereby evaluated whether higher initial levels and/or lower reductions during winter of energy storage (measured as fat content) and investment in immune function (measured as the activity of phenoloxidase), and/or stronger compensatory responses in food intake contributed to the higher winter survival in high-latitude populations. To this end, we simulated a long high-latitude winter at 4°C under manipulated food conditions. Across food levels, winter survival was highest in Swedish larvae, intermediate in Belgian larvae and lowest in Spanish larvae, indicating latitude-specific thermal adaptation that could be partly linked to differences in voltinism. The semivoltine Swedish larvae were growing slower before winter and as a result accumulated the highest fat content and PO activity when the winter started compared to the univoltine, faster growing Belgian and Spanish larvae. Fat content and PO activity declined during the winter, yet equally across latitudes, and were not buffered by compensatory food intake. Our data identified possible underlying physiological mechanisms of winter survival and support the hypothesis that widespread latitude-associated voltinism shifts may be a selective factor contributing to latitudinal shifts in winter survival.

Keywords: Cold tolerance, overwintering strategies, range limits, thermal evolution, voltinism shifts

Introduction

Species that span large latitudinal gradients typically show strong differences in life history and are adapted to the latitude-specific thermal conditions (Stoks et al. 2015). Given that latitudinal gradients in winter temperatures are steeper than those in summer temperatures (Bradshaw and Holzapfel 2006), latitudinal gradients in winter cold tolerance are much more prominent than those in summer heat tolerance (Addo-Bediako et al. 2000; Sunday et al. 2014). In line with this, several intraspecific studies identified latitudinal gradients in the capacity to deal with winter conditions (Schultz et al. 1998; Fullerton et al. 2000; Wilson and Cooke 2004; Krehenwinkel and Tautz 2013; Rooke and Fox 2014; Williams et al 2015).

Despite the importance of winter survival to be able to maintain populations at higher latitudes, we have little knowledge on the physiological mechanisms underlying latitudinal variation in winter survival. The current insights are limited to a few studies (Schultz et al. 1998; Fullerton et al. 2000; Finstad et al. 2010; Rooke and Fox 2014; Williams et al 2015). Moreover, only Schultz et al. (1998) and Williams et al (2015) were able to identify genetic rather than plastic differences in physiological mechanisms among populations.

While in terrestrial systems the capacity to survive subzero winter temperatures is crucial, in aquatic systems buffered by ice cover winter survival is determined by the capacity to endure above freezing winter temperatures (Danks 2007; De Block et al. 2007). Aquatic species inhabiting ponds and lakes therefore experience mainly a latitudinal gradient in the duration of winter temperatures rather than in the magnitude of minimum winter temperatures. When dealing with long-term above freezing winter temperatures, both energy storage (Sinclair 2015) and immune function (Krams et al. 2011) have been determined as key determinants of survival. This is true despite the fact that many species enter a state of metabolic suppression (diapause, Hahn and Denlinger 2011) during winter. Indeed, only insects with extreme metabolic suppression and deep diapause may have relatively little energy use over the duration of winter (Sinclair 2015). Given low food intake rates during winter, overwintering animals fuel energy demands mainly by using their lipid stores (Sinclair 2015). Therefore, animals with higher fat levels at the start of winter and/or lower fat depletion rates during winter can better endure winter conditions (Sinclair 2015). In addition, cold winter temperatures can cause immunosuppression which may result in increased susceptibility to pathogens and an increased winter mortality (Lepak and Kraft 2008; Krams et al. 2011; Ferguson et al. 2016). This is especially true as investment in immune function is energetically costly (De Block and Stoks 2008a). While differences in energy storage and/or depletion have been associated with latitudinal patterns in winter survival in fish species (Schultz et al. 1998; Fullerton et al. 2000; Finstad et al. 2010; Rooke and Fox 2014), no studies investigated this with regard to the investment in immune function. This is especially relevant as energy storage and investment in immune function can be traded off against each other (Houston et al. 2007; Franke and Fischer 2013). The importance of both physiological mechanisms in shaping (latitudinal patterns in) winter survival can critically depend on food intake rates both before and during winter (e.g., Finstad et al. 2010).

Many insects change voltinism (the number of generations per year) across latitudinal gradients (Chown and Gaston 1999; Gaston 2003) which may shape their ability to deal with winter conditions. While at transition zones between voltinism types a mixture of voltinism may occur in populations, across large latitudinal gradients there is a clear gradient in voltinism with zones where only one voltinism type dominates (Nilsson-Örtman et al. 2012, Zeuss et al. 2017). Latitude-associated changes in voltinism are driving changes in many life history traits, including growth and development (Blanckenhorn and Demont 2004). Typically, at low latitudes species experience a longer thermally favourable larval growth season, hence have surplus time available for growth and development prior to emergence. In response to this, many insect species add extra generations per year at low latitudes. The somewhat counterintuitive result of this is that each generation ends up with less time to complete its life cycle, which selects for a faster larval life history to deal with the increased time constraints (e.g., Ragland and Kingsolver 2007; Shama et al. 2011). Given that faster growth rates are traded off against functions such as energy storage and investment in immune function (Dmitriew 2011), we hypothesize that the voltinism gradient may contribute in shaping a gradient in the ability to deal with winter conditions. Under time constraints animals may compensate the higher energy needs by increasing food intake (Abrams et al. 1996). To get a better picture how winter survival changes across latitudes we therefore need integrated studies considering changes in voltinism that quantify energy storage, immune function and food intake.

We tested for latitude-associated changes in winter survival under a simulated high-latitude winter in a freshwater insect that shows changes in voltinism across a north-south gradient in Europe. To better understand any patterns in winter survival we also quantified growth rates, two key physiological mechanisms underlying winter survival (energy storage and immune function), and food intake. We measured the activity of the enzyme phenoloxidase (PO), a key immune enzyme in arthropods (González-Santoyo and Córdoba-Aguilar 2012). As study taxon we chose damselflies, which are common inhabitants of ponds and overwinter as aquatic larvae under the ice (Corbet 1999; De Block et al. 2007). The study species *Enallagma cyathigerum* (Odonata, Coenagrionidae) spans a large latitudinal gradient in Europe occurring from southern Spain to northern Sweden (Boudot and Kalkman 2015), along which it shifts voltinism (Corbet et al. 2006). While some variation in voltinism may occur at a given latitude, in general the species is univoltine (has one generation per year) at the low and central latitudes and is semivoltine (has one generation every two years) at the high latitude (Johansson 2003; Corbet et al. 2006); this is associated with changes in larval growth and development (De Block et al. 2008). We expected a lower growth rate and a higher winter survival in the semi-voltine high-latitude populations and tested whether this was associated with a higher build-up before winter and/or lower overwinter reductions in fat storage and phenoloxidase activity, potentially mediated by changes in food intake.

Materials and methods

Study populations and rearing experiment

We studied populations of the damselfly *E. cyathigerum* from three latitudes spanning the species' latitudinal range in Europe (Boudot and Kalkman 2015). The north-south distance between the high-latitude (central Sweden) and the central-latitude (Belgium) populations is ca. 1700 km, and between the central-latitude and the low-latitude (northern Spain) ca. 1400 km. Two randomly chosen populations from shallow lakes were sampled at each latitude: Bjånnsjön (+63°47'27"N, +20°01'05"E) and Stöcksjön (+63°46'29"N, +20°11'32"E) for central Sweden, Kalmthoutse heide (+51°24'19"N, +4°26'26"E) and Ruiterskuilen (+51°02'54"N, +5°32'36"E) for Belgium, and Trema (+42°41'15"N, +8°41'31"W) and Doniños (+43°29'26"N, +8°18'55"W) for northern Spain.

End of June – early July 2011, eight mated females were collected per population. Females were placed individually in small plastic vials with wet filter paper as substrate for oviposition. In each country, filter paper with eggs was placed in sealed plastic bags containing water to keep the eggs wet. Plastic bags were packed in brown envelopes and shipped with regular mail to Belgium. Belgian eggs were handled in the same way but remained in Belgium. To equalize handling of the eggs across countries envelopes were not opened before all packages had arrived, which took 5 days. Other studies using similar transportation methods for damselfly eggs found that these did not result in observable effects on life history (e.g. Sniegula et al. 2012). Given that all eggs were treated similarly this transportation is unlikely to have introduced any bias in our results.

Egg hatching was synchronous and only eggs hatched within a period of five days were used in the experiment. After hatching, larvae were placed individually in 200 mL plastic cups filled with 100 mL of aged tap water. Cups were placed in incubators at 20°C and a photoperiod of 16:8 L:D with a light intensity of ca. 680 lux. This temperature matches mean July water temperatures in central Sweden in ponds inhabited by the study species (F. Johansson, unpublished data); the photoperiod corresponds to the mean photoperiod of July and August in central Sweden. Larvae were fed *Artemia* nauplii *ad libitum* twice per day, five days per week. When larvae were one month old, they were randomly divided between two food treatments: larvae at high food received six days per week one portion of *Artemia* nauplii while larvae at low food received this portion only three days per week. One portion consisted of 1347 ± 102 nauplii (mean ± SE, n = 15 food portions). Throughout the experiment larvae were kept individually (damselfly larvae are cannibalistic, De Block and Stoks 2004).

Experimental design

We exposed *E. cyathigerum* larvae from the three latitudes to simulated mean high-latitude (central Sweden) fall and winter conditions. Therefore, we gradually adjusted the temperature and photoperiod to mimic the natural progress of water temperatures and photoperiods in the natural Swedish populations (F. Johansson, unpublished data; for details see Online Resource 1). Starting from 20°C the water temperatures were lowered to 15°C (26 August), 10°C (24 September) and finally to 4°C (22 October). Damselfly larvae typically stop growing (enter diapause) at temperatures in the range 8-12°C (Suhling et al. 2015); in our study larvae entered diapause below 10°C (see results). A water temperature of 4°C was retained during the rest of the winter period (6 months) until 26 April, simulating the situation where larvae are under the ice (De Block et al. 2007). We gradually adjusted the photoperiod from 18:6 L:D to 11:13 L:D during the pre-winter period (August till October), and during the winter period (October to April) we first reduced the photoperiod to 4.5:19.5 L:D and later on we led it increase again to 13.5:10.5 L:D (see Online Resource 1). During the winter period the mean light intensity in the incubators was lowered to ca. 150 lux because during the winter period lakes in Sweden are covered with snow and ice. The frequency of feeding in the two food treatments was retained during the winter period (22 October till 26 April) but daily food portions of *Artemia* nauplii were halved because nutritional needs are lower at 4°C (De Block et al. 2007). We reared 150 larvae per combination of latitude and food treatment (total of 900 larvae).

Response variables

We quantified pre-winter survival (from hatching until the start of winter at 22 October) and winter survival (from 22 October to the end of winter, 26 April). To obtain estimates of growth rate per temperature period, we successively measured larval head width of a subset of larvae at regular moments in time (see e.g. Nilsson-örtman et al. 2012), namely just before the water temperatures were lowered at 26 August, 24 September and 22 October, and when the larvae were 4 weeks in their winter period at 19 November. The final measurement of larval head width took place on 26 April, when the winter ended. Head widths were taken on 210 larvae (15 to 40 larvae per treatment combination). Given that hatchlings were too small to handle, we took the initial head width at hatching on a separate sample of 10 randomly chosen larvae per population that were stored on ethanol. Head width was quantified from images taken using a stereomicroscope connected to the program Streampix v3 (NorPix, Inc., Montreal, Quebec, Canada). Afterwards, images were analysed by Image Pro Plus 5.0 (Photometrics UK, Marlow, UK). Individual growth rates were estimated for four periods: the 20°C growth period (from hatching till 26 August), the 15°C growth period (from 26 August till 24 September), the 10°C growth period (from 24 September till 22 October) and the winter 4°C growth period (from 22 October till 26 April). Growth rates during each period were calculated as [ln(head width at time t_2) – ln(head width at time t_1)] / (t_2-t_1). Growth rates were estimated based on individual increases in growth, except for the 20°C growth period where we used the mean head width of the ten hatchlings of a given population as the initial head width of larvae for that population.

Food intake was measured on the same 210 larvae we used to measure head width, at each of the following experimental temperatures: 20°C (just before the water temperature was lowered on 26 August), 15°C (24 September), 10°C (22 October) and 4°C (after a winter period of 4 weeks on 19 November). Larvae were not fed 24 hours before each foraging trial to standardise the hunger level. The measurement took place in 200 mL plastic cups filled with 30 mL dechlorinated tap water, 20 *Artemia* nauplii and one damselfly larva. After the trial, the damselfly larvae were removed and the remaining *Artemia* nauplii were counted. Based on pre-trials per period, trial duration was adjusted to avoid depletion of the nauplii. Trials were ended after 60 minutes at 20°C and 4°C, and after 30 minutes at 15°C and 10°C. Food intake was expressed as the number of *Artemia* nauplii eaten per minute divided by head width (to correct for larval size).

To document changes in physiology over the winter period, a subset of larvae per treatment combination were stored at -80°C at the start (22 October) and at the end (26 April) of the winter period. The sample size was 24 larvae per treatment combination at the start and between 14 and 30 (depending on mortality) at the end of the winter period; exact sample sizes are shown on the figures. Before freezing, larvae were gently blotted dry with tissue paper to ensure that no water remained on the larvae and their wet mass was taken to the nearest 0.01 mg using an electronic balance (Mettler Toledo® AB135-S). This gives a reliable wet mass estimate that strongly correlates with dry mass (Stoks et al. 2005).

Before the assays, the body supernatant was obtained by homogenizing the larvae in a body-mass adjusted volume (the final mass x 10 µL) of phosphate buffer saline (50mM, pH

7.4). The mixture was centrifuged for 8 min (10,000 g and 4°C) and the resulting supernatant was used to quantify fat content and the activity of phenoloxidase (PO). We spectrophotometrically quantified two important condition-related traits in damselflies (Rolff and Joop 2002; Stoks and Córdoba-Aguilar 2012): the total fat content (the most important long-term storage molecule) and the activity of the enzyme phenoloxidase (PO, a key component of an insect's immune system: González-Santoyo and Córdoba-Aguilar 2012). Fat content was quantified based on a modified protocol of Marsh and Weinstein (1966), which was optimized for damselfly larvae. We added 8 µL of the supernatant and 56 µL sulphuric acid (98%) to glass tubes. The tubes were heated for 20 minutes at 150°C and after cooling we added 64 µL of milliQ water. We pipetted 30 µL of the samples in a well of a 384-well microtiter plate and measured absorbance in duplicate at 340 nm using an Infinite M2000 (TECAN) plate reader. The average of the duplicate readings per sample was used for statistical analyses. To convert absorbances in fat contents we used a standard curve of glyceryl tripalmitate (Postle & Goodland 1978).

The activity of phenoloxidase (PO) was measured using a modified version of the protocol of Stoks et al. (2006a). We pipetted 75 µL PBS-buffer (Phosphoric Buffered Saline), 5 µL chymotrypsin (5 mg mL^{-1} milliQ water) and 20 µL supernatant in wells of a 384-well microtiter plate. A 5 minute incubation period at room temperature allowed the inactive form proPO to get activated to PO. Thereafter, we added 100 µL L-Dopa (1.97 mg mL^{-1} PBS). The reaction, where PO catalyses the transition from L-Dopa to dopachrome (Sugumaran 2002), proceeded for 30 minutes at 30°C. We photometrically measured the absorbance of dopachrome at 490 nm every 20 seconds during 30 minutes at 30°C. PO activity was quantified as the slope of the linear part of the reaction curve (time interval 1000-2000s). The average of the duplicate readings per sample was used for statistical analyses. PO activity was expressed in nmol dopachrome formed per minute. To correct PO activity, protein content in the supernatant was measured per sample using the Bradford (1976) method.

Statistical analyses

We tested for effects of the food treatment and latitude on the response variables with linear mixed models using the ‘lme4’ package (Bates et al. 2014) in R 3.1.1. for Windows (R Core Team 2014). The ‘car’ package was used to calculate Wald chi-square statistics and p-values for fixed effects (Fox and Weisberg 2011). In each model we added two random effects, namely family nested within population and population nested within latitude. We tested for effects on the binary response variables, pre-winter survival and winter survival, using a generalized linear mixed model (GLMM) with a binomial error distribution and a logit-link function. We analysed the successive measurements of head width, growth rate and food intake using repeated-measures analyses of variance (RM-ANOVAs) with a normal error structure and the identity link. We did not include head width at hatching in the RM-ANOVA as this measurement was obtained on another subset of larvae; head width at hatching was analysed separately in an ANOVA. As our focus was on latitudinal patterns in growth rate (and their link to voltinism) we report the growth data in the results section and the associated temporal patterns in head widths in Online Resource 2. We ran separate analyses of variance (ANOVAs) to test for effects of latitude, food treatment and winter (before vs after winter) on larval mass, fat content and PO activity. Fat content was log-transformed to meet ANOVA assumptions. We included larval mass as covariate in the model to test for effects on fat content, and protein content to test for effects on PO activity. The least-square means were computed using the ‘lsmeans’ package and were compared using Tukey posthoc tests for further analysis of significant effects and interactions (Lenth 2015).

Results

Survival

Pre-winter survival was ca. 60% and did not differ among latitudes (χ^2_2 = 1.40, P = 0.50, Fig. 1a) and was not influenced by the food treatment (GLMM, χ^2_2 = 0.06, P = 0.81, Fig. 1a). While winter survival also did not differ between food treatments (χ^2_2 = 0.13, P = 0.72, Fig. 1b), it differed among latitudes (χ^2_2 = 14.60, P < 0.001, Fig. 1b): Spanish larvae (67%) had a lower winter survival than the Swedish larvae (86%) (Tukey, P < 0.001) and tended to have a lower survival than the Belgian larvae (78%) (P = 0.076); survival did not differ between Belgian and Swedish larvae (P = 0.17). A more detailed analysis per month showed that differences in larval survival among latitudes increased as winter progressed (Fig. 1c). During the first months of the winter period (November till January) larval survival did not differ among latitudes (all P > 0.13). From February onwards, latitudinal differences in survival became apparent with lower survival in Spanish larvae and higher survival in Swedish larvae (Effect of latitude, February: χ^2_2 = 10.27, P = 0.0059; March: χ^2_2 = 15.95, P < 0.001; April: χ^2_2 = 14.60, P < 0.001, Fig. 1c).

Larval growth rate and food intake

Larval growth rates based on head width measurements strongly decreased through time with the lowering of the temperature and growth stopped during the 4°C winter period (RM-ANOVA, Period: χ^2_3 = 7502.21, P < 0.001, Fig. 2). While Swedish larvae had overall the lowest growth rate (RM-ANOVA, χ^2_2 = 217.75, P < 0.001, Fig. 2a-d), larval growth differences among latitudes changed through time (Latitude × Period interaction: χ^2_6 = 406.19, P < 0.001, Fig. 2a-d). During the 20°C pre-winter period (Fig. 2a) Belgian larvae grew faster than Spanish and Swedish larvae (Tukey, both P < 0.001) and Spanish larvae grew faster than Swedish larvae (P < 0.001). However, during the 15°C pre-winter period (Fig. 2b) Spanish larvae had a higher growth rate than Belgian and Swedish larvae (both P < 0.001) and there was no difference in growth rate between Belgian and Swedish larvae ($P = 0.95$). At the much lower growth rates during the 10°C pre-winter period and the 4°C winter period, differences between latitudes were no longer present (all $P > 0.17$, Fig. 2c-d). Larvae grew faster at high food than at low food but only in the 15°C pre-winter period (Food × Period interaction: $\chi^2_3 = 10.85$, $P = 0.013$, Tukey tests at 15°C: $P < 0.001$, other periods: all $P > 0.50$).

Food intake strongly changed through time (RM-ANOVA, Period: $\chi^2_3 = 433.11$, $P < 0.001$, Fig. 2e-h): strongly increasing from the 20°C to the 15°C pre-winter periods (Tukey, $P < 0.001$), then slightly decreasing in the 10°C pre-winter period ($P < 0.001$), and further strongly decreasing in the 4°C winter period ($P < 0.001$). While Belgian larvae had overall the highest food intake ($\chi^2_2 = 20.69$, $P < 0.001$, Fig 2e-h), differences among latitudes disappeared through time (Latitude × Period interaction: $\chi^2_6 = 12.23$, $P = 0.057$). Belgian larvae tended to have a higher food intake than Swedish ($P = 0.051$) and Spanish larvae ($P = 0.066$) during the 20°C pre-winter period and had a higher food intake during the 15°C pre-winter period (both $P < 0.01$), while Spanish and Swedish larvae did not differ in these periods (both $P > 0.49$). During the 10°C pre-winter period and the 4°C winter period latitudinal differences in food intake disappeared. Overall, larvae in the low food treatment ate more *Artemia* than larvae in the high food treatment ($\chi^2_1 = 5.27$, $P = 0.022$, Fig 2e-h).

Larval mass, fat content and PO activity

Larval mass did not change during the winter period ($\chi^2_1 = 0.75$, $P = 0.39$). Overall, Belgian larvae had the highest mass and Swedish larvae the lowest mass ($\chi^2_2 = 15.92$, $P < 0.001$, Fig. 3a-b), this tended to be more pronounced at high food before winter (Latitude × Food × Winter interaction: $\chi^2_2 = 5.06$, $P = 0.080$). Larval mass was higher in the high food treatment ($\chi^2_1 = 4.49$, $P = 0.034$, Fig. 3a-b), but only at the start and not at the end of the winter period (Food × Winter interaction: $\chi^2_1 = 4.52$, $P = 0.033$, Fig. 3a-b).

Swedish larvae had the highest fat content ($\chi^2_2 = 84.42$, $P < 0.001$, Fig. 3c-d), while fat content did not differ between Belgian and Spanish larvae (Tukey, $P = 0.70$). There was no main effect of the food treatment ($\chi^2_1 = 0.22$, $P = 0.64$, Fig. 3c-d). The fat content decreased during the winter period ($\chi^2_1 = 20.84$, $P < 0.001$, Fig. 3c-d), and this decrease did not depend on food level and latitude (interactions with winter, all $P > 0.064$).

PO activity decreased during the winter ($\chi^2_2 = 10.87$, $P < 0.001$, Fig. 3e-f) and this decrease was consistent across latitudes and food levels (interactions with Period: all $P > 0.31$). PO activity was highest in Swedish larvae ($\chi^2_2 = 150.89$, $P < 0.001$, Fig. 3e-f) and equally low in Belgian and Spanish larvae (Tukey, $P = 0.61$). PO activity was higher in the high food treatment than in the low food treatment, but only in Swedish larvae, generating a Latitude × Food interaction ($\chi^2_2 = 6.08$, $P = 0.048$, Fig. 3e-f).

Discussion

As expected, the 4°C winter period that mimicked a natural winter in Central Sweden caused differential mortality among latitudes. Winter mortality was lower in the high-latitude larvae, indicating latitude-specific thermal adaptation that could be linked to differences in voltinism. The lower winter mortality in the Swedish populations was associated with a higher build-up of fat storage and investment in immune function (measured as PO activity) before winter. The winter conditions caused reductions in fat content and PO activity, yet these reductions were similar across latitudes. Overwinter reductions in energy storage are widely documented (e.g., Biro et al. 2004; Bemani et al. 2012; Wasielewski et al. 2013), yet have only been rarely observed for immune function (but see two studies on fish: Le Morvan et al. 1998; Lepak and Kraft 2008). Despite several negative effects of the low food treatment, it did not magnify the observed lethal and sublethal effects of winter.

A limitation of our study is that we exposed larvae from all three populations not only to the temperature but also the photoperiod conditions that are experienced at the high latitude. Photoperiods differ between latitudes and many species are adapted to local photoperiods to adjust their life history to the progress of the seasons (Bradshaw and Holzapfel 2006). As a result, the northern photoperiod may have affected life history in central and southern populations differently than in northern populations. The only study in coenagrionid damselflies that explicitly tested the effects of latitude-specific photoperiod on life history in northern and southern populations (Sniegula et al. 2012) showed that the latitudinal differences in growth were for the two damselfly species tested much larger than the effect of the latitude-specific photoperiod on growth. Moreover, when the southern populations of both species were reared at the northern photoperiod (as we did) this resulted in a somewhat lower growth rate compared to when the southern populations were reared at their local (southern) photoperiod, yet this effect was small. This may suggest that the southern larvae of our study species when reared under the southern photoperiod would even have been growing a bit faster than we observed under the northern photoperiod used in our experiment. The rather subtle changes in growth rate when switching the photoperiod as detected in the study by Sniegula et al. (2012) seem, however, unlikely to translate in changes in voltinism. It is also important to note that besides adjusting the photoperiod we also gradually lowered the temperature providing direct information about the arrival of winter for each population. In this context it is relevant that in a study on another damselfly species we showed that foraging activity, growth and development were much more sensitive to temperature than photoperiod, and that the temperature response was not modulated by photoperiod (De Block and Stoks 2003).

Larval survival did not differ among latitudes before winter and during the first months of the winter period, but as the winter progressed the Swedish larvae became gradually better in withstanding the northern winter than the Belgian and especially the Spanish larvae. This indicates local thermal adaptation to deal with the northern winter conditions along the latitudinal gradient. Indeed, winters are in general very mild and short in Spain (ca. one month of about 5°C), intermediate in harshness in Belgium (ca. four months of about 4°C), and more severe and long in Sweden (ca. six months of about 4°C) (based on the Flake model, Simmons et al. 2007). This latitude-specific pattern in winter survival has been found before in a few other taxa: terrestrial spiders (Krehenwinkel and Tautz 2013), lizards (Wilson and Cooke 2004), freshwater fishes (Fullerton et al. 2000; Rooke and Fox 2014) and marine fishes (Schultz et al. 1998) . Given that we reared animals from the egg stage under common garden conditions, these latitudinal differences likely reflect genetic differences. While our setup cannot fully exclude maternal effects playing a role, these have been shown to play a minor role in shaping life history in damselflies (Shama et al. 2011; Sniegula et al. 2016, including the study species: Strobbe and Stoks 2004).

Our data suggest that the higher winter survival of Swedish larvae may partly be explained by voltinism- associated latitudinal differences in life history and an allocation trade-off between growth and the physiological traits energy storage and investment in immune function (Dmitriew 2011). The semivoltine Swedish larvae were growing slower and had the highest fat content and PO activity when the winter started compared to the univoltine Spanish and Belgian larvae that were growing faster to complete their life cycle in one year. Costs of rapid growth in terms of reduced energy storage and immune function are widespread (Dmitriew 2011) and previously observed in damselfly larvae (Stoks et al. 2006a, b; De Block and Stoks 2008b). The shorter winter periods in Spain and Belgium (that imply less need to invest in energy storage) may have further shifted the balance of the energy allocation trade-off away from energy storage in Spanish and Belgian larvae. In contrast, we did not detect support for the second type of physiological mechanisms driving winter survival: a lower depletion of the fat content and lower reduction in immune function during winter in the Swedish larvae compared to Belgian and Spanish larvae. A lower lipid depletion rate has been observed in northern populations of two fish species (salmon: Finstad et al. 2010 and pumpkinseeds: Rooke and Fox 2014), yet, in these species no higher fat content was accumulated in the northern populations at the start of winter (Finstad et al. 2010; Rooke and Fox 2014).

Spanish larvae had a lower survival during winter than Belgian larvae, yet this could not be explained by patterns in fat storage and immune function. Indeed, larvae from both latitudes did not differ in fat content and PO activity before winter or in how fat content and PO activity decreased during winter. Given the lower overwinter survival of Spanish compared to the Belgian larvae, any survival selection, if present, would have more strongly affected the Spanish larvae. Possibly, larvae with the lowest levels of fat storage and immune function at the start of winter and/or the highest reductions in these physiological variables were more likely to die during winter. If such survival selection was present, it may have masked any physiological differences between both latitudes in the survivors.

The low food treatment negatively affected larvae (in terms of lower growth at 15°C, lower mass at the start of winter and a lower PO activity in Swedish larvae), yet did not affect survival during winter and fat content at the start and end of winter. Likely, animals prioritized investment in energy storage above growth before winter to keep fat content and consequently winter survival as high as in the high food group. This was supported by the lower growth rate at 15°C of larvae reared at low food than at high food. The absence of a food effect on winter survival and fat content at the end of winter may be explained by the fact that aquatic ectotherms strongly lower their metabolism and food intake during the winter period (Denlinger and Lee 2010), which may lead to no differences in survival and fat content between both food treatments. This was supported by the observation that food intake during winter did not differ between high and low food levels.

Food intake was predictably shaped by the food and temperature treatments and differed among latitudes but did not fully match the patterns in growth rates. Overall, food intake during the feeding test was higher in the low than in the high food treatment, independent of the latitude. This indicates that larvae in the low food treatment needed to forage more to prevent starvation (Abrams 1991). As predicted, low temperatures of 10°C and especially 4°C decreased food intake and consequently growth rate in larvae from all latitudes, confirming the general pattern that insects are less active, feed less and grow less during the winter period (Denlinger and Lee 2010; Sinclair 2015). We also expected size-corrected food intake to be higher at 20°C than at 15°C (De Block and Stoks 2003; Dinh Van et al. 2014), especially since growth rate decreased with decreasing temperatures. Yet, we found the opposite pattern with a higher food intake at 15°C. Possibly, the decrease in temperature to 15°C was perceived as indicating the arrival of winter and therefore caused the increase in food intake. We hypothesize that the increased energy intake was mainly allocated to energy storage to prepare for winter thereby not generating a faster growth at 15°C. A higher food intake under time constraints has been observed before in damselfly larvae (Stoks et al. 2005) and other animals (e.g. amphibians: Orizaola et al. 2014). Time constraints may also explain why univoltine Belgian larvae have a higher food intake at 20°C and 15°C, as they have less time to complete their life cycle within one growing season compared to the univoltine Spanish (that have a longer thermally favorable growing season) and the semivoltine Swedish larvae. This may also explain the higher growth rate of Belgian larvae at 20°C.

To conclude, we demonstrated latitude-specific thermal adaptation to deal with winter survival in a pond insect. Our data support the novel hypothesis that the widespread occurrence of latitude-associated voltinism shifts in insects may be an important selective factor contributing to latitudinal shifts in winter survival. We thereby identified possible mechanisms driving voltinism-linked latitudinal differences in winter survival: a higher buildup of energy storage and investment in immune function in the semivoltine high-latiude populations, while reductions in these variables and compensatory food intake played no role. This suggests that winter survival may be an important, yet overlooked, selective factor contributing to latitudinal patterns in immune function as been observed for phenoloxidase activity in crickets (Fedorka et al. 2012) and other damselfly species (Janssens et al. 2014).

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Electronic Supplemental Material

Online Resource 1. Overview of the applied photoperiod and temperature treatments during the experiment.

Online Resource 2. Results larval head width.

References

Abrams PA (1991) Life history and the relationship between food availability and foraging effort. Ecology 72:1242–1252. doi: 10.2307/1941098

Abrams PA, Leimar O, Nylin S, Wiklund C (1996) The effect of flexible growth rates on optimal sizes and development times in a seasonal environment. Am Nat 147:381–395.

Addo-Bediako A, Chown SL, Gaston KJ (2000) Thermal tolerance, climatic variability and latitude. Proc R Soc London B Biol Sci 267:739–745. doi: 10.1098/rspb.2000.1065

Bates D, Maechler M, Bolker B, Walker S (2014) Lme4: linear mixed-effects models using S4 classes. R package version 1. 1-4. R.

Bemani M, Izadi H, Mahdian K, Khani A, Amin samih M (2012) Study on the physiology of diapause, cold hardiness and supercooling point of overwintering pupae of the pistachio fruit hull borer, *Arimania comaroffi*. J Insect Physiol 58:897–902. doi: 10.1016/j.jinsphys.2012.04.003

Biro PA, Morton AE, Post JR, Parkinson EA (2004) Over-winter lipid depletion and mortality of age-0 rainbow trout (*Oncorhynchus mykiss*). Can J Fish Aquat Sci 61:1513–1519. doi: 10.1139/f04-083

Blanckenhorn WU, Demont M (2004) Bergmann and converse Bergmann latitudinal clines in Arthropods: two ends of a continuum? Integr Comp Biol 44:413–424. doi: 10.1093/icb/44.6.413.

Boudot JP, Kalkman VJ (2015) Atlas of the dragonflies and damselflies of Europe. KNNV uitgeverij, Netherlands.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein, utilizing the principle of protein-dye landing. Anal Biochem 72:248–254. doi: 10.1006/abio.1976.9999

Bradshaw WE, Holzapfel CM (2006) Evolutionary response to rapid climate change. Science 312:1477–1478. doi: 10.1126/science.1127000

Chown SL, Gaston KJ (1999) Exploring links between physiology and ecology at macro-scales: the role of respiratory metabolism in insects. Biol Rev Camb Philos Soc 74:87–120. doi: 10.1111/j.1469-185X.1999.tb00182.x

Corbet PS (1999) Dragonflies: behaviour and ecology of Odonata. Cornell University Press, Ithaca, NY.

Corbet PS, Suhling F, Soendgerath D (2006) Voltinism of Odonata: a review. Int J Odonatol 9:1–44. doi: 10.1080/13887890.2006.9748261

Danks HV (2007) How aquatic insects live in cold climates. Can Entomol 139:443–471. doi: 10.4039/N06-100

De Block M, McPeek MA, Stoks R (2007) Winter compensatory growth under field conditions partly offsets low energy reserves before winter in a damselfly. Oikos 116:1975–1982. doi: 10.1111/j.2007.0030-1299.16186.x

De Block M, Slos S, Johansson F, Stoks R (2008) Integrating life history and physiology to understand latitudinal size variation in a damselfly. Ecography (Cop) 31:115–123. doi: 10.1111/j.2007.0906-7590.05313.x

De Block M, Stoks R (2003) Adaptive sex-specific life history plasticity to temperature and photoperiod in a damselfly. J Evol Biol 16:986–995. doi: 10.1046/j.1420-9101.2003.00581.x

De Block M, Stoks R (2004). Cannibalism-mediated life history plasticity to combined time and food stress. Oikos, 106:587–597. doi: 10.1111/j.0030-1299.2004.12974.x

De Block M, Stoks R (2008a) Short-term larval food stress and associated compensatory growth reduce adult immune function in a damselfly. Ecol Entomol 33:796–801. doi: 10.1111/j.1365-2311.2008.01024.x

De Block M, Stoks R (2008b) Compensatory growth and oxidative stress in a damselfly. Proc R Soc B 275:781–785. doi: 10.1098/rspb.2007.1515

Denlinger DL, Lee REJ (2010) Low temperature biology of insects., 1st edn. Cambridge University Press, Gillingham, UK.

Dinh Van K, Janssens L, Debecker S, Stoks R (2014) Temperature- and latitude-specific individual growth rates shape the vulnerability of damselfly larvae to a widespread pesticide. J Appl Ecol 51:919–928. doi: 10.1111/1365-2664.12269

Dmitriew CM (2011) The evolution of growth trajectories: what limits growth rate? Biol Rev 86:97–116. doi: 10.1111/j.1469-185X.2010.00136.x

Fedorka KM, Winterhalter WE, Shaw KL, Brogan WR, Mousseau TA (2012) The role of gene flow asymmetry along an environmental gradient in constraining local adaptation and range expansion. J Evol Biol 25:1676–1685. doi: 10.1111/j.1420-9101.2012.02552.x

Ferguson LV, Heinrichs DE, Sinclair BJ (2016) Paradoxical acclimation responses in the thermal performance of insect immunity. Oecologia 181:77–85. doi: 10.1007/s00442-015-3529-6

Finstad AG, Berg OK, Forseth T, Ugedal O, Naesje TF (2010) Adaptive winter survival strategies: defended energy levels in juvenile Atlantic salmon along a latitudinal gradient. Proc R Soc B 277:1113–1120. doi: 10.1098/rspb.2009.1874

Fox J, Weisberg S (2011) An {R} Companion to Aplied Regression, Second. Sage Publications, Thousand Oaks CA

Franke K, Fischer K (2013) Effects of inbreeding and temperature stress on life history and immune function in a butterfly. J Evol Biol 26:517–528. doi: 10.1111/jeb.12064

Fullerton AH, Garvey JE, Wright RA, Stein RA (2000) Overwinter growth and survival of largemouth bass: interactions among size, food, origin, and winter severity. Trans Am Fish Soc 129:1–12. doi: 10.1577/1548-8659(2000)129<0001:OGASOL>2.0.CO;2

Gaston KJ (2003) The structure and dynamics of geographic ranges. Oxford Series. Oxford University Press., Oxford.

González-Santoyo I, Córdoba-Aguilar A (2012) Phenoloxidase: a key component of the insect immune system. Entomol Exp Appl 142:1–16. doi: 10.1111/j.1570-7458.2011.01187.x

Hahn DA, Denlinger DL (2011) Energetics of insect diapause. Annu Rev Entomol 56:103–121. doi: 10.1146/annurev-ento-112408-085436

Houston A, McNamara J, Barta Z, Klasing K (2007) The effect of energy reserves and food availability on optimal immune defence. Proc R Soc B Biol Sci 274:2835–2842. doi: 10.1098/rspb.2007.0934

Janssens L, Dinh Van K, Stoks R (2014) Extreme temperatures in the adult stage shape delayed effects of larval pesticide stress: a comparison between latitudes. Aquat Toxicol 148:74–82. doi: 10.1016/j.aquatox.2014.01.002

Johansson F (2003) Latitudinal shifts in body size of *Enallagma cyathigerum* (Odonata). J Biogeogr 30:29–34. doi: 10.1046/j.1365-2699.2003.00796.x

Krams I, Daukšte J, Kivleniece I, Krama T, Rantala MJ (2011) Overwinter survival depends on immune defence and body length in male *Aquarius najas* water striders. Entomol Exp Appl 140:45–51. doi: 10.1111/j.1570-7458.2011.01132.x

Krehenwinkel H, Tautz D (2013) Northern range expansion of European populations of the wasp spider *Argiope bruennichi* is associated with global warming-correlated genetic admixture and population-specific temperature adaptations. Mol Ecol 22:2232–2248. doi: 10.1111/mec.12223

Le Morvan C, Troutaud D, Deschaux P (1998) Differential effects of temperature on specific and nonspecific immune defences in fish. J Exp Biol 201:165–168.

Lenth RV (2015) Package “lsmeans.” http://cran.r-project.org/web/packages/lsmeans/lsmeans.pdf

Lepak JM, Kraft CE (2008) Alewife mortality, condition, and immune response to prolonged cold temperatures. J Great Lakes Res 34:134–142. doi: 10.3394/0380-1330(2008)34[134:AMCAIR]2.0.CO;2

Marsh JB, Weinstein DB (1966). Simple charring method for determination of lipids. J Lipid Res 7:574–576.

Nilsson-örtman V, Stoks R, De Block M, Johansson F (2012) Generalists and specialists along a latitudinal transect: patterns of thermal adaptation in six species of damselflies. Ecology 93:1340–1352. doi: 10.1890/11-1910.1

Orizaola G, Dahl E, Laurila A (2014) Compensatory growth strategies are affected by the strength of environmental time constraints in anuran larvae. Oecologia 174:131–137. doi: 10.1007/s00442-013-2754-0

Postle AD, Goodland FC (1978) Comparison of three automated methods of serum triglyceride analysis. Ann Clin Biochem 15:18–24.

R Core Team (2014) R: A language and environment for statistical computing. R Found. Stat. Comput.

Ragland GJ, Kingsolver JG (2007) Influence of seasonal timing on thermal ecology and thermal reaction norm evolution in *Wyeomyia smithii*. J Evol Biol 20:2144–2153. doi: 10.1111/j.1420-9101.2007.01428.x

Rolff J, Joop G (2002) Estimating condition: pitfalls of using weight as a fitness correlate. Evol Ecol Res 4:931–935.

Rooke AC, Fox MG (2014) Living a century in warm thermal conditions: introduced populations of Pumpkinseed (*Lepomis gibbosus*) inhabiting the Iberian Peninsula (Spain) show reduced adaptation to North American winter conditions. Aquat Sci 76:497–509. doi: 10.1007/s00027-014-0349-4

Schultz ET, Conover DO, Ehtisham A (1998) The dead of winter: size-dependent variation and genetic differences in seasonal mortality among Atlantic silverside (Atherinidae: *Menidia menidia*) from different latitudes. Can J Fish Aquat Sci Aquat Sci 55:1149–1157. doi: 10.1139/f97-320

Shama LNS, Campero-Paz M, Wegner KM, De Block M, Stoks R (2011) Latitudinal and voltinism compensation shape thermal reaction norms for growth rate. Mol Ecol 20:2929–2941. doi: 10.1111/j.1365-294X.2011.05156.x

Simmons A, Uppala SM, Dee D, Kobayashi S (2007) ERA-Interim: New ECMWF reanalysis products from 1989 onwards. ECMWF Newsl 110:25–35.

Sinclair BJ (2015) Linking energetics and overwintering in temperate insects. J Therm Biol 54:5–11. doi: 10.1016/j.jtherbio.2014.07.007

Sniegula S, Golab MJ, Drobniak SM, Johansson F (2016) Seasonal time constraints reduce genetic variation in life-history traits along a latitudinal gradient. J Anim Ecol 85:187–198. doi: 10.1111/1365-2656.12442

Sniegula S, Johansson F, Nilsson-Örtman V (2012) Differentiation in developmental rate across geographic regions: a photoperiod driven latitude compensating mechanism? Oikos 121: 1073–1082. doi: 10.1111/j.1600-0706.2011.20015.x

Stoks R, Córdoba-Aguilar A (2012) Evolutionary ecology of Odonata: a complex life cycle perspective. Annu Rev Entomol 57:249–265. doi: 10.1146/annurev-ento-120710-100557

Stoks R, Debecker S, Dinh Van K, Janssens L (2015) Integrating ecology and evolution in aquatic toxicology: insights from damselflies. Freshw Sci 34:1032–1039. doi: 10.1086/682571

Stoks R, De Block M, McPeek MA (2006a) Physiological costs of compensatory growth in a damselfly. Ecology 87:1566–1574. doi: 10.1890/0012-9658(2006)87[1566:PCOCGI]2.0.CO;2

Stoks R, De Block M, Slos S, Van Doorslaer W, Rolff J (2006b) Time constraints mediate predator-induced plasticity in immune function, condition, and life history. Ecology 87:809–815. doi: 10.1890/0012-9658(2006)87[809:TCMPPI]2.0.CO;2

Stoks R, De Block M, Van De Meutter F, Johansson F (2005) Predation cost of rapid growth: behavioural coupling and physiological decoupling. J Anim Ecol 74:708–715. doi: 10.1111/j.1365-2656.2005.00969.x

Strobbe F, Stoks R (2004) Life history reaction norms to time constraints in a damselfly: differential effects on size and mass. Biol J Linn Soc 83:187–196. doi: 10.1111/j.1095-8312.2004.00379.x

Sugumaran H (2002) Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Res 15:2–9. doi: 10.1034/j.1600-0749.2002.00056.x

Suhling F, Suhling I, Richter O (2015) Temperature response of growth of larval dragonflies - an overview. Int J Odonatol 18:15-30. doi: 10.1080/13887890.2015.1009392

Sunday JM, Bates AE, Kearney MR, Colwell RK, Dulvy NK, Longino JT, Huey RB (2014) Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. Proc Natl Acad Sci USA 111:5610–5615. doi: 10.1073/pnas.1316145111

Wasielewski O, Wojciechowicz T, Giejdasz K, Krishnan N (2013) Overwintering strategies in the red mason solitary bee - physiological correlates of midgut metabolic activity and turnover of nutrient reserves in females of *Osmia bicornis*. Apidologie 44:642–656. doi: 10.1007/s13592-013-0213-x

Williams, CM, Chick, WD, Sinclair BJ (2015) A cross-seasonal perspective on local adaptation: metabolic plasticity mediates responses to winter in a thermal-generalist moth. Funct Ecol 29:549–561. doi: 10.1111/1365-2435.12360

Wilson BS, Cooke DE (2004) Latitudinal variation in rates of overwinter mortality in the lizard *Uta stansburiana*. Ecology 85:3406–3417. doi: 10.1890/03-4075

Zeuss D, Brunzel S, Brandl R (2017) Environmental drivers of voltinism and body size in insect assemblages across Europe. Global Ecol Biogeogr 26:154-165. doi: 10.1111/geb.12525

Figure legends

Fig. 1 Overall survival (percentage) of *Enallagma cyathigerum* larvae of three latitudes across the (a) pre-winter and (b) winter periods, and (c) detailed survival curves (Kaplan-Meier) throughout the winter period

Fig. 2 Mean (with 95% confidence interval) growth rate (a-d) and food intake (e-h) of *Enallagma cyathigerum* larvae during the pre-winter periods at 20°C, 15°C, 10°C and during the winter period at 4°C as a function of latitude and food treatment. Note that the food treatment started after 26 August, hence after the 20°C pre-winter period. Food intake was expressed as the number of *Artemia* eaten per minute and was corrected for head width. Numbers above bars represent sample sizes

Fig. 3 Mean (with 95% confidence interval) larval mass (a-b), fat content (c-d) and PO activity (e-f) of *Enallagma cyathigerum* larvae at the start and at the end of the 4°C winter period as a function of latitude and food treatment. Fat content was corrected for larval mass and PO activity was corrected for protein content. Numbers above bars represent sample sizes

Figure 1

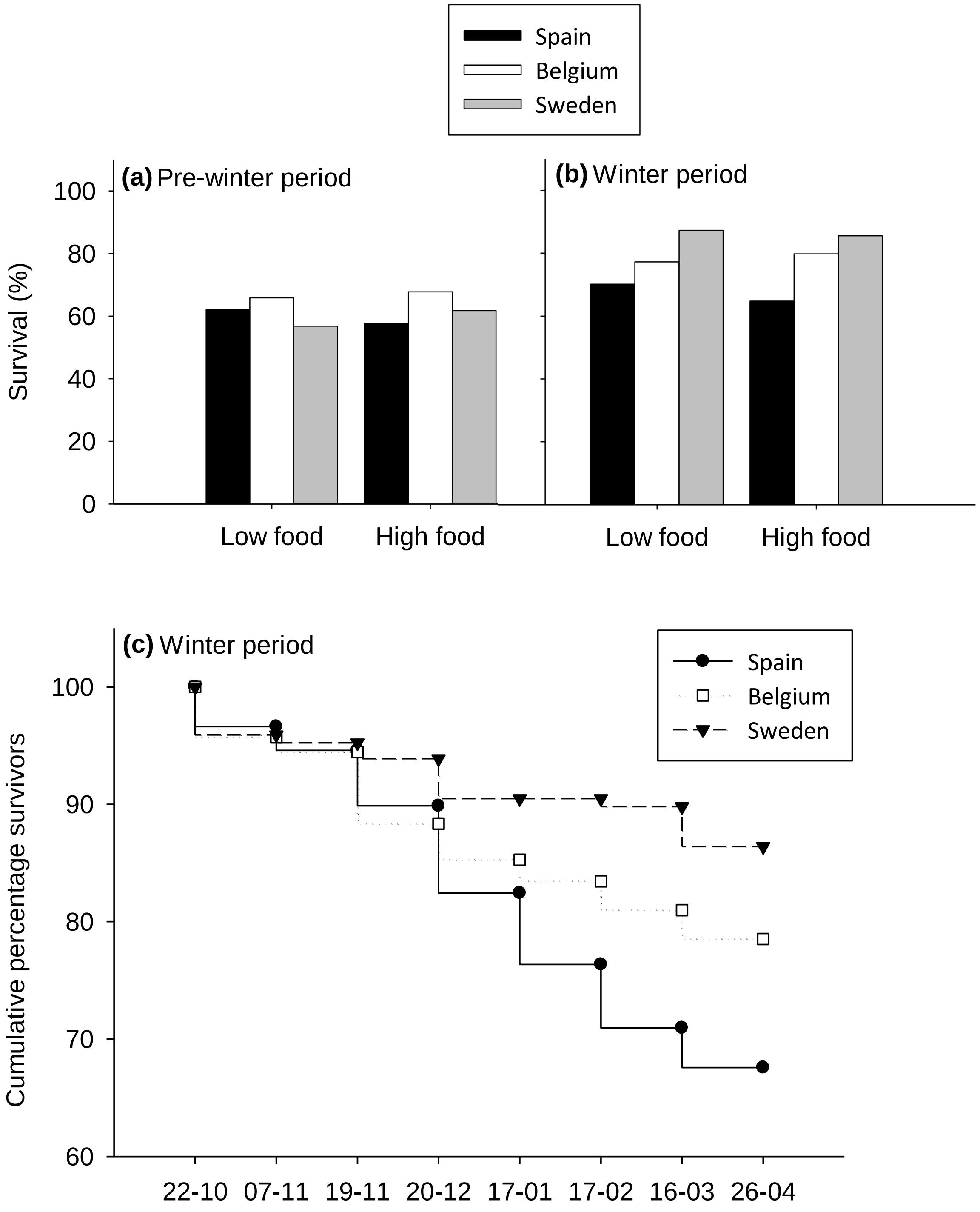

Spain
Belgium
Sweden
(a) Pre-winter period
(b) Winter period
Survival (%)
0
20
40
60
80
100
Low food
High food
Low food
High food
(c) Winter period
Spain
Belgium
Sweden
Cumulative percentage survivors
60
70
80
90
100
22-10
07-11
19-11
20-12
17-01
17-02
16-03
26-04

Figure 2

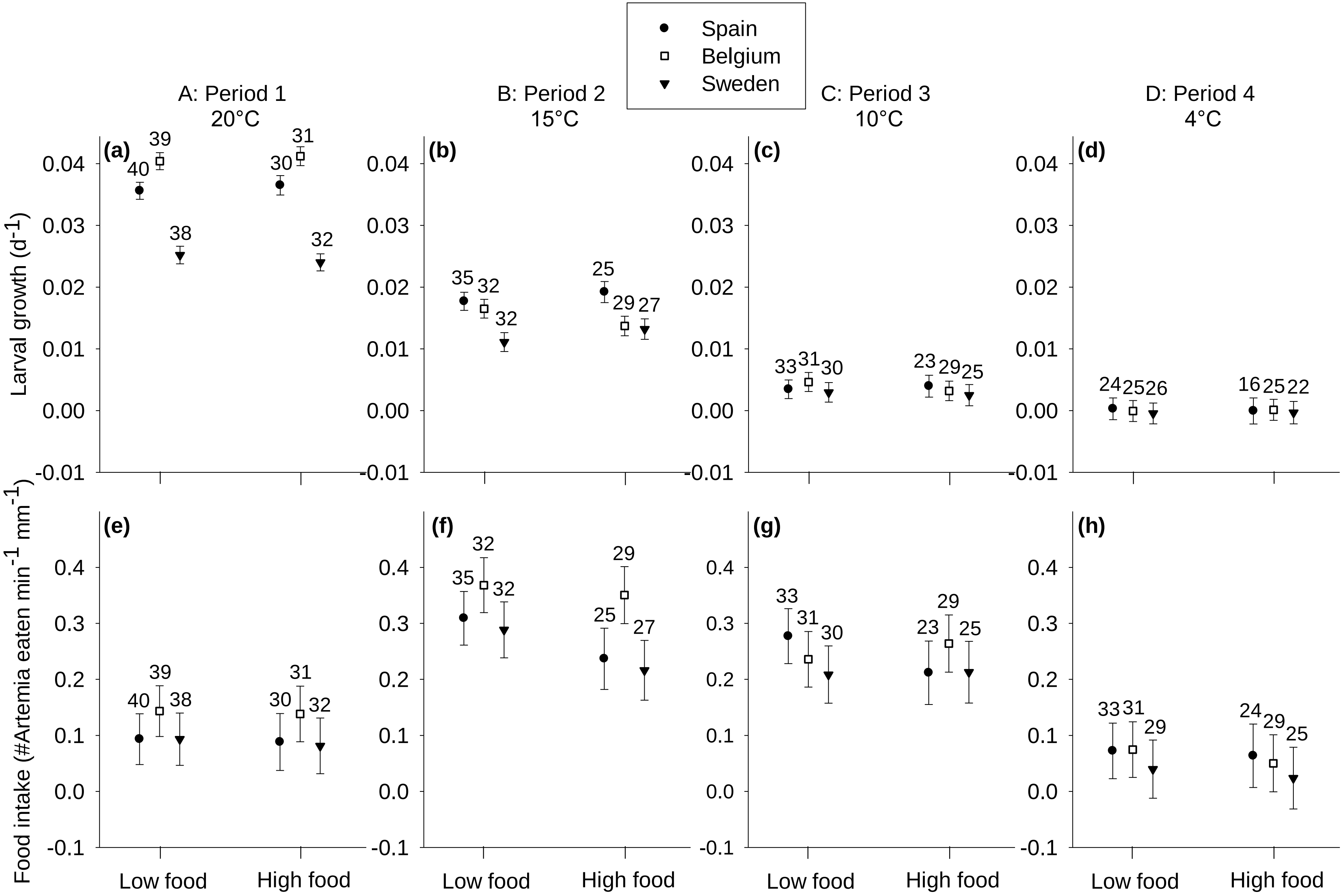
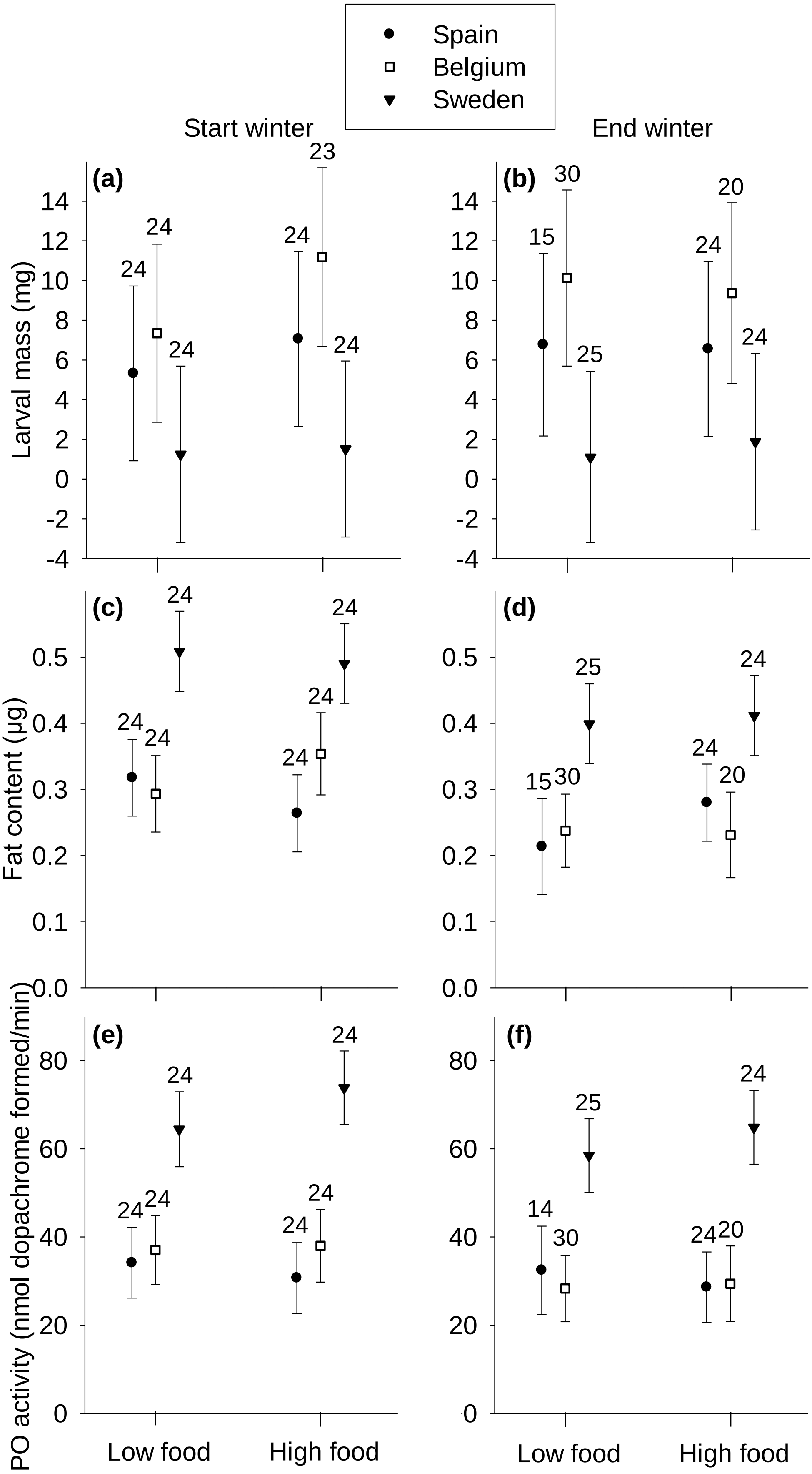

● Spain
□ Belgium
▼ Sweden
A: Period 1
20°C
B: Period 2
15°C
C: Period 3
10°C
D: Period 4
4°C
Larval growth (d-1)
(a)
(b)
(c)
(d)
0.04
0.03
0.02
0.01
0.00
-0.01
40 39 38 30 31 32
35 32 32 25 29 27
33 31 30 23 29 25
24 25 26 16 25 22
Food intake (#Artemia eaten min-1 mm-1)
(e)
(f)
(g)
(h)
0.4
0.3
0.2
0.1
0.0
-0.1
40 39 38 30 31 32
35 32 32 25 29 27
33 31 30 23 29 25
33 31 29 24 29 25
Low food
High food

Figure 3


● Spain
□ Belgium
▼ Sweden
Start winter
End winter
(a)
(b)
(c)
(d)
(e)
(f)
Larval mass (mg)
Fat content (µg)
PO activity (nmol dopachrome formed/min)
14
12
10
8
6
4
2
0
-2
-4
0.5
0.4
0.3
0.2
0.1
0.0
80
60
40
20
0
24
24
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23
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15
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24
24
15
30
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24
24
24
14
30
25
24
20
24
Low food
High food
Low food
High food