Temperature variation makes an ectotherm more sensitive to global warming unless thermal evolution occurs

Julie Verheyen and Robby Stoks

Evolutionary Stress Ecology and Ecotoxicology, University of Leuven, Charles Deberiotstraat 32, B-3000 Leuven, Belgium

Corresponding author. Tel.: +3216376436; fax: +3216324575

E-mail address: julie.verheyen@kuleuven.be

Abstract

1. To assess long-term impacts of global warming on species there is growing interest in latitudinal intraspecific patterns in thermal adaptation. Yet, while both mean temperatures and daily temperature fluctuations (DTFs) are expected to increase under global warming, latitudinal differences in the effects of DTFs have not been documented.
2. We tested whether low-latitude populations of an ectotherm deal better with greater DTF than high-latitude populations, especially at a high mean temperature close to the optimal temperature for growth where DTF causes exposure to extreme high temperatures. We evaluated the impact of DTFs when assessing the effect of gradual thermal evolution at the high latitude with a space-for-time substitution.
3. We compared effects of both mean temperatures (20°C and 24°C) and DTFs (constant = 0°C, low = 5°C and high = 10°C) on growth rates between low-latitude and high-latitude populations of the damselfly *Ischnura elegans* in a common-garden experiment.
4. DTFs , if anything, reduced growth and were generally stressful as indicated by reductions in body condition, antioxidant defense and metabolic rate, and increases in oxidative damage. Most negative effects of DTFs were only present at a mean of 24°C when too high temperatures were reached during a daily cycle. Notably, while 4°C warming was beneficial in terms of growth rate at both latitudes at a constant temperature regime, this changed in a negative effect at high DTF. Moreover, this modulating effect of the mean temperature by DTF differed between latitudes indicating local thermal adaptation. While 4°C warming at low DTF still caused faster growth in low-latitude larvae, it already slowed growth in high-latitude larvae. This supports the emerging insight that warming would increase growth in high-latitude larvae in absence of DTF, yet would decrease growth in the more realistic scenarios with DTF. In contrast, a space-for-time substitution

approach suggested that under gradual thermal evolution, the evolved high-latitude larvae would no longer suffer a growth reduction in the presence of DTF.

5. Our study provided important proof-of-principle that jointly integrating gradual thermal evolution and the expected increase in DTF generates opposing predictions of effects of global warming on this ectotherm.

Keywords: Climate warming, Geographic differences, Latitudinal gradient, Life-history trait, Space-for-time substitution, Temperature variability, Thermal evolution.

Introduction

There is increasing interest in latitudinal differences in thermal adaptation to mean temperatures to address the long-term effects of global warming on species (Woodward, Perkins, & Brown, 2010; Stoks, Geerts, & De Meester, 2014). Within temperate regions, ectotherms from lower latitudes typically have their maximal performance at a higher temperature compared to ectotherms from higher latitudes (Angilletta, Huey, & Frazier, 2009; Conover, Duffy, & Hice, 2009). This is directly relevant to assess the potential of gradual thermal evolution in shaping the performance of high-latitude populations under global warming through a so-called space-for-time substitution (Fukami & Wardle, 2005). This approach compares populations at strategically choosing latitudes that differ in mean temperatures corresponding to an IPCC warming scenario, and assumes thermal genetic adaptation of these populations to the current local thermal regime. In such case, the performance of the low-latitude populations at their current higher environmental temperature can then be used as a proxy for the performance of the high-latitude populations when they gradually evolve (hence genetically adapt) under future warming (De Frenne et al., 2013). While this approach should be done cautiously as time scales differ and it assumes that the drivers of trait change in space are the same as those that drive trait change through time, this

approach has been shown reliable to infer micro-evolutionary processes (Wogan & Wang, 2017).

The majority of global warming studies focused on the effects of an increase in mean temperatures (Thompson, Beardall, Beringer, Grace, & Sardina, 2013; Vázquez, Gianoli, Morris, & Bozinovic, 2015). Yet, also an increase in the magnitude of daily temperature fluctuations (DTFs, the difference between the absolute maximum and absolute minimum day temperatures) is expected (Colinet, Sinclair, Vernon, & Renault, 2015; IPCC, 2013; Thompson et al., 2013; Vázquez et al., 2015). In general, increased DTFs at the same mean temperature will result in a changed frequency of extreme warm and cold daily temperatures (Estay, Lima, & Bozinovic, 2014). The more realistic climate scenario, where both the mean temperature and the DTF around this mean increase, will result in a higher frequency and intensity of extreme warm temperatures (scenario 3 in Estay et al., 2014). Notably, recent studies indicate that it may be more challenging for ectotherms to deal with the increase in DTFs than with the increase in mean temperatures (Paaijmans et al. 2013; Vasseur et al. 2014). DTFs at a given mean temperature can indeed negatively affect fitness-related traits such as growth rate and development rate, and decrease survival (Bozinovic, Medina, Alruiz, Cavieres, & Sabat, 2016; Colinet et al., 2015; Paaijmans et al., 2013). These deleterious effects occur when the range of temperatures encountered during a daily cycle exceeds the optimal temperature, above which performance rapidly falls (Colinet et al., 2015; Estay et al., 2014; Martin & Huey, 2008; Stoks, Verheyen, Van Dievel, & Tüzün, 2017). A reduction in performance under DTF can be explained because of the higher allocation of energy to the increased metabolic demands for cell maintenance (Colinet et al., 2015; Kern, Cramp, & Franklin, 2015), which may include the production of stress proteins (McMillan, Fearnley, Rank, & Dahlhoff, 2005). Moreover, DTFs can reduce the thermal optimum (Bozinovic,

Sabat, Rezende, & Canals, 2016) and the heat and cold tolerance (Bozinovic, Medina, et al., 2016, but see Nyamukondiwa, Kleynhans, & Terblanche, 2010).

Besides mean temperatures, also DTFs are higher at low than at high latitudes in temperate regions (30°-60° range in both hemispheres, Figure 1f in Wang & Dillon, 2014; Manenti, Sørensen, & Loeschcke, 2017). Note this pattern is the opposite for climate variability (the difference between the absolute maximum and absolute minimum year temperatures), which is higher at high latitudes because of lower extreme winter temperatures (Addo-Bediako, Chown, & Gaston, 2000). Whether populations from low latitudes also are better adapted to deal with the higher daily temperature variation compared to high latitudes is largely unknown. Surprisingly, only three empirical studies tested for geographic differences in the effects of DTF on performance (all on invertebrates studied at different latitudes), and none of these studies detected it (for development rate in Ragland and Kingsolver, 2008; intrinsic population growth rate in Hong and Shurin, 2015; multiple life-history and stress-resistance traits in Manenti et al., 2017). Moreover, only one of these studies (Ragland and Kingsolver, 2008) exposed animals both to their local mean temperature and DTF and those of populations at different latitudes, which is important to assess adaptation to the local thermal regime.

Despite the absence of empirical studies demonstrating it, latitudinal differences in the effect of DTFs can be expected because of the widespread patterns of adaptation to the local mean temperatures. Especially at high mean temperatures, DTFs can be predicted to be more deleterious for high-latitude populations. This is because at a high mean temperature the high DTFs will more often include temperatures exceeding the optimum temperature in the high-latitude populations (as these have a lower optimum temperature than the low-latitude populations). In other words, one can expect deleterious effects of DTFs on high-latitude individuals because of the extreme high temperatures that these fluctuations impose. The

potential for latitudinal differences in how populations deal with DTV is a crucial but neglected aspect in space-for-time substitutions. Indeed, space-for-time studies assume that the current response to the higher mean temperature in low-latitude populations can be used as a proxy for the future response to the predicted higher mean temperature under thermal evolution at the high-latitude populations (De Frenne et al., 2013; Stoks et al., 2014). These studies thereby ignore that besides the mean temperatures also the DTFs differ between latitudes and will increase under warming.

In the current study, we investigated differences in the response of individual growth rate to both mean temperature and daily temperature fluctuations between high- and low-latitude populations of an aquatic insect in a common-garden rearing experiment. Given the higher mean temperature and greater DTF at the low latitude, we expected low-latitude populations to deal better with greater DTF than high-latitude populations, especially at a high temperature close to the optimal temperature for growth. This is because (i) low-latitude populations are experiencing (hence likely are better adapted to cope with) both a higher mean temperature and a higher DTF around that mean compared to high-latitude populations (Wang & Dillon 2014), and (ii) because when a high mean temperature is combined with a high DTF the range of temperatures encountered often exceeds the optimal temperature which will cause sharp decreases in performance as performance rapidly falls above the thermal optimum (Estay et al., 2014). In other words, under large temperature fluctuations at a high mean temperature, high-latitude larvae are more likely to suffer as they will more often experience extreme high temperatures (above their thermal optimum) compared to low-latitude populations.

We studied damselfly larvae as these are particular vulnerable to global warming as they cannot escape exposure during their obligate aquatic life (Hassall & Thompson, 2008). Moreover, the study species *Ischnura elegans* prefers shallow freshwater ponds and therefore

is subjected to high daily temperature fluctuations. This species has a wide European distribution, occurring from northern Spain till central Sweden (Boudot & Kalkman, 2015). While high-latitude populations (Denmark/Sweden) are semivoltine (one generation every two years), low-latitude populations (southern France) are multivoltine (3-4 generations per year) (Corbet, Suhling, & Soendgerath, 2006). We capitalized on the observation that the mean water temperature experienced during summer is 4°C warmer at the studied low- compared to high-latitude populations (De Block, Pauwels, Van Den Broeck, De Meester, & Stoks, 2013; Dinh Van, Janssens, Debecker, & Stoks, 2014), thereby matching the expected increase in temperature by 2100 under IPCC (2013) scenario RCP 8.5. Importantly, we not only included the two mean water temperatures at both latitudes (20°C and 24°C), but also the maximum daily water temperature fluctuations during summer at both latitudes (5°C and 10°C), allowing for the first time a space-for-time substitution including DTF. Hence, we can use the current performance of the low-latitude populations at 24°C and 10°C DTF (their current thermal summer regime to which they are genetically adapted) as a proxy for the future performance of the high-latitude populations when they would gradually evolve when their thermal regime (current thermal summer regime of 20°C with 5°C DTF) under global warming shifts to the current regime at the low latitude.

To better understand patterns on growth rate we measured multiple physiological mechanisms to independently evaluate whether thermal regimes were stressful (Folguera et al. 2011). We measured two important condition-related traits in damselflies (Rolff & Joop, 2002; Stoks & Córdoba-Aguilar, 2012): the activity of phenoloxidase (PO), a key enzyme of the insect immune function, and total fat content, the main energy storage molecule in insects. To assess oxidative stress, we determined the activity of the two key anti-oxidant enzymes in insects (Korsloot, van Gestel, & van Straalen, 2004), superoxide dismutase (SOD) and catalase (CAT), and the level of oxidative damage to lipids measured as lipid peroxidation

(malondialdehyde, MDA) (Monaghan, Metcalfe, & Torres, 2009). As an estimate of metabolic rate, we measured the activity of the electron transport system (ETS) (De Coen & Janssen, 2003).

Materials and methods

Study populations and pre-experimental rearing

We studied three low-latitude (southern France) and three high-latitude (Denmark and southern Sweden) populations within the European range of *I. elegans* (Boudot & Kalkman, 2015). The low-latitude populations were St. Martin de Crau (43°37'56.77"N; 4°46'58.20"E), Camaret-sur-Aigues (44°08'56"N; 4°51'17"E) and Bassin du Réaltor (43°28'1.85"N; 5°19'35.51"E) in southern France. The high-latitude populations were Lund (55°44'5.4"N; 13°9'13.4"E) in southern Sweden, and Laesoe (57°15'12.14"N; 10°54'19.75"E) and Roskilde (55°39'09.80"N; 12°08'01.68"E) in Denmark. All populations are situated at shallow lakes.

In each population 20-25 mated females were collected between the end of June and the beginning of July 2015. The females were placed individually in small plastic vials with wet filter paper to oviposit. After oviposition, the filter papers carrying eggs were transferred to the laboratory in Leuven (Belgium) and incubated at 20°C. In the first two months, larvae were reared in groups of 15 in 2 L containers. Thereafter, larvae were placed individually in plastic cups (7.5 cm height, 3.5 cm diameter) filled with 100 mL dechlorinated tap water. Throughout this period larvae were kept in a temperature-controlled room at a water temperature of 20°C and a photoperiod of 14:10h (L:D). Larvae were fed *Artemia* nauplii ad libitum once per day, five days per week.

To mimic realism all larvae were given the same artificial winter. At the end of September, we started simulating the natural fall and winter temperature and photoperiod conditions in Belgium (ca. halfway between both latitudes). Therefore, cups with larvae were

placed on shelves in an outdoor cage where the water temperature ranged between 4 and 12°C. Winter temperatures of 4°C occur at both the high latitude (Denmark, southern Sweden) and the low latitude (southern France) (data derived from the Flake model, Simmons, Uppala, Dee, & Kobayashi, 2007). Mid-January, larvae were placed back inside at 10°C in incubators. Thereafter, water temperatures were gradually raised: to 13°C (27 January), 15°C (29 January), 18°C (2 February) and finally to 22°C (4 February). From the simulated fall and winter onwards, larvae were fed *Artemia* nauplii ad libitum three days per week. We checked every two days for final instar larvae, starting 12 February.

Experimental design

We exposed final instar larvae from both latitudes for 13 days to all six combinations of 2 mean water temperatures (20°C and 24°C) × 3 daily water temperature fluctuations (0°C, 5°C, 10°C). The mean temperatures represent the mean summer water temperatures in the high-latitude (20°C) and low-latitude (24°C) ponds inhabited by the study species (De Block et al., 2013; Dinh Van et al., 2014). The daily water temperature fluctuations were chosen based on the maximum daily water temperature fluctuations during July in shallow clear freshwater bodies (< 1m) at the high latitude (5°C) and the low latitude (10°C). These temperature fluctuations were derived from the Flake model (Simmons et al., 2007) and validated with actual temperature data from shallow ponds at both latitudes (unpublished data). During the entire experiment water temperature data were collected using Hobo onset data loggers (TidbiT v2 Temp logger). In each temperature treatment, water temperatures were logged every 10 minutes throughout the whole experiment. The realized thermal regimes are shown in figure S1 in Appendix S1.

Under global warming a mean temperature increase of 4°C is predicted by 2100 under IPCC (2013) scenario RCP 8.5. Therefore, the current mean temperature (24°C) at the low latitude corresponds to the future mean temperature at the high latitude. Following Paaijmans

et al. (2013), expected patterns in DTF were estimated based on daily maximum and minimum temperatures predicted for 2080 under the RCP 8.5 scenario (IPCC 2013) using BCC_CSM 1.1 (Beijing Climate Center Climate System Model 1.1) and Delta Method IPCC AR5 at a spatial resolution of 2.5 min (consulted from http://www.ccafs-climated.org/Data). To obtain latitude-specific data, temperature data were imported in ArcGIS Pro 2.2. This model predicted an increase of DTF with 5°C by 2080. Therefore, we can conclude that the current maximum DTF (10°C) at the low latitude matches the future maximum DTF at the high latitude by 2100.

The thermal regimes started one day after the larvae molted into the final instar and ran for 13 days. At the start, larvae were transferred into transparent cups (4.4 cm height, 5.0 cm diameter) filled with 90 mL dechlorinated tap water. During the 13-day period, larvae received ad libitum *Artemia* nauplii seven days per week.

Response variables

Mortality during the 13-day period was low and varied between 0% and 2.5% among treatments. To estimate growth rates we weighed each larva two times (at the start and at the end of the 13-day growth experiment) to the nearest 0.01 mg using an electronic balance (Mettler Toledo® AB135-S, Zaventem, Belgium). Before weighing, we gently blotted the larvae dry with tissue paper; this gives reliable wet mass estimates which are strongly correlated with dry mass (Stoks, De Block, Van De Meutter, & Johansson, 2005). We estimated growth rates as [ln (final mass) – ln (initial mass)] / 13 days. The sample size for growth rate per treatment combination was 30 larvae (total of 360 larvae). After the final weighing, all larvae were stored at -80°C for further physiological analyses. Sample sizes for phenoloxidase (PO) and superoxide dismutase (SOD) varied between 28 and 30 larvae. The other physiological variables were measured on a subset of 18-20 larvae per treatment combination. For all physiological variables, exact sample sizes are given in the figures.

We quantified a set of physiological traits on the body supernatants using spectrophotometry. To obtain the body supernatant we homogenized the larvae (without head) in PBS buffer (Phosphoric Buffered Saline, 90% of the final mass x 15 µL) and centrifuged the mixture. To correct the enzyme activities, we measured protein content in the body supernatant of every sample using the Bradford (1976) method.

As a measure of immune function we quantified phenoloxidase (PO) activity using a modified protocol of Stoks, De Block, Slos, Van Doorslaer, and Rolff (2006). The enzyme PO plays a major role in the immune function of insects since it is involved in eliminating a variety of pathogens (Braun, Hoffmann, & Meister, 1998) and in wound repair (Sugumaran, 2002). We added 5 µL of the body supernatant, 15 µL PBS buffer and 3 µL chymotrypsin (1 mg mL^{-1} milliQ water) to wells of a 384-well microtiter plate. After the mixture incubated 5 minutes at room temperature, allowing the conversion of proPO to PO, we added 17 µL L-Dopa (1.97 mg mL^{-1} PBS). PO catalyses the transition from L-Dopa to dopachrome (Sugumaran, 2002). This reaction proceeded at 30°C for 30 minutes; we measured the absorbance of dopachrome every 20 seconds at 490 nm. We used the slope of the linear part of the reaction curve (time interval 1000-2000s) to quantify the PO activity. For statistical analyses we used the average of the duplicate readings per larva. One unit of PO activity is expressed in nmol dopachrome formed per minute per mg protein.

To quantify fat content we used a protocol of Marsh and Weinstein (1966), which was modified and optimized for damselfly larvae (Verheyen, Temmerman, De Block, & Stoks, 2018). After filling glass tubes with 8 µL body supernatant and 56 µL sulphuric acid (100%), tubes were first heated at 150°C for 20 minutes and then cooled down to add 64 µL of milliQ water. We filled a 380-well microtiter plate with 30 µL of the final mixture in triplicate per larva and measured the absorbance at 490 nm. Means of the triplicate readings were used in

255 statistical analyses and a standard curve of glyceryl tripalmitate was used to convert
256 absorbances into fat contents. Fat content was expressed in µg per mg wet mass.

257 As antioxidant enzymes we measured superoxide dismutase (SOD) and catalase
258 (CAT). SOD dismutates superoxide anions into hydrogen peroxide, which is further reduced
259 to water by CAT. We measured SOD activity using the SOD assay kit WST (Fluka, Buchs,
260 Austria) following the protocol of (De Block & Stoks, 2008a). We pipetted 20 µL of the body
261 supernatant, 2 µL of enzyme working solution and 40 µL of WST working solution in wells
262 of a 96-well microtiter plate. We measured the absorbance of formazan, which is formed by
263 the reduction of the tetrazolium salt WST-1 with superoxide anion, at 440 nm after a 20-
264 minute incubation at 37°C. SOD activity units were derived from inhibition rates that were
265 calculated using an inhibition curve made with commercial SOD from bovine erythrocytes (>
266 97% purity). One unit SOD is defined as the amount of SOD needed to inhibit the WST-1
267 reduction with 50%. SOD activity was expressed as units per mg protein. We measured CAT
268 activity using an established protocol for damselfly larvae (De Block & Stoks, 2008a;
269 Janssens & Stoks, 2013). First, we diluted the body supernatant by adding 75 µL PBS buffer
270 to 5 µL supernatant. We added 20 µL of the diluted body supernatant, 80 µL PBS and 100 µL
271 of 20 mM hydrogen peroxide in a well of a 96-well microtiter plate. We measured the
272 degradation of hydrogen peroxide every 15 seconds during 2.5 minutes at 240 nm. The slope
273 of the linear part of the absorbance reaction curve was used to calculate the CAT activity
274 (measured in duplicate). One CAT unit is defined as the amount of enzyme needed to degrade
275 1 nmol hydrogen peroxide per min per mg protein.

276 To measure the amount of malondealdehyde (MDA), a measure of oxidative damage
277 to lipids, we used the thiobarbituric acid assay (TBA assay) based on a modified protocol of
278 (Miyamoto, Almeida, Nogueira, Gennari de Medeiros, & Di Mascio, 2011). The mixture
279 existing of 50 µL TBA solution (0.4 %, in 0.1 M HCl) and 50 µL of body supernatant was

incubated at 90°C for 60 minutes. Afterwards, we added 165 µL butanol, mixed and centrifuged the final mixture at 845 g during 4 minutes. We pipetted 30 µL of the final mixture in triplicate in a 384-well microtiter plate and we measured fluorescence at an excitation/emission wavelength of 535/550 nm. We used the standard curve of 1,1,3,3-tetramethoxypropan 99% malonaldehyde bis (dimethyl acetol) 99% to calculate the concentration of MDA. MDA levels were expressed as nmol MDA per mg fat.

The electron transport system (ETS) activity is an estimate of metabolic rate and was measured using the protocol of De Coen and Janssen (2003). The ETS is a multi-enzyme complex that is localized in the inner membrane of the mitochondria, where it functions as a bridge between oxidizing organic matter and oxygen (G.-Tóth, Szabo, & Webb, 1995). We loaded wells of a 384-well microtiter plate in duplicate with 5 µL of the body supernatant and 15 µL buffered substrate solution (0.13 M Tris-HCl, 0.3% Triton X-100, 1.7 mM NADH, 250 µM NADPH, pH 8.5). Afterwards, we added 10 µL iodonitrotetrazolium (INT, 8 mM p-iodonitrotetrazolium), which replaces O_2 as electron acceptor and receives electrons from NADPH via NADH-cytochrome oxidoreductase with the formation of formazan as a result. We measured the increase in absorbance of formazan every 30 seconds during 5 minutes at 490 nm and 20°C. We calculated the formazan concentrations based on the Lambert-Beer law using a molecular extinction coefficient of 15.9 $mM^{-1}cm^{-1}$. These concentrations were converted to cellular oxygen consumption rates using the stoichiometric relationship that 1 µmol O_2 is used to form 2 µmol formazan in the ETS system. ETS activity was expressed as nmol O_2 per minute per mg protein.

Statistical analyses

All analyses were run in R 3.4.0. for Windows (R Core Team, 2014). We tested for effects of mean temperature, daily temperature fluctuation (DTF) and latitude on the different response variables using linear mixed models using the 'lme4' package (Bates, Mächler, Bolker, &

Walker, 2015). We calculated Wald chi-square statistics and p-values for fixed effects using the 'car' package (Fox & Weisberg, 2011). In each model we added population nested in latitude as random effect. Growth rate was square root transformed, while MDA levels, CAT and ETS activity were log transformed to meet ANOVA assumptions. We further analyzed significant effects of the daily temperature fluctuation (three levels) and its interactions by comparing the least-square means using Tukey posthoc tests with the 'lsmeans' package (Lenth, 2018).

Results

General patterns in larval growth rate

Low-latitude larvae had higher growth rates than high-latitude larvae across all thermal regimes (Table 1, Fig. 1). Mean temperature and DTF interacted (MeanT × DTF) and this interaction differed between latitudes (MeanT × DTF × Lat, Table 1, Fig. 1). While at both latitudes growth rate was higher at 24°C than at 20°C at 0°C DTF, and lower at 24°C than at 20°C at 10°C DTF, at the intermediate 5°C DTF growth of low-latitude larvae was faster at 24°C than at 20°C (Tukey $P = 0.037$), while growth of high-latitude larvae tended to be lower at 24°C than at 20°C ($P = 0.052$). This 3-way interaction also indicated different effects of DTF and its interaction with latitude between mean temperatures. Indeed, at a mean of 20°C DTF had no effect on growth rate (DTF: $\chi^2_2 = 3.92$, $P = 0.14$, DTF × Lat, $\chi^2_2 = 4.52$, $P = 0.10$, Fig. 1). Yet, at a mean of 24°C larval growth rate strongly decreased with increasing DTF ($\chi^2_2 = 104.97$, $P < 0.001$), and this further depended on latitude (DTF × Lat: $\chi^2_2 = 20.88$, $P < 0.001$, Fig. 1). In high-latitude larvae, the decrease in growth with increase in DTF at 24°C was stronger and already occurring at 5°C DTF (Tukey $P < 0.001$), while in low-latitude larvae the growth decrease was smaller and not yet present at 5°C DTF ($P = 0.39$).

Estimated effects of global warming on growth rate at the high latitude

Assuming only plastic thermal responses, 4°C warming will increase the growth rate of high-latitude larvae when DTF is absent (Tukey $P = 0.007$). When considering DTF, however, high-latitude larvae will grow slower at the predicted 24°C with 10°C DTF under global warming than at their current mean temperature of 20°C with 5°C DTF (Tukey $P < 0.001$).

Estimated effects of global warming on growth change considerably when we simulate thermal evolution by applying a space-for-time substitution where the growth of the high-latitude larvae gradually evolves into the current growth of the low-latitude larvae. In the absence of DTF, evolved high-latitude larvae will grow faster at 24°C compared to the current growth of the high-latitude larvae at 20°C and no DTF (Tukey $P < 0.001$), and more so than when they would show only a plastic thermal response (Tukey $P < 0.001$). When considering DTF, the increase in growth of high-latitude larvae under warming (24°C with 10°C DTF) will still be present compared to the not evolved high-latitude larvae at their current temperature conditions (20°C with 5°C DTF, Tukey $P = 0.005$).

Condition-related traits

Of the two condition-related traits, PO activity but not fat content was affected by the thermal treatments and their interactions (fat content: all $P > 0.36$, Table 1, Fig. 2a). Mean temperature and DTF interacted for PO activity (MeanT × DTF, Table 1, Fig. 2b), and this further depended on latitude (MeanT × DTF × Lat, Table 1, Fig. 2b). In high-latitude larvae, PO activity was lower at 24°C than at 20°C, but only at 10°C DTF (Tukey $P = 0.011$). In low-latitude larvae, however, PO activity was lower at 24°C than at 20°C at 0°C DTF ($P = 0.038$) and nearly so at 5°C DTF ($P = 0.097$). This 3-way interaction also indicated different effects of DTF and its interaction with latitude between mean temperatures. Indeed, PO activity was not affected by DTF and latitude at 20°C (Tukey, all $P > 0.44$), while at 24°C PO activities were higher in high-latitude larvae than in low-latitude larvae but only at 0°C DTF ($P = 0.005$) and 5°C DTF ($P = 0.036$). Furthermore, at 24°C PO activity in high-latitude larvae

decreased at 10°C DTF compared to 0°C (trend: $P = 0.10$) and 5°C DTF ($P = 0.036$), while in low-latitude larvae PO activity instead increased at 10°C DTF compared to 0°C DTF ($P = 0.036$).

Oxidative stress and damage

The effect of mean temperature on both antioxidant enzymes depended on DTF (MeanT × DTF, Table 1, Fig. 3). CAT activity was higher at 24°C than at 20°C but only at 0°C DTF (Tukey $P = 0.027$, at other DTFs: $P > 0.13$, Fig. 3a). Furthermore, CAT activity at 24°C tended to be lower at 10°C DTF than at 0°C DTF ($P = 0.098$). SOD activity was higher at 24°C than at 20°C but only at 10°C DTF (Tukey $P = 0.018$, at other DTF: $P > 0.20$, Fig. 3b). Furthermore, SOD activity at 24°C was higher at 10°C DTF than at 0°C DTF (Tukey $P = 0.046$). At 20°C, DTF did not affect CAT (all $P > 0.46$, Fig. 3a) and SOD activity (all $P > 0.53$, Fig. 3b). MDA levels were higher in low-latitude than in high-latitude larvae but only at 10°C DTF (Tukey $P = 0.045$, DTF × Lat, Table 1, Fig. 3c). Furthermore, MDA levels did not differ among DTFs in high-latitude larvae ($P > 0.46$), while in low-latitude larvae MDA levels were higher at 10°C DTF compared to 0°C DTF ($P = 0.033$).

Metabolic rate

Low-latitude larvae tended to have a higher ETS activity than high-latitude larvae (Table 1, Fig. 4). ETS activity did not differ between mean temperatures but was lower at 10°C DTF than at 0°C DTF (Tukey $P = 0.024$, main effect DTF, Table 1, Fig. 4).

Discussion

We found widespread effects of daily temperature fluctuation (DTF) on nearly all traits measured (except for fat content) confirming the biological importance of the daily temperature cycles that animals encounter in natural populations (Colinet et al., 2015). DTF, if anything, reduced growth and was stressful as indicated by reductions in body condition

(activity of phenoloxidase), antioxidant defense (activity of catalase) and metabolic rate (activity of the electron transport system), and an increase in oxidative damage (measured as malondialdehyde). Notably, most effects of DTF were strongly dependent on the mean temperature. Indeed, for four traits (growth rate, and the activity levels of PO, CAT and superoxide dismutase (SOD)), the effect of DTF was only present at 24°C and not at 20°C, indicating the thermal performance curve was linear at 20°C. Vice versa, the effect of 4°C warming strongly depended on DTF. Our findings indicate that while a 4°C increase in mean temperature was beneficial in the absence of DTF, this critically changed when DTFs were present. An important novel finding of our study was that, as expected, the effects of DTF also differed for several traits between latitudes, mostly supporting the idea that DTF was more costly for high-latitude larvae. Based on these latitudinal differences, the long-term effects of warming in the high-latitude population may strongly differ when taking into account gradual evolution and DTF.

General latitudinal patterns

Low-latitude larvae grew faster than high-latitude larvae in all thermal regimes. This confirms the latitudinal pattern in growth rate in the study species when using constant rearing temperatures (Dinh Van et al., 2014; Shama, Campero-Paz, Wegner, De Block, & Stoks, 2011; Stoks, Swillen, & De Block, 2012), and extends it to thermal regimes with DTF. Low-latitude populations have multiple generations per year (multivoltine), which selects for a faster life history (including growth rate) due to higher time constraints experienced per generation (Shama et al., 2011, for other insects: e.g. Ragland & Kingsolver, 2007). In contrast, the slower growing high-latitude populations complete one generation every two years (semivoltine) (Corbet et al., 2006), thereby experiencing less time constraints per generation. The faster life history of low-latitude larvae may also explain the trend for a higher metabolic rate (measured as ETS activity), confirming a higher respiration rate in the

low-latitude larvae (Debecker & Stoks, in press). Related to this, low-latitude larvae experienced more oxidative damage to lipids (measured as MDA) than high-latitude larvae at 10°C DTF. Oxidative damage occurs when antioxidant defense mechanisms fail to eliminate the produced ROS (reactive oxygen species) in time, causing an imbalance towards ROS (Monaghan et al., 2009). Associated with prioritizing rapid growth, low-latitude larvae also invest less energy in costly immune defense mechanisms (Stoks & De Block, 2011), observed here as a trend for a lower PO activity.

DTF shapes the effects of mean temperature

A key finding was that while larval growth rates were higher at 24°C than at 20°C in the absence of DTF, this pattern reversed at high DTF. The higher growth rates at 24°C and no DTF match previous studies where *I. elegans* larvae were reared at constant temperatures (Dinh Van et al., 2014; Shama et al., 2011; Stoks et al., 2012). This confirms that growth rates at 24°C are situated in the rising part of the thermal performance curve for growth. In contrast, larvae from both latitudes grew slower at 24°C than at 20°C when DTF was large (10°C) suggesting that during the high DTFs at 24°C temperatures regularly exceeded the optimal temperature for growth rate. Our results match the stronger reduction in survival in *A. stephensi* mosquito larvae when a higher DTF (12°C) was imposed around the thermal optimum (32°C) than a smaller DTF (8°C) (Paaijmans et al., 2013).

A growth reduction under DTF at a high mean temperature has been explained by the higher allocation of energy to the increased metabolic demands for cell maintenance (Colinet et al., 2015; Ruel & Ayres, 1999). However, in our study metabolic rate (measured as ETS activity), was instead lower at 10°C DTF compared to 0°C DTF. This matches the pattern that oxygen consumption in Chinese shrimp (*Fenneropenaeus chinensis*, Tian, Dong, Wang, & Wu, 2004) and in sea cucumbers (*Apostivhopus japonicas*, Dong & Dong, 2006) was lower when temperatures fluctuated than when constant at the same mean temperature. Intriguingly,

428 in both studies the decrease in oxygen consumption was assumed to underlie the opposite
429 growth pattern than we observed, namely a higher growth rate under fluctuating compared to
430 constant temperatures (Dong & Dong, 2006; Tian et al., 2004). The relationship between
431 metabolic rate and growth rate can, however, be positive, absent or negative (Glazier, 2015).
432 Apparently, in our study when too high temperatures were reached for a given acclimation
433 temperature during a daily cycle, larvae reduced both their metabolism and when at 24°C also
434 their growth rate. This resembles the hypometabolic response that is shown in many animals,
435 including damselfly larvae (Dinh Van, Janssens, & Stoks, 2016), at stressful temperatures
436 (Storey, 2015).

437 To the best of our knowledge, we provide the first demonstration that the modulation
438 of the effect of the mean temperature by DTF has a geographic component indicating local
439 thermal adaptation. Indeed, the increase in growth rate going from a mean temperature of
440 20°C to 24°C at a constant temperature (0°C DTF), was still present at low (5°C) DTF in low-
441 latitude larvae but no longer in high-latitude larvae. At high (10°C) DTF growth rate was
442 lower at a mean of 24°C than at 20°C at both latitudes. This suggests that stressful (above the
443 thermal optimum for growth) upper temperatures during a day-night cycle at the mean
444 temperature of 24°C were already reached at lower DTF in high-latitude larvae than in low-
445 latitude larvae. This observation is consistent with local thermal adaptation as the mean
446 summer water temperature is lower in the high-latitude populations (20°C) than in the low-
447 latitude populations (24°C) (De Block et al., 2013). In support of this, in the study by Shama
448 et al. (2011), growth rate of the low-latitude larvae was still steadily increasing at the highest
449 test temperature used in that study (24°C) while growth rate of the high-latitude larvae was
450 already considerably levelling off at 24°C, indicating 24°C to be close to the thermal optimum
451 for high-latitude populations and the thermal optimum to be higher in low-latitude
452 populations than in high-latitude populations. At high (10°C) DTF, the upper temperatures

453 came close to 30°C, which apparently was also stressful in low-latitude larvae. This is
454 supported by the fact that low-latitude *I. elegans* adults suffered a reduced flight ability when
455 reared as larvae at 30°C (Arambourou, Sanmartín-Villar, & Stoks, 2017). Geographic
456 differences in the effect of DTF have been largely ignored. The three other studies could not
457 find geographic differences in the effect of DTV on performance traits despite differences in
458 both mean temperature and DTF across populations from different latitudes (Hong & Shurin,
459 2015; Manenti et al. 2017; Ragland & Kingsolver, 2008). Our results suggest that one reason
460 for differences among studies is that the geographic signal may only be detected at low DTF
461 and not at high DTF.

462 DTF also differently shaped the effect of 4°C warming on immune function between
463 latitudes. Indeed, 4°C warming reduced the PO activity only at 10°C DTF in high-latitude
464 larvae, while it reduced the PO activity only at 0°C (and trend at 5°C) DTF in low-latitude
465 larvae. For the high-latitude larvae that experience colder temperatures in their ponds of
466 origin, a mean temperature 24°C with 10°C likely resulted in temperatures crossing the
467 thermal optimum for immune function. Apparently, the optimum temperature for PO activity
468 is slightly higher than for growth rate as a mean temperature of 24°C with 5°C DTF already
469 caused a reduction in growth rate. Differences in thermal sensitivity among traits are a general
470 phenomenon (Colinet et al., 2015, Sinclair et al., 2016). In contrast, the lower PO activity of
471 the low-latitude larvae at a mean temperature of 24°C compared to 20°C when no or 5°C DTF
472 was present, likely was driven by a trade-off with their high investment in growth rate under
473 low DTF. Such trade-off between rapid growth and lower PO activity has been
474 experimentally demonstrated in damselfly larvae (e.g. De Block and Stoks, 2008b). This
475 trade-off is less expected at 24°C combined with 10°C DTF where low-latitude larvae
476 reduced their growth rate.

477 *Possible implications for effects of global warming in the high-latitude populations*

The interactive effects between mean temperature, DTF and latitude have important implications for the predicted effect of 4°C warming (based on IPCC (2013) scenario RCP8.5) at the high latitude. Indeed, while mild warming is thought to be beneficial for temperate ectotherms (Deutsch et al., 2008), our results indicate this may critically depend on the degree of DTF. This supports similar findings based on simulation studies (Estay et al., 2014; Vasseur et al., 2014) and on an empirical study on mosquito larvae (Paaijmans et al., 2013). Because under global warming both increases in mean temperature and in DTF are expected (models: Easterling et al., 2000; empirical data: Katz, Brush, & Parlange, 2005), considering this interaction is highly relevant to arrive at more realistic predictions of the effects of global warming on fitness-related traits (Colinet et al., 2015; Folguera et al., 2011; Vasseur et al., 2014). Assuming no thermal evolution, 4°C warming would plastically increase growth in high-latitude larvae in the absence of DTF, yet would decrease growth in the more realistic scenarios with 5°C and 10°C DTF. DTFs around a warmer mean temperature daily generate extreme temperatures which may neutralize any performance advantages established by the increase in mean temperature alone (Vasseur et al., 2014). This strong interaction between mean temperature and DTF confirms that caution is needed when using constant temperatures to study the plastic responses to warming because this may not reflect changes in performance under more realistic conditions (Carrington, Armijos, Lambrechts, Barker, & Scott, 2013; Kingsolver, Higgins, & Augustine, 2015; Paaijmans et al., 2013; Ragland & Kingsolver, 2008; Vasseur et al., 2014).

These predictions based on current plastic thermal responses in the high-latitude population strongly changed when we assessed the impact of the more realistic scenario of gradual thermal evolution at the high latitude using a space-for-time substitution (De Frenne et al., 2013; Stoks et al., 2014). We thereby built on the latitudinal pattern in thermal adaptation and assumed that by 2100 the mean temperature at the high latitude will raise to

503 24°C and the DTF to 10°C (based on IPCC (2013) scenario RCP8.5), thereby matching the
504 current thermal regime at the low latitude. This approach predicts that under gradual thermal
505 evolution where the high-latitude larvae evolve to have the same ability as the low-latitude
506 larvae to deal with a mean of 24°C and 10°C DTF, the evolved high-latitude larvae would no
507 longer suffer a growth reduction, but instead would increase growth rate. Hence, our results
508 suggest that gradual evolution has the potential to reverse the emerging insight that
509 temperature variation will make ectotherms more vulnerable to future warming (Paaijmans et
510 al., 2013; Vasseur et al., 2014; this study). Note that the high levels of gene flow in the study
511 species (Shama et al., 2011) may facilitate the gradual evolution in northern populations by
512 providing alleles from low-latitude populations that are pre-adapted to warmer conditions
513 (Paul, Sheth, & Angert, 2011). Taken together, our combined study of the effects of increases
514 in mean temperature and DTF at two strategically chosen latitudes provided important proof-
515 of-principle that integrating gradual thermal evolution and the expected increase in DTF may
516 generate different and even opposing predictions of the effect of global warming.

517 To conclude, our results add to the increasing insight that mean temperature and DTF
518 can not only strongly impact individual performance and physiology but may do so in an
519 interactive way (Colinet et al., 2015; Estay et al., 2014; Stoks et al., 2017). This supports the
520 call for their integrated study when assessing the impact of climate change on species (Boher,
521 Trefault, Estay, & Bozinovic, 2016; Estay et al., 2014). We added an important dimension to
522 this topic by showing predictable latitudinal differences in how species react to such
523 integrated warming scenarios that consider both increases in mean temperature and in DTF.
524 This highlights the importance of jointly considering gradual thermal evolution and DTF
525 when predicting the impact of global warming on species.

526 **Acknowledgements**

We thank Philippe Lambret, Dinh Van Khuong, Kent Olsen and Ulf Norling for collecting the damselfly eggs, Ria Van Houdt for the physiological analyses and assistance during the experiment, Geert Neyens and Rony Van Aerschot for technical support and Vienna Delnat for assistance during the experiment. The associate editor and three anonymous reviewers provided feedback that improved our manuscript. The authors declare no conflict of interest. JV is a PhD fellow of the Fund for Scientific Research Flanders (FWO). Financial support came from FWO grant G.0524.17 and KU Leuven grant C16/17/002.

Authors' contributions

JV and RS conceived and designed the experiments. JV performed the experiments and analyzed the data. Both authors wrote the manuscript and gave final approval for publication.

Data accessibility

Data are available from Dryad Digital Repository https://doi.org/10.5061/dryad.90473mt (Verheyen & Stoks, 2018).

References

Addo-Bediako, A., Chown, S. L., & Gaston, K. J. (2000). Thermal tolerance, climatic variability and latitude. *Proceedings of the Royal Society of London B: Biological Sciences*, *267*, 739–745. doi:10.1098/rspb.2000.1065

Angilletta, M. J., Huey, R. B., & Frazier, M. R. (2009). Thermodynamic effects on organismal performance: is hotter better? *Physiological and Biochemical Zoology*, *83*, 197–206. doi:10.1086/648567

Arambourou, H., Sanmartín-Villar, I., & Stoks, R. (2017). Wing shape-mediated carry-over effects of a heat wave during the larval stage on post-metamorphic locomotor ability. *Oecologia*, *184*, 279–291. doi:10.1007/s0044

Bates, D., Mächler, M., Bolker, B., & Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*, 1–48.

Boher, F., Trefault, N., Estay, S. A., & Bozinovic, F. (2016). Ectotherms in variable thermal landscapes: A physiological evaluation of the invasive potential of fruit flies species. *Frontiers in Physiology*, *7*, 1–6. doi:10.3389/fphys.2016.00302

Boudot, J. P., & Kalkman, V. J. (2015). *Atlas of the dragonflies and damselflies of Europe*. Netherlands: KNNV uitgeverij.

Bozinovic, F., Bastías, D. A., Boher, F., Clavijo-Baquet, S., Estay, S. A., & Angilletta, M. J. J. (2011). The mean and variance of environmental temperature interact to determine physiological tolerance and fitness. *Physiological and Biochemical Zoology*, *84*, 543–552. doi:10.1086/662551

Bozinovic, F., Medina, N. R., Alruiz, J. M., Cavieres, G., & Sabat, P. (2016). Thermal tolerance and survival responses to scenarios of experimental climatic change: changing thermal variability reduces the heat and cold tolerance in a fly. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, *186*, 581–587. doi:10.1007/s00360-016-0980-6

Bozinovic, F., Sabat, P., Rezende, E. L., & Canals, M. (2016). Temperature variability and thermal performance in ectotherms: Acclimation, behaviour, and experimental considerations. *Evolutionary Ecology Research*, *17*, 111–124.

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

Braun, A., Hoffmann, J. A., & Meister, M. (1998). Analysis of the Drosophila host defense in

573 domino mutant larvae, which are devoid of hemocytes. *Proceedings of the National*
574 *Academy of Sciences*, *95*, 14337–14342. doi:10.1073/pnas.95.24.14337

575 Carrington, L. B., Armijos, M. V., Lambrechts, L., Barker, C. M., & Scott, T. W. (2013).
576 Effects of fluctuating daily temperatures at critical thermal extremes on Aedes aegypti.
577 *PLoS ONE*, *8*, e58824. doi:10.1371/journal.pone.0058824

578 Colinet, H., Sinclair, B. J., Vernon, P., & Renault, D. (2015). Insects in fluctuating thermal
579 environments. *Annual Review of Entomology*, *60*, 123–140. doi:10.1146/annurev-ento-
580 010814-021017

581 Conover, D. O., Duffy, T. A., & Hice, L. A. (2009). The covariance between genetic and
582 environmental influences across ecological gradients: Reassessing the evolutionary
583 significance of countergradient and cogradient variation. *Annals of the New York*
584 *Academy of Sciences*, *1168*, 100–129. doi:10.1111/j.1749-6632.2009.04575.x

585 Corbet, P. S., Suhling, F., & Soendgerath, D. (2006). Voltinism of odonata: A review.
586 *International Journal of Odonatology*, *9*, 1–44. doi:10.1080/13887890.2006.9748261

587 De Block, M., Pauwels, K., Van Den Broeck, M., De Meester, L., & Stoks, R. (2013). Local
588 genetic adaptation generates latitude-specific effects of warming on predator-prey
589 interactions. *Global Change Biology*, *19*, 689–696. doi:10.1111/gcb.12089

590 De Block, M., & Stoks, R. (2008a). Compensatory growth and oxidative stress in a damselfly.
591 *Proceedings of The Royal Society B*, *275*, 781–785. doi:10.1098/rspb.2007.1515

592 De Block, M., & Stoks, R. (2008b). Short-term larval food stress and associated
593 compensatory growth reduce adult immune function in a damselfly. *Ecological*
594 *Entomology*, *33*, 796–801. doi:10.1111/j.1365-2311.2008.01024.x

595 De Coen, W. M., & Janssen, C. R. (2003). The missing biomarker link: relationships between

effects on the cellular energy allocation biomarker of toxicant-stressed Daphnia magna and corresponding population characteristics. *Environmental Toxicology and Chemistry*, *22*, 1632–1641. doi:10.1002/etc.5620220727

De Frenne, P., Graae, B. J., Rodríguez-Sánchez, F., Kolb, A., Chabrerie, O., Decocq, G., … Verheyen, K. (2013). Latitudinal gradients as natural laboratories to infer species' responses to temperature. *Journal of Ecology*, *101*, 784–795. doi:10.1111/1365-2745.12074

Debecker, S., & Stoks, R. (n.d.). Pace of life syndrome under warming and pollution: integrating life history, behavior and physiology across latitudes. *Ecological Monographs, in press*.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., & Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Sciences USA*, *105*, 6668–6672. doi:10.1073/pnas.0709472105

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. *Journal of Applied Ecology*, *51*, 919–928. doi:10.1111/1365-2664.12269

Dinh Van, K., Janssens, L., & Stoks, R. (2016). Exposure to a heat wave under food limitation makes an agricultural insecticide lethal: a mechanistic laboratory experiment. *Global Change Biology*, *22*, 3361–3372. doi:10.1111/gcb.13415

Dong, Y., & Dong, S. (2006). Growth and oxygen consumption of the juvenile sea cucumber Apostichopus japonicus (Selenka) at constant and fluctuating water temperatures. *Aquaculture Research*, *37*, 1327–1333. doi:10.1111/j.1365-2109.2006.01570.x

620 Easterling, D. R., Meehl, G. A., Parmesan, C., Changnon, S. A., Karl, T. R., & Mearns, L. O.
621 (2000). Climate Extremes: Observations, Modeling, and Impacts. *Science*, *289*, 2068–
622 2075. doi:10.1126/science.289.5487.2068

623 Estay, S. A., Lima, M., & Bozinovic, F. (2014). The role of temperature variability on insect
624 performance and population dynamics in a warming world. *Oikos*, *123*, 131–140.
625 doi:10.1111/j.1600-0706.2013.00607.x

626 Folguera, G., Bastías, D. a, Caers, J., Rojas, J. M., Piulachs, M.-D., Bellés, X., & Bozinovic,
627 F. (2011). An experimental test of the role of environmental temperature variability on
628 ectotherm molecular, physiological and life-history traits: implications for global
629 warming. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative*
630 *Physiology*, *159*, 242–246. doi:10.1016/j.cbpa.2011.03.002

631 Fox, J., & Weisberg, S. (2011). *An {R} Companion to Aplied Regression* (Second). Thousand
632 Oaks CA: Sage Publications. Retrieved from
633 http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

634 Fukami, T., & Wardle, D. A. (2005). Long-term ecological dynamics: reciprocal insights from
635 natural and anthropogenic gradients. *Proceedings of the Royal Society B: Biological*
636 *Sciences*, *272*, 2105–2115. doi:10.1098/rspb.2005.3277

637 G.-Tóth, L., Szabo, M., & Webb, D. J. (1995). Adaptation of the tetrazolium reduction test for
638 the measurement of the electron transport system (ETS) activity during embryonic
639 development of medaka. *Journal of Fish Biology*, *46*, 835–844. doi:10.1111/j.1095-
640 8649.1995.tb01606.x

641 Glazier, D. S. (2015). Is metabolic rate a universal “pacemaker” for biological processes?
642 *Biological Reviews*, *90*, 377–407. doi:10.1111/brv.12115

643 Hassall, C., & Thompson, D. J. (2008). The effects of environmental warming on Odonata: a
644 review. *International Journal of Odonatology*, *11*, 131–153.
645 doi:10.1080/13887890.2008.9748319

646 Hong, B. C., & Shurin, J. B. (2015). Latitudinal variation in the response of tidepool
647 copepods to mean and daily range in temperature. *Ecology*, *96*, 2348–2359.
648 doi:10.1890/14-1695.1

649 IPCC. (2013). *Climate Change 2013: The Physical Science Basis. Contribution of Working*
650 *Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate*
651 *Change.* Cambridge (United Kingdom) and New York (USA).

652 Janssens, L., & Stoks, R. (2013). Synergistic effects between pesticide stress and predator
653 cues: Conflicting results from life history and physiology in the damselfly Enallagma
654 cyathigerum. *Aquatic Toxicology*, *132–133*, 92–99. doi:10.1016/j.aquatox.2013.02.003

655 Katz, R. W., Brush, G. S., & Parlange, M. B. (2005). Statistics of Extremes:Moldeling
656 Ecological Disturbances. *Ecology*, *86*, 1124–1134. doi:10.1890/04-0606

657 Kern, P., Cramp, R. L., & Franklin, C. E. (2015). Physiological responses of ectotherms to
658 daily temperature variation. *Journal of Experimental Biology*, *218*, 3068–3076.
659 doi:10.1242/jeb.123166

660 Kingsolver, J. G., Higgins, J. K., & Augustine, K. E. (2015). Fluctuating temperatures and
661 ectotherm growth: distinguishing non-linear and time-dependent effects. *Journal of*
662 *Experimental Biology*, *218*, 2218–2225. doi:https://doi.org/10.1242/jeb.120733

663 Korsloot, A., van Gestel, C. A. M., & van Straalen, N. M. (2004). *Environmental stress and*
664 *cellular response in arthropods*. CRC, Boca Raton.

665 Lenth, R. V. (2018). *Package "lsmeans."* Retrieved from http://cran.r-

666 project.org/web/packages/lsmeans/lsmeans.pdf. Accessed 11 May 2018

667 Manenti, T., Sørensen, J. G., & Loeschcke, V. (2017). Environmental heterogeneity does not
668 affect levels of phenotypic plasticity in natural populations of three Drosophila species.
669 *Ecology and Evolution*, *7*, 2716–2724. doi:10.1002/ece3.2904

670 Marsh, J. B., & Weinstein, D. B. (1966). Simple charring method for determination of lipids.
671 *Journal of Lipid Research*, *7*, 574–576.

672 Martin, T. L., & Huey, R. B. (2008). Why "Suboptimal" Is Optimal: Jensen's Inequality and
673 Ectotherm Thermal Preferences. *The American Naturalist*, *171*, e102–e118.
674 doi:10.1086/527502

675 McMillan, D. M., Fearnley, S. L., Rank, N. E., & Dahlhoff, E. P. (2005). Natural temperature
676 variation affects larval survival, development and Hsp70 expression in a leaf beetle.
677 *Functional Ecology*, *19*, 844–852. doi:10.1111/j.1365-2435.2005.01031.x

678 Miyamoto, S., Almeida, E. A., Nogueira, L., Gennari de Medeiros, M. H., & Di Mascio, P.
679 (2011). *Evaluation of malondialdehyde levels. Oxidative Stress in Aquatic Ecosystems*.
680 Chichester, UK: John Wiley & Sons.

681 Monaghan, P., Metcalfe, N. B., & Torres, R. (2009). Oxidative stress as a mediator of life
682 history trade-offs: Mechanisms, measurements and interpretation. *Ecology Letters*, *12*,
683 75–92. doi:10.1111/j.1461-0248.2008.01258.x

684 Nyamukondiwa, C., Kleynhans, E., & Terblanche, J. S. (2010). Phenotypic plasticity of
685 thermal tolerance contributes to the invasion potential of Mediterranean fruit flies
686 (*Ceratitis capitata*). *Ecological Entomology*, *35*, 565–575. doi:10.1111/j.1365-
687 2311.2010.01215.x

688 Paaijmans, K. P., Heinig, R. L., Seliga, R. A., Blanford, J. I., Blanford, S., Murdock, C. C., &

689 Thomas, M. B. (2013). Temperature variation makes ectotherms more sensitive to
690 climate change. *Global Change Biology*, *19*, 2373–2380. doi:10.1111/gcb.12240

691 Paul, J. R., Sheth, S. N., & Angert, A. L. (2011). Quantifying the Impact of Gene Flow on
692 Phenotype-Environment Mismatch: A Demonstration with the Scarlet Monkeyflower
693 Mimulus cardinalis. *The American Naturalist*, *178*, S62–S79. doi:10.1086/661781

694 R Core Team. (2014). R: A language and environment for statistical computing. *R*
695 *Foundation for Statistical Computing*. Vienna, Austria. Retrieved from http://www.r-
696 project.org/

697 Ragland, G. J., & Kingsolver, J. G. (2007). Influence of seasonal timing on thermal ecology
698 and thermal reaction norm evolution in Wyeomyia smithii. *Journal of Evolutionary*
699 *Biology*, *20*, 2144–2153. doi:10.1111/j.1420-9101.2007.01428.x

700 Ragland, G. J., & Kingsolver, J. G. (2008). The effect of fluctuating temperatures on
701 ectotherm life-history traits: Comparisons among geographic populations of Wyeomyia
702 smithii. *Evolutionary Ecology Research*, *10*, 29–44. doi:10.1111/j.1558-
703 5646.2008.00367.x

704 Rolff, J., & Joop, G. (2002). Estimating condition: pitfalls of using weight as a fitness
705 correlate. *Evolutionary Ecology Research*, *4*, 931–935.

706 Ruel, J. J., & Ayres, M. P. (1999). Jensen's inequality predicts effects of environmental
707 variation. *Trends in Ecology and Evolution*, *14*, 361–366. doi:10.1016/S0169-
708 5347(99)01664-X

709 Shama, L. N. S., Campero-Paz, M., Wegner, K. M., De Block, M., & Stoks, R. (2011).
710 Latitudinal and voltinism compensation shape thermal reaction norms for growth rate.
711 *Molecular Ecology*, *20*, 2929–2941. doi:10.1111/j.1365-294X.2011.05156.x

712 Simmons, A., Uppala, S. M., Dee, D., & Kobayashi, S. (2007). ERA-Interim: New ECMWF
713 reanalysis products from 1989 onwards. *ECMWF Newsletter*, *110*, 25–35.
714 doi:10.21957/pocnex23c6

715 Sinclair, B. J., Marshall, K. E., Sewell, M. A., Levesque, D. L., Willett, C. S., Slotsbo, S., …
716 Huey, R. B. (2016). Can we predict ectotherm responses to climate change using thermal
717 performance curves and body temperatures? *Ecology Letters*, *19*, 1372–1385.
718 doi:10.1111/ele.12686

719 Stoks, R., & Córdoba-Aguilar, A. (2012). Evolutionary ecology of Odonata: a complex life
720 cycle perspective. *Annual Review of Entomology*, *57*, 249–265. doi:10.1146/annurev-
721 ento-120710-100557

722 Stoks, R., & De Block, M. (2011). Rapid growth reduces cold resistance: Evidence from
723 latitudinal variation in growth rate, cold resistance and stress proteins. *PLoS ONE*, *6*,
724 e16935. doi:10.1371/journal.pone.0016935

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

728 Stoks, R., De Block, M., Van De Meutter, F., & Johansson, F. (2005). Predation cost of rapid
729 growth: Behavioural coupling and physiological decoupling. *Journal of Animal Ecology*,
730 *74*, 708–715. doi:10.1111/j.1365-2656.2005.00969.x

731 Stoks, R., Geerts, A. N., & De Meester, L. (2014). Evolutionary and plastic responses of
732 freshwater invertebrates to climate change: Realized patterns and future potential.
733 *Evolutionary Applications*, *7*, 42–55. doi:10.1111/eva.12108

734 Stoks, R., Swillen, I., & De Block, M. (2012). Behaviour and physiology shape the growth

735 accelerations associated with predation risk, high temperatures and southern latitudes in
736 Ischnura damselfly larvae. *Journal of Animal Ecology*, *81*, 1034–1040.
737 doi:10.1111/j.1365-2656.2012.01987.x

738 Stoks, R., Verheyen, J., Van Dievel, M., & Tüzün, N. (2017). Daily temperature variation and
739 extreme high temperatures drive performance and biotic interactions in a warming world.
740 *Current Opinion in Insect Science*, *23*(F), 35–42. doi:10.1016/j.cois.2017.06.008

741 Storey, K. B. (2015). Regulation of hypometabolism: insights into epigenetic controls.
742 *Journal of Experimental Biology*, *218*, 150–159. doi:10.1242/jeb.106369

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

746 Thompson, R. M., Beardall, J., Beringer, J., Grace, M., & Sardina, P. (2013). Means and
747 extremes: Building variability into community-level climate change experiments.
748 *Ecology Letters*, *16*, 799–806. doi:10.1111/ele.12095

749 Tian, X., Dong, S., Wang, F., & Wu, L. (2004). The effects of temperature changes on the
750 oxygen consumption of juvenile Chinese shrimp Fenneropenaeus chinensis Osbeck.
751 *Journal of Experimental Marine Biology and Ecology*, *310*, 59–72.
752 doi:10.1016/j.jembe.2004.04.002

753 Vasseur, D. A., Delong, J. P., Gilbert, B., Greig, H. S., Harley, C. D. G., McCann, K. S., …
754 O'Connor, M. I. (2014). Increased temperature variation poses a greater risk to species
755 than climate warming. *Proceedings of the Royal Society B*, *281*, 1–8.
756 doi:10.1098/rspb.2013.2612

757 Vázquez, D. P., Gianoli, E., Morris, W. F., & Bozinovic, F. (2015). Ecological and

758 evolutionary impacts of changing climatic variability. *Biological Reviews*, *7*, 1–21.
759 doi:10.1111/brv.12216

760 Verheyen, J., & Stoks, R. (2018). Data from: Temperature variation makes an ectotherm more
761 sensitive to global warming unless thermal evolution occurs. *Dryad Digital Repository*,
762 https://doi.org/10.5061/dryad.90473mt

763 Verheyen, J., Temmerman, K., De Block, M., & Stoks, R. (2018). Voltinism-associated
764 differences in winter survival across latitudes: integrating growth, physiology, and food
765 intake. *Oecologia*, *186*, 919–929. doi:10.1007/s00442-018-4079-5

766 Wang, G., & Dillon, M. E. (2014). Recent geographic convergence in diurnal and annual
767 temperature cycling flattens global thermal profiles. *Nature Climate Change*, (11), 988–
768 992. doi:10.1038/nclimate2378

769 Wogan, G. O. U., & Wang, I. J. (2017). The value of space-for-time substitution for studying
770 fine-scale microevolutionary processes. *Ecography*, *40*, 1–12. doi:10.1111/ecog.03235

771 Woodward, G., Perkins, D. M., & Brown, L. E. (2010). Climate change and freshwater
772 ecosystems: impacts across multiple levels of organization. *Philosophical Transactions*
773 *of the Royal Society of London B*, *365*, 2093–2106. doi:10.1098/rstb.2010.0055

774

Table 1. The results of linear mixed models testing for the effects of mean temperature (MeanT), daily temperature fluctuation (DTF) and latitude (Lat) on larval growth rate and a set of physiological traits in larvae of *Ischnura elegans*. Physiological traits analyzed were phenoloxidase (PO) activity, fat content, activities of catalase (CAT) and superoxide dismutase (SOD), malondialdehyde (MDA) levels and electron transport system (ETS) activity. Significant *P*-values (< 0.05) are indicated in bold, trends (*P* < 0.10) are underlined.

	Growth rate		Fat content		PO activity		CAT activity		SOD activity		MDA levels		ETS activity	
Effect	χ^2_2	*P*	χ^2_2	*P*	χ^2_2	*P*	χ^2_2	*P*	χ^2_2	*P*	χ^2_2	*P*	χ^2_2	*P*
MeanT	12.61	**<0.001**	0.0002	0.99	0.011	0.92	2.74	0.098	1.22	0.27	1.74	0.19	0.018	0.89
DTF	4.10	0.13	2.05	0.36	1.90	0.39	1.49	0.48	0.80	0.67	1.29	0.52	7.49	**0.024**
Lat	24.87	**<0.001**	0.15	0.70	2.77	0.096	1.19	0.27	0.13	0.71	1.39	0.24	2.76	0.097
MeanT × DTF	34.19	**<0.001**	1.59	0.45	7.97	**0.019**	5.22	0.074	6.74	**0.034**	3.24	0.20	0.62	0.73
MeanT × Lat	1.09	0.29	0.74	0.39	2.42	0.12	0.067	0.80	1.34	0.25	2.01	0.16	0.35	0.55
DTF × Lat	4.13	0.13	1.08	0.58	1.59	0.45	2.44	0.30	0.071	0.97	4.91	0.086	0.73	0.69
MeanT × DTF × Lat	9.13	**0.010**	0.19	0.91	6.45	**0.040**	0.66	0.72	4.47	0.11	1.52	0.47	1.12	0.57

Figure legends

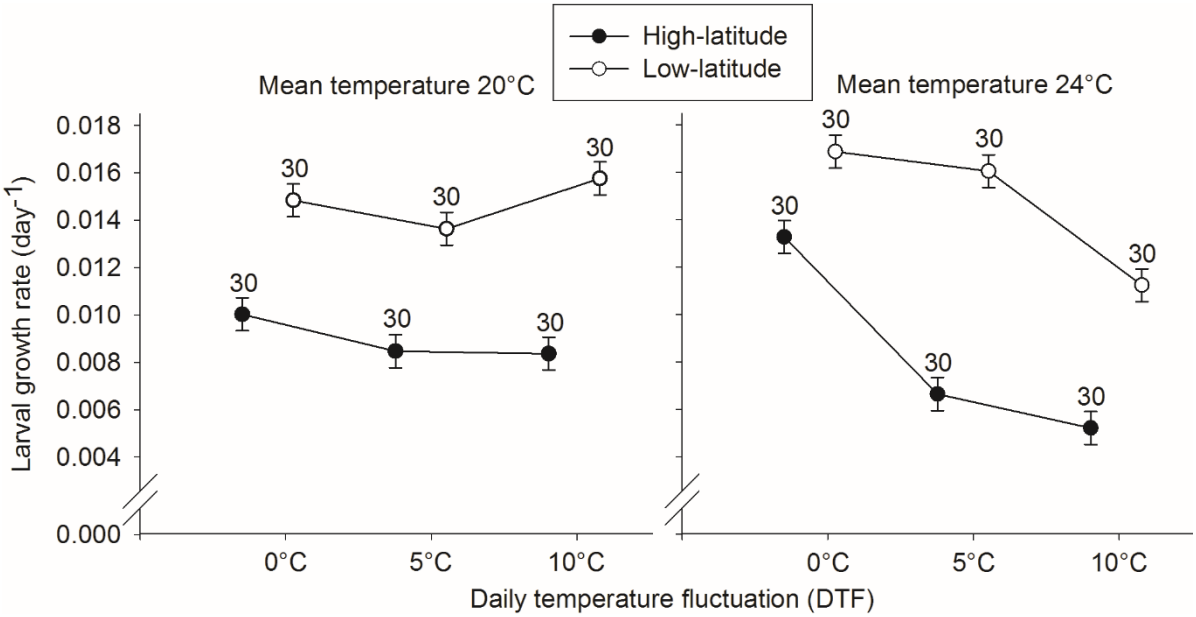
Figure 1. Mean (+ 1 SE) larval growth rate of *Ischnura elegans* as a function of mean temperature, daily temperature fluctuation (DTF) and latitude. Numbers denote sample sizes.

Figure 2. Mean (+ 1 SE) phenoloxidase (PO) activity (a-b) and fat content (c-d) of *Ischnura elegans* as a function of mean temperature, daily temperature fluctuation (DTF) and latitude. Numbers denote sample sizes.

Figure 3. Mean (+ 1 SE) catalase (CAT) activity (a-b), superoxide dismutase (SOD) activity (c-d) and levels of oxidative damage to lipids (MDA) (e-f) of *Ischnura elegans* as a function of mean temperature, daily temperature fluctuation (DTF) and latitude. Numbers denote sample sizes.

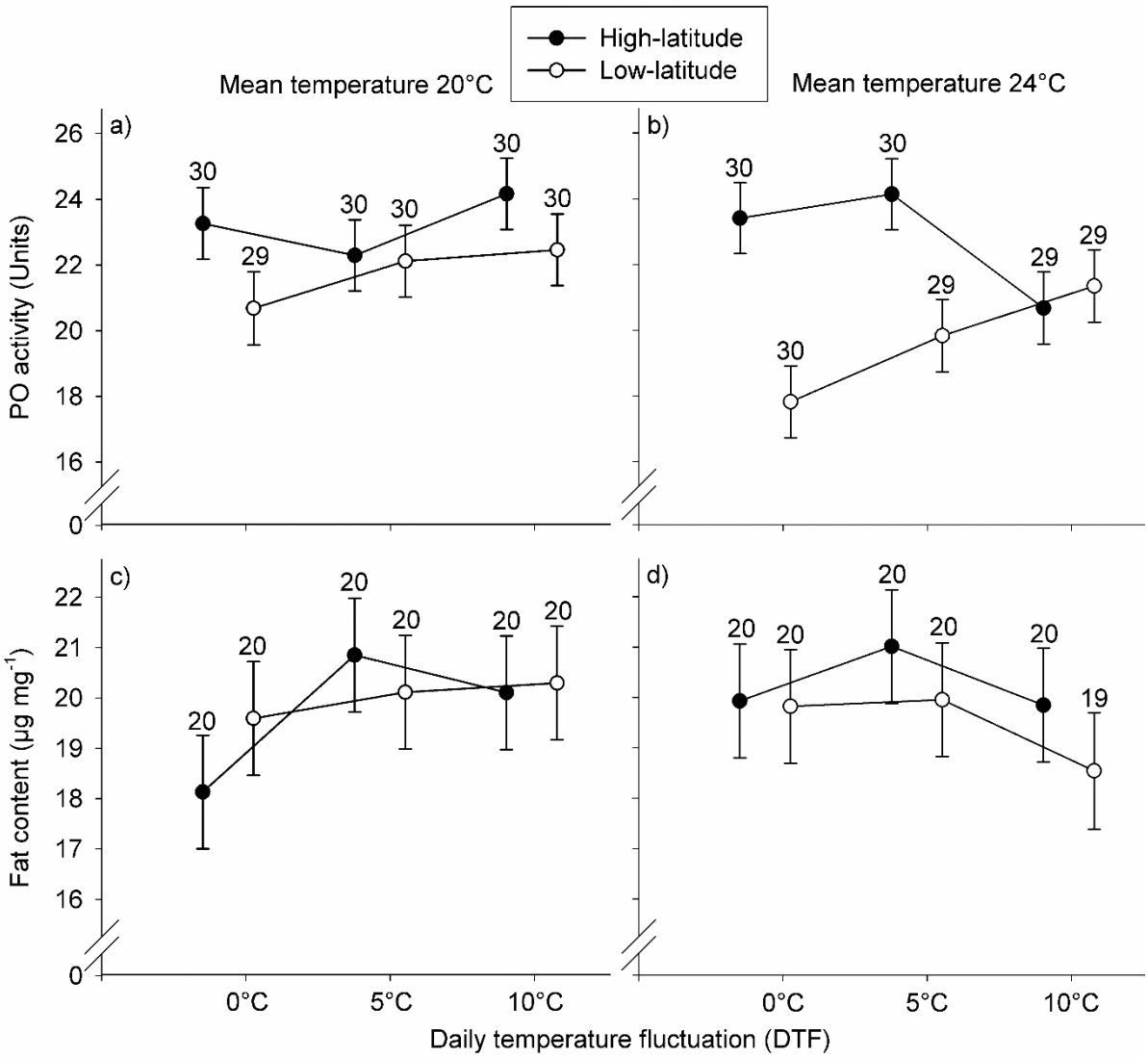
Figure 4. Mean (+ 1 SE) electron transport system (ETS) activity (a-b) of *Ischnura elegans* as a function of mean temperature, daily temperature fluctuation (DTF) and latitude. Numbers denote sample sizes.

790 **Figure 1**



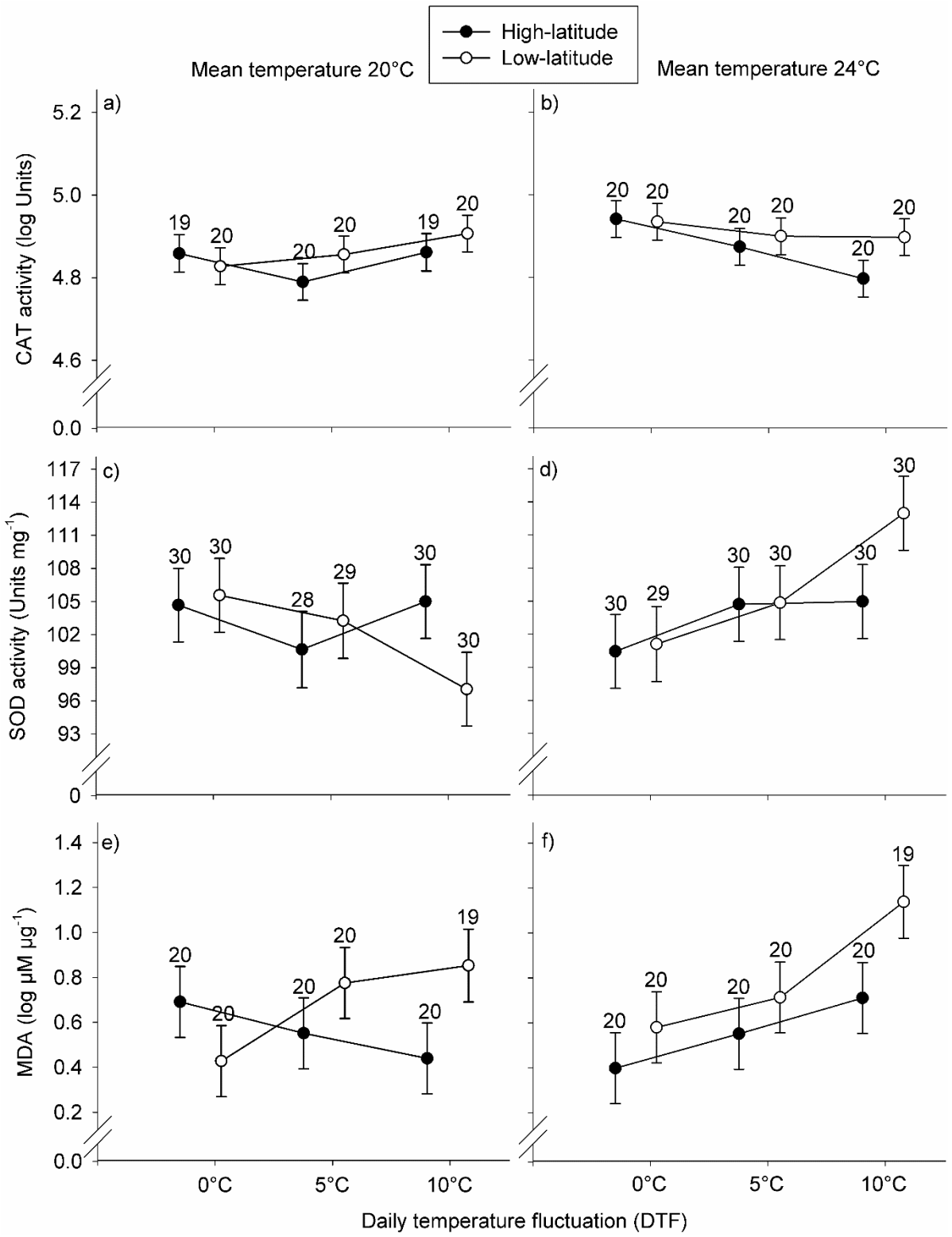
791

792 **Figure 2**



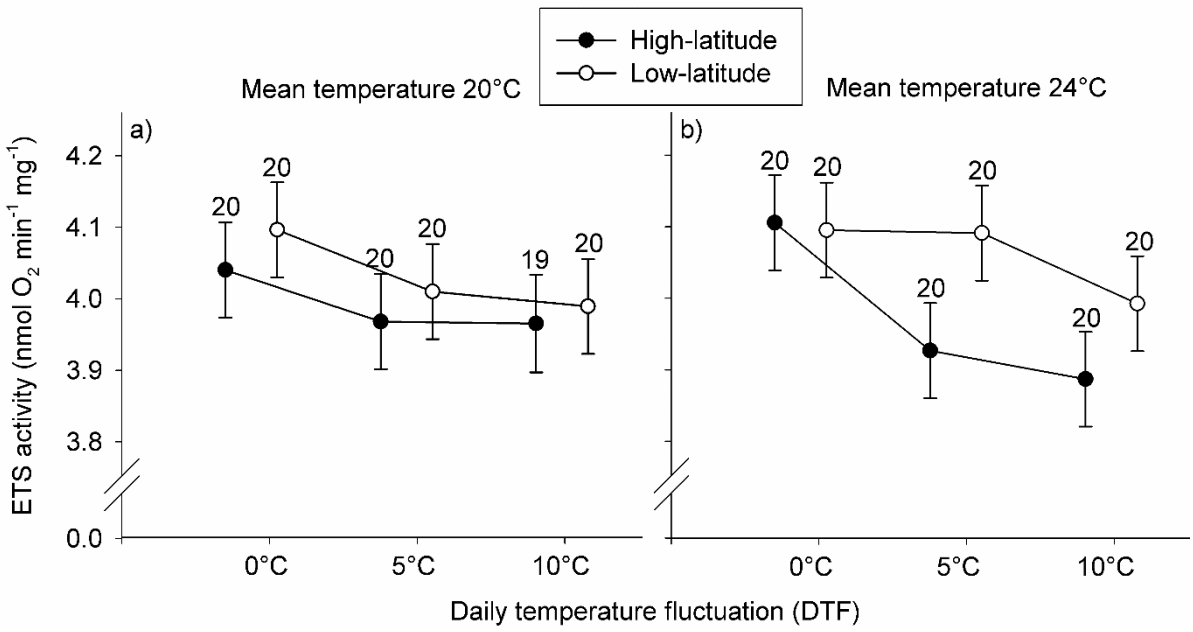
793

794 **Figure 3**



795

796 **Figure 4**



797