Title**: Is thermal tolerance in non-biting midges driven by phenotypic plasticity or adaptation?**

Author names and affiliations:

Quentin Foucault[1,2], Andreas Wieser[1,2], Ann-Marie Oppold[2], Barbara Feldmeyer[2] , Markus Pfenninger[1,2]

[1] Goethe University, Max-von-Laue-Straße 9, 60438 Frankfurt am Main, Germany

[2] Molecular Ecology Group, Senckenberg Biodiversity and Climate Research Centre, Senckenberganlage 25, 60325 Frankfurt am Main, Germany

Corresponding author: QF, quentin.foucault@senckenberg.de; phone +33(0)603221891

Email addresses : QF: quentin.foucault@senckenberg.de AW: andreas.wieser@senckenberg.de AMO : ann-marie.oppold@senckenberg.de BF: barbara.feldmeyer@senckenberg.de MP: pfenninger@bio.uni-frankfurt.de

Author contributions: QF, AW and MP conceived the project, MP supplied animal, facilities, material and equipment, QF AMO and MP designed the experiments QF conducted the experiments, analysed the data and drafted the manuscript. AW modelled thermal performance, developed Fig.7 and helped conducting the experiments. BF provided feedback on the analysis. All authors contributed to manuscript writing and approved the final version of the manuscript.

Abstract

Effects of seasonal or daily temperature variation on fitness and physiology of ectothermic organisms have been widely studied, but the effect of long term climatic modification is still not well understood and complex to predict. With the current climatic change, it becomes more important to investigate the adaptive potential and underlying mechanisms of organisms in response to temperature changes.

The aim of this study is to investigate whether the multi-voltine (multiple generations per year) midge *Chironomus riparius* Meigen (1803) responds mainly via acclimatization or by long term modification using adaptation mechanism. A mix of larvae from five European populations was raised at three different pre-exposure temperatures (PET) : 14, 20, 26°C. After three and five generations in laboratory cultures larvae were exposed to three treatment temperatures (TT) 14, 20, 26°C. Mortality was monitored for the first 48h and after emergence.

After three generations a significant mortality rate differences dependent on an interaction between PET and TT could be observed. This finding supports the hypothesis that *Chironomid* midges respond to climatic variation via adaptive mechanisms. Moreover, these results accordingly show that three generations were sufficient to already adapt to warm temperature, increasing the resilience and decreasing the mortality rate.

Keywords: mortality, rearing temperature, ectotherm, chironomids, climate

Introduction

Ambient temperature variation is a major factor affecting the fitness of organisms by regulating the speed of metabolic processes, and thus, everything from development to reproduction, often leading to genetic adaptation (Atkinson 1994, Merilä et al. 2014). This factor especially impacts ectothermic organisms, due to their dependence of body temperature from ambient temperature (Clarke et al. 2004, Deutsch et al. 2008). Moreover, elevated temperatures can have a strong effect on ectotherms due to the asymmetric shape of the thermal performance curve (TPC), which is typically used to characterize the relationship between temperature and fitness (Fig. 1, Sinclair et al. 2016). The TCP shows a steady increase of fitness with temperature up to an optimal temperature and then a quick deterioration of fitness (Fig. 1), leading eventually to the death of the organism (Paaijmans et al. 2013, Sinclair et al. 2016). Even though the effects of short term thermal variations on physiology and fitness have been well studied, both effects of seasonal temperature variations and long term climate change on ectothermic organisms is still not fully understood and therefore complex to predict (Paaijmans et al. 2013). To cope with temperature variation, ectothermic organisms can use different mechanisms, such as (i) behavioural changes, allowing organisms to escape or mitigate the environmental pressure (Hutchison et al. 1979, Lencioni 2004), (ii) phenotypic plasticity, allowing them to cope with temperature variation and resulting environmental modifications by changes in gene expression (Johnston et al. 2006) and/or (iii) by genetic adaptation (Bergland et al. 2014). Distinguishing whether phenotypic change is genetically based or results from phenotypic plasticity is difficult and needs to be experimentally tested over several generations in order to identify which of those mechanisms are involved (Merilä et al. 2014).

For phenotypically plastic organisms, we expect that the fitness of each generation will depend only on the temperature that they experience themselves, independent of the temperature their parents were pre-exposed to. If organisms are genetically adapted, the fitness response to different temperatures will also depend on the temperature experienced by the previous generation(s), i.e. the interaction between temperature experience by former generations and actual temperatures experienced by the individuals (Fig. 2). Multi-voltine (multiple generations per year) aquatic insects often have limited migration/movement capacities, during larval stage they mostly respond through phenotypic plasticity or genetic adaptation to temperature variation. Indeed larvae have low velocity crawling possibility and energetically wasteful swimming activity (Brackenbury 2000). Therefore, these organisms often have strong phenotypic responses to deal with temperature variation. Those responses can be morphological, like the modification of body size depending on temperature to adjust energy requirement, known as Bergmann's rule (French et al. 1998, Gardner et al. 2011), even if the physiological cause of the Bergmann's rule can be linked to both plasticity and adaptation (Atkinson et al. 1997, Angilletta Jr et al. 2004). However, responses to both cold and heat stresses after short periods of exposition to these temperatures, can also be purely physiological with hardening mechanisms involving protein modifications at the cell level (Bowler 2005, Overgaard et al. 2005). The effect of temperature variation on the life history traits of these organisms such as growth (Hauer et al. 1991, Frouz et al. 2002), reproduction (Péry et al. 2006), metabolic processes (Edwards 1958, Sankarperumal et al. 1991, Hirthe et al. 2001) and sensibility to various toxins (Fisher et al. 1985, Lydy et al. 1999) has been thoroughly studied. However, under the current climatic change, it becomes increasingly important to investigate by which mechanisms organisms deal with temperature changes. This is an important issue considering the ecological role of invertebrate ectotherms being at

the base of the food chain, as well as often a pest for humans (Pinder 1986) and a vector of diseases (Broza et al. 2001, Halpern 2011).

The aim of this study is to determine whether ectothermic invertebrates cope with short term temperature variations over a few generations via phenotypic plasticity or genetic adaptation and what impact on the fitness can be expected. For this purpose, chironomids were used as a model for investigating the potential impact of climate change on the aquatic ectothermic fauna due to their large thermal range and repartition all across the Northern hemisphere (Pinder 1986). We specifically used *Chironomus riparius* (MEIGEN 1803), a multi-voltine species with an aquatic larval stage (Armitage et al. 1995), as an experimental model because of its capacity to cope with culture conditions (Downe and Caspary 1973) as well as a good knowledge of its genome (Oppold et al. 2017). Moreover, the adaptive potential of this species to local climate conditions along a climatic gradient has already been shown (Nemec et al. 2013, Oppold et al. 2017). In this study we expect C. riparius to genetically adapt to temperature variation based on previous finding. (Oppold et al. 2017)

Material and Methods

This experiment was performed on an admixed C. riparius culture composed of individuals from five different populations originating from Metz (NMF) and Lyon (MF) in France, Hasselroth in Germany (MG), Collobiano in Italy (SI) and Las Vegas in Spain (SS)(Oppold et al. 2016) (Supporting information 1). We decided to mix individuals from multiple populations in order to avoid the expected loss of genetic diversity during the experiment (Nowak et al. 2007), and to make sure that alleles involved in adaptation to the complete temperature gradient are present in the gene pool. From this admixed base population, three sub-populations were raised following the OECD guideline 219 (OECD 2004, Oppold et al. 2016) at three different temperatures: 14 °C, 20 °C and 26 °C with light–dark rhythm of 16:8

h at 60% humidity. These temperatures were calculated by adding the expected increase of temperature to current temperature measurement for the five populations (Supporting information 2). Larvae were raised in medium constituted of purified water with sea salt (TropicMarin) adjusted to a conductivity of 520–540 µS cm-1, and pH 8. The ground of the glass bowl (20 cm diameter) was covered with washed sand. Populations were raised at these temperatures for 5 generations; this phase will hereafter be referred to as "Pre-Exposure Temperature" (PET).

SURVIVAL TEST

Survival tests were performed in the third and fifth generation, i.e. they were exposed to PET for three and five generations, respectively. These two different time points were used to investigate possible acclimation or adaptation with a longer exposition to a stable temperature. Generation time at the respective temperature was determined during the first generation, as time from the hatching of the eggs until the death of the adults. This was necessary, because generations started overlapping with the second generation, leaving us unable to infer the exact beginning and end of a generation. The generation times used were 58 days at 14°, 34 days at 20 °C and 26 days at 26 °C (Oppold et al. 2016).

For each population, two replicates of five egg clutches were put to hatch. Individuals coming from the same egg clutch will be referred to as families. 18 larvae from each family for each replicate were raised at three different experimental temperatures called "Treatment Temperature" (TT) for the analysis (18 larvae x five families x three temperatures x two replicates). Each larva was individually raised in six well plates (Ø3.5x2cm) filled with six mL of medium for 48 hours without feeding. Larval mortality rates were measured after 24 and 48 hours. After 48 hours, the surviving larvae were pooled by families in glass bowls (Ø20 x10 cm) with sediment and medium and reared until emergence. The adult mortality

was calculated as the number of individuals not emerged per family, PET and TT when imagines were removed from bowls. During this stage, larvae were fed daily with dried fish food (0.5 mg/individual of grounded TetraMin® flakes) and the water level adjusted daily with deionized water in order to conserve the water parameters.

STATISTICS

Statistical analyses were performed using R (Version 3.2.3) in addition with Rstudio (Version 0.99.903). The normality of the data set was tested using qqplot, Kolmogorov-Smirnov tests and homoscedasticity as well with Levenes test. A stepwise linear generalized model was used to investigate the effect of the experimental factors on the mortality with the families as random factor followed by one-way ANOVA tests. In case of significant interactions of two or more factors, each instance of the interaction was analysed separately. In order to investigate significant differences between the TT, ANOVA followed by Tukey *post-hoc* test were used for data following the assumptions of normality and homoscedasticity. Kruskal-Wallis tests followed by Dunn *post-hoc* tests were used if the one or both of the previous assumptions were rejected.

Additionally, using the R package thermPerf, we compared fits to ten different nonlinear models of thermal performance that produce curves mirroring the expected trajectory of development rate. We used data from the experiment and from the literature (Vogt et al. 2007, Oetken et al. 2009, Nemec et al. 2013, Oppold et al. 2016) in order to determine if our populations deviate from the expected values.

Results

LARVAL MORTALITY

Larval mortality was significantly affected by PET and TT, with a significant interaction between these two factors. However, mortality did not differ significantly between generations. Based on this result, both generations were grouped for downstream statistical analysis (table1).

Concerning the mortality rates according to the delta temperature (ΔT°=TT– PET) (Fig. 3), the results showed an increase in mortality concomitantly with the increase in temperature. On the opposite, mortality decreased concomitantly with decreasing temperatures until a difference of -6°C. After this threshold, the mortality did not significantly differ anymore.

After 48 hours, significant differences were found between mortality depending on the PET (Kruskal-Wallis Chi-squared = 76.392, p-value < 0.001). The overall mortality of the larvae was significantly lower for individuals coming from 26 °C PET compared to a 14 °C PET (post-hoc Dunn's-test p-value < 0.001) or 20 °C PET (*post-hoc* Dunn's-test p-value < 0.001) (Fig. 4), independently of the TT. By looking at mortality in detail, larvae coming from 14 °C PET showed different mortality between TT (ANOVA F value = 8.189, p-value = 0.000748): mortality was significantly higher at 26 °C TT than at both 20 °C TT (*post-hoc* Tukey's-test p-value = 0.0012641) and 14 °C TT (*post-hoc* Tuckey's-test p-value =0.0058708) (Fig.5a). For the larvae from 20 °C PET none of the TT showed a significant difference for larval mortality compared to the PET (ANOVA F value = 2.936, p-value = 0.0611) (Fig.5b). Finally, for the larvae coming from the 26 °C PET, no significant difference was found between the different TT, even though the means showed a trend for lower mortality at higher temperatures (ANOVA F value = 1.453 , p-value = 0.242) (Fig. 5c).

TOTAL MORTALITY

Mortality was significantly affected by PET, as well as TT. However unlike the larval mortality, the analysis did not show significant interactions between PET and TT temperature (table2).

The mortality of adults showed the same trend as the larval mortality (ANOVA F value= 56.56, p-value < 0.001). Indeed, individuals coming from the 26 °C PET showed a significantly lower mortality than individuals coming from 20° PET (post-hoc Tukey's test p-value < 0.001) and 14 °C (post-hoc Tukey's test p-value < 0.001) when all TT were merged. Regarding the results of each experiment, we see that, for the individuals at PET 14 °C (Kruskal-Wallis Chi-squared = 15.65, p-value < 0.001) (Fig.6a), a lower mortality on average is observed when they are exposed to 14 °C TT compared to TT 26 °C (post-hoc Dunn's-test p-value < 0.001). No significant differences were found when comparing the 20 °C TT to the two others. For the individuals coming from the 20 °C PET the 26 °C TT induced significantly higher mortality (ANOVA F value = 4.734, p-value = 0.0125), compared to the two other TT temperatures (26 °C-14 °C post-hoc Tukey's test p-value = 0.043 and 26 °C-20 °C post-hoc Tukey's test p-value= 0.018) (Fig.6b). Finally for 26 °C PET (Fig.6c), no significant differences were found between the different TT (ANOVA F value = 1.796, p-value = 0.175).

From the fits of the data on development rate models, AIC weight comparison showed a slight preference for the model Brière 2 (Brière et al. 1999). Brière defines development rate *R(T)* as:

$$\boldsymbol{R(T) = \begin{cases} 0 & T \leq T_L \\ aT(T - T_0)(T_L - T)^{\frac{1}{m}} & T_0 \leq T \leq T_L \\ 0 & T \geq T_L \end{cases}}$$

Where T_0 equals low temperature development threshold, T_L equals lethal temperature (upper threshold), a is an empirical constant, and m = 2 in Brière 2. But since none of the data, either from our experiment or the literature, reached the model boundaries (Fig. 7) we will not be able to use this result according to Brière (1999).

Discussion

The aim of this study was to investigate whether the response of chironomids to various temperature regimes is rather driven by phenotypic plasticity or genetic adaptation. We exposed C. riparius to three different temperatures for three and five generations and then determined the fitness of the offspring under reciprocal temperature regimes. Our results show indication for effects of both responses, the chironomids adapted to new temperature regimes and/or coped with temperature change via phenotypical plasticity.

Regarding the results of the larval mortality after 48 hours of experiment, the two factors, PET temperature and TT temperature, have a significant impact on the mortality and an interaction of both. Upon this finding, we can hypothesise that the PET had an influence on the TPC by shifting the optimal temperature of the organism (Fig.1, Sinclair et al. 2016). In our experiment, 26 °C is the maximum temperature, if the optimal temperature of the TPC for larvae has been redefined to 26 °C, the overall mortality would not increase further before the new critical maximal temperature (lethal CTmax) is reached. This hypothesis could explain the low mortality recorded for the individuals of PET 26 °C. This shifting of the TPC depending on the PET suggests that the larvae inherited adaption to 26 °C via genetic mechanisms. We can also infer that three generations of pre-exposure is sufficient for this adaptation since the mortality after three and five generations of PET did not differ significantly. However, we cannot answer at this stage whether the genetic adaptation may be based on standing genetic variation, or was caused by a new mutation.

We also see that the mortality of the 26 °C pre-exposed individuals is the only one low in all TT which suggests that no strong selection event occurred at PET of 20 °C and 14 °C explaining why none of them showed a low mortality rate when subjected to the same TT. This result indicates that we did not reach the critical high temperature (CTmax) with PET 20 °C and thus selection pressure at this temperature was too low to lead to adaptations; this finding was expected since 20 °C is an intermediate temperature. In the same way, the critical low temperature (CTmin) may not have been reached with PET 14 °C. If the thermal performance curves display a decrease of fitness at decreasing temperatures, the mortality in ectotherms is mostly driven by physiological limitations causing the mortality curve to be U-shaped with a falling of the survival at a certain low temperature (Pörtner 2001, Paaijmans et al. 2013). This decrease of fitness might then be only due to lower developmental rates at low temperatures since this parameter is an important part of the models generating TCPs (Huey et al. 1976, Deutsch et al. 2008, Amarasekare et al. 2012). Indeed thermal performance models can show the relationship of development rate and temperature. Development rate is determined by the reciprocal of time to emergence in constant temperature regimes. Generally, in ectotherms, rising temperatures translate to higher development rates until a certain lethal threshold is surpassed, when performance rapidly plummets and mortality increases. Thereby the development rate collected from own experiments and literature (Vogt et al. 2007, Oetken et al. 2009, Nemec et al. 2013, Oppold et al. 2016) fitted to the temperature dependent development model from Briere et al. (1999) (Fig. 7), displays the same increase with temperature as the TCP. Then in the same way, we can expect mortality at lower temperature to only increase when the population will reaches the physiologic limitation temperature which would correspond to the critical minimal temperature on the TPC. Since we do not observe a drop/decrease survival (data not shown) in our experiment at 14 °C, we suppose that 14 °C is not an extreme low temperature for our species.

Despite the effect of the 26 °C PET, the two other PET revealed variations in mortality that cannot be explained by adaptive mechanisms. Indeed for pre–exposure at 14 °C and 20 °C the mortality variance is very high indicating that a lot of variation occurred within replicates at the individual level; this high variance is not present for the 26° PET. These individual differences could be linked to phenotypical plasticity since within these two TT no extreme temperature has been reached.

Our results on adaptation to high temperature with low phenotypical plasticity are consistent with the literature: in *Drosophila melanogaster* populations reared at 28°C, survival was greatest when compared to populations reared at lower temperature (Cavicchi et al. 1995). The same individuals showed fixation of alleles on the heat shock gene Hsp70, but also a level of inducible heat shock proteins which was lower at 28°C than at the lower TT (Bettencourt et al. 2002). This lower level of inducible heat shock proteins indicate a lowering of the possibility to cope with temperature increase via phenotypical plasticity (Bettencourt et al. 1999). A similar pattern has also been found in a natural D. *melanogaster* strain from Africa displaying an exceptional thermotolerance (Zatsepina et al. 2001). If those mechanisms are the same in our species, they can have an important impact on populations of C. *riparius* since climatic models have shown that we can expect an increase in temperature between 2.6 to 4.8°C in Europe for the next 100 years (under the RCP8.5 scenario) (Prein et al. 2011, Pachauri et al. 2014).

We need to take into account that the experiments here have been realized on a mixture of populations from different populations across Europe. This mixture resulted in an artificial increase of genetic diversity composed of warm and cold temperature adapted individuals (Supporting information 2). Therefore, even if it was shown that many of these haplotypes may occur together in natural populations because of gene flow (Oppold et al. 2017), the adaptation noticed during our experiment would possibly not be as fast under natural

conditions. Previous studies showed that field populations have a high genetic variability lowering the chance of allele fixation, which we might have forced in our experiment due to smaller population size (Vogt et al. 2007). Another possibility is that the adaptation capacity could have been underestimated in our experiment because of a possible outbreeding depression, and adaptation to changing temperatures might even occur quicker in natural populations. Indeed it has been shown by Oppold et al. (2017) that crossing distant populations can result in a decrease of fitness in hybrids caused by Cla-element incompatibilities.

Conclusion

In this study we were able to show that C. *riparius* is coping with temperature changes by adaptation mechanism more than phenotypical plasticity depending on the mean temperature experienced by the previous generations. Our results also indicate that C. *riparius* is able to adapt to warm temperature in a short time, modifying the thermal tolerance of the organism not only for warm but also for colder temperatures. These results are in line with previous findings in D. *melanogaster* and we expect a similar mechanism to take place in C. *riparius.* However, further studies on genetic and molecular levels are needed to clearly identify the process involved.

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Compliance with ethical standards

Ethics approval: Not applicable.

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Fig. 1 Thermal performance curve (TPC) modified from Sinclair et al (2016) to show only relevant parameter for our study. CTmin and CTmax: critical minimal and maximal temperature; Topt: optimal temperature

Fig. 2 Expected fitness curves in case of organisms only responding to temperature change by genetic adaptation mechanisms. Every panel correspond to a temperature at which previous generations of the populations were subjected, the temperature within a panel is the temperature at which individuals of those population are currently exposed to

Fig. 3 Mean mortality of the larvae after 48h depending on the delta temperature (°C) (delta temperature = TT– PET). Different letters denote significant mortality differences between TT (Kruskal-Wallis Chi-squared = 57.253, df = 4, p-value < 0.001)

Fig. 4 Boxplot of the larval mortality after 48h depending on the PET (°C), the red crosses shows the mean mortality for each PET. The letters denote significant differences between mortality (Kruskal-Wallis Chi-squared = 76.392, p-value < 0.001)

Fig. 5 Boxplot displaying the larval mortality after 48h by TT (°C) for (A) PET 14 °C (ANOVA F value = 8.189, p-value = < 0.001), (B) PET 20 °C (ANOVA F value = 2.936, p-value = 0.0611), (C) PET 26 °C (ANOVA F value = 1.453 , p-value = 0.242). Red crosses illustrate mean mortality for each PET. Different letters denote significant differences in mortality rates between groups

Fig. 6 Boxplot displaying the total mortality after emergence by TT (°C) for (A) PET 14 °C (Kruskal-Wallis Chi-squared = 15.65, p-value < 0.001), (B) PET 20 °C (ANOVA F value = 4,734, p-value 0.0125), (C) PET 26 °C (ANOVA F value = 1.796, p-value = 0.175). Red crosses show the mean mortality rate for each PET. The letters denote significant differences between mortality rate

Fig. 7 Development rate [1/day] in respect to temperature (°C). data point from different studies on C. riparius fitting to a development rate model by Briere et al. 1999 using the Thermperf R package. Solid line represents linear fit. The circles represent data obtained through experiment (data not shown), the triangles represent data from Nemec et al. 2013, squares represent data from Oetken et al. 2009 and crosses represent data from Vogt et al. 2007

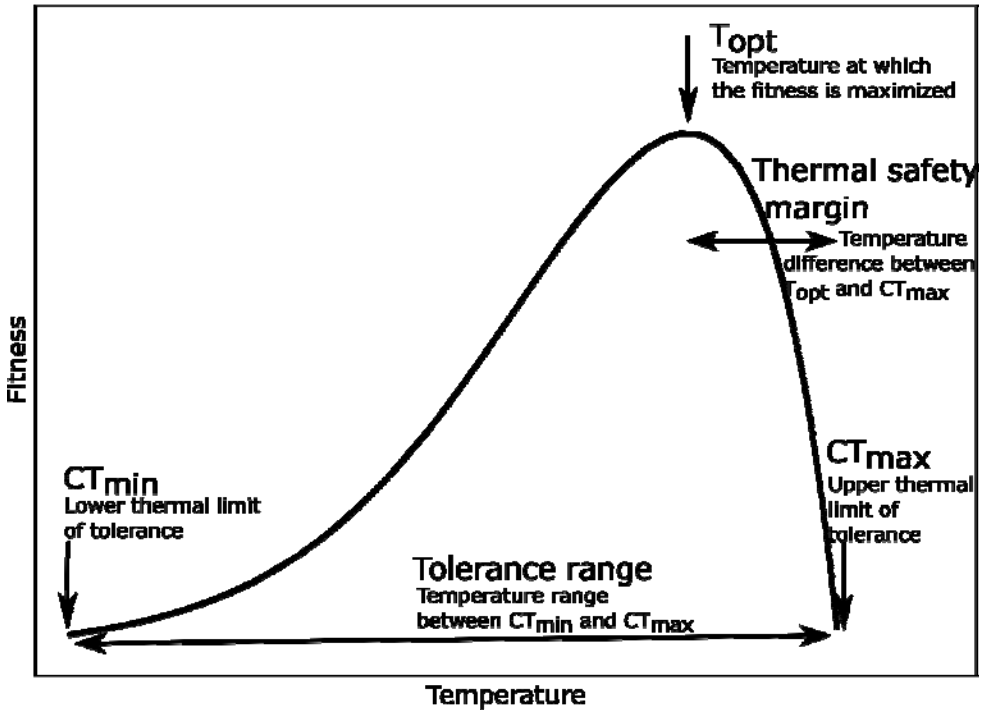
Topt
Temperature at which the fitness is maximized
Thermal safety margin
Temperature difference between Topt and CTmax
Fitness
CTmax
Upper thermal limit of tolerance
CTmin
Lower thermal limit of tolerance
Tolerance range
Temperature range between CTmin and CTmax
Temperature

Figure 1

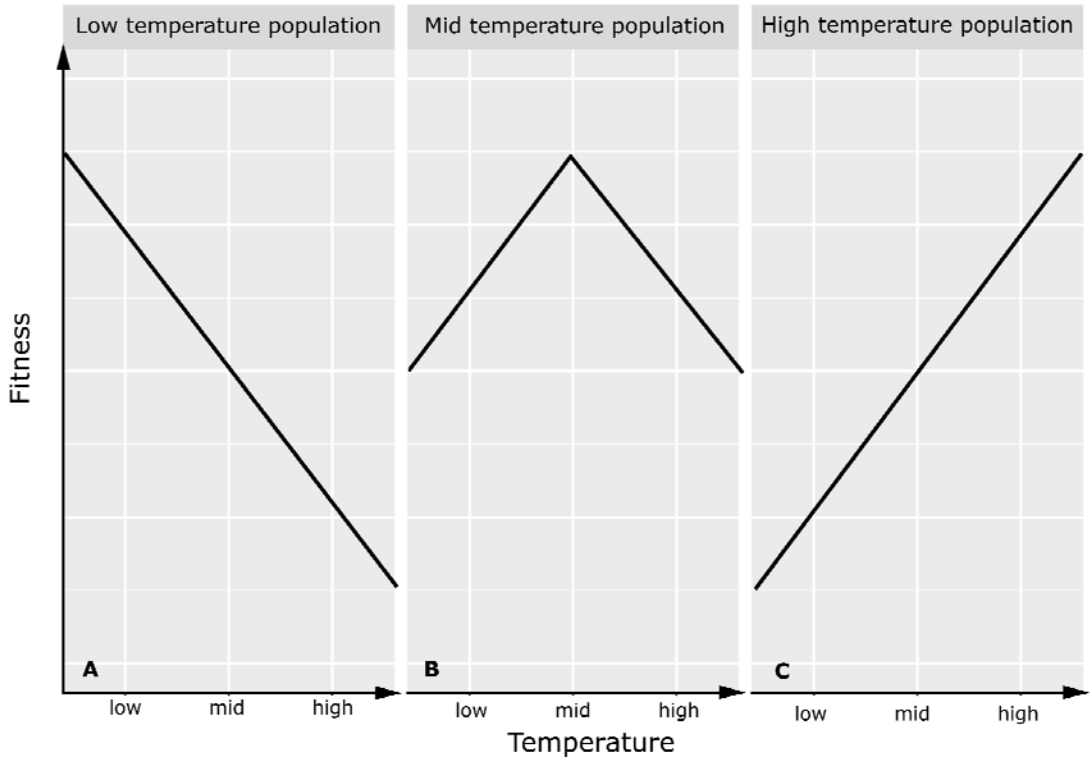
Low temperature population
Mid temperature population
High temperature population
Fitness
A
B
C
low
mid
high
low
mid
high
low
mid
high
Temperature

Figure 2

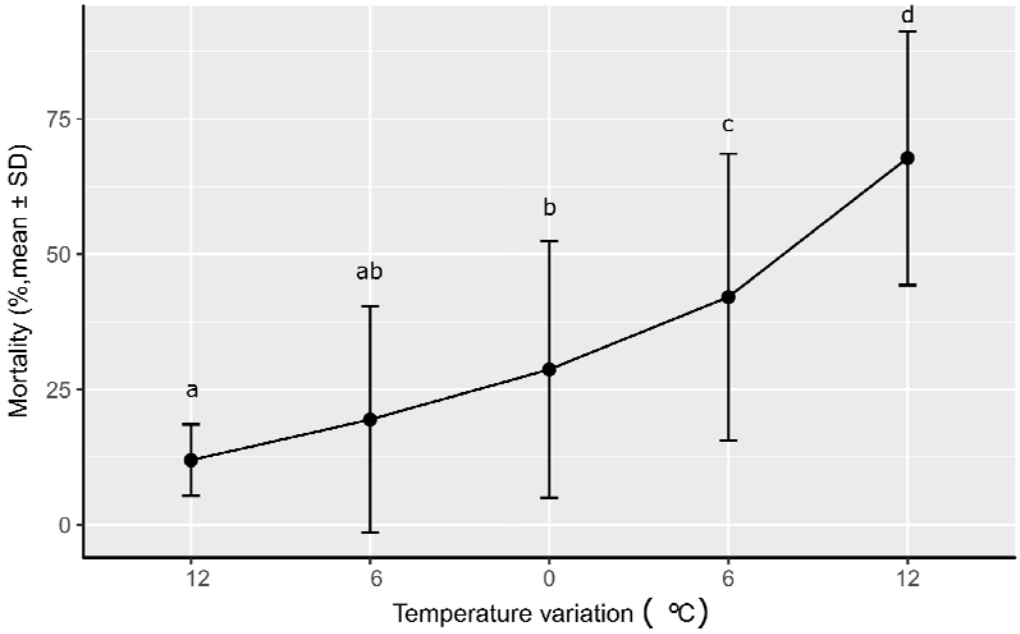
Mortality (%,mean ± SD)
75
50
25
0
a
ab
b
c
d
12
6
0
6
12
Temperature variation (℃)

Figure 3

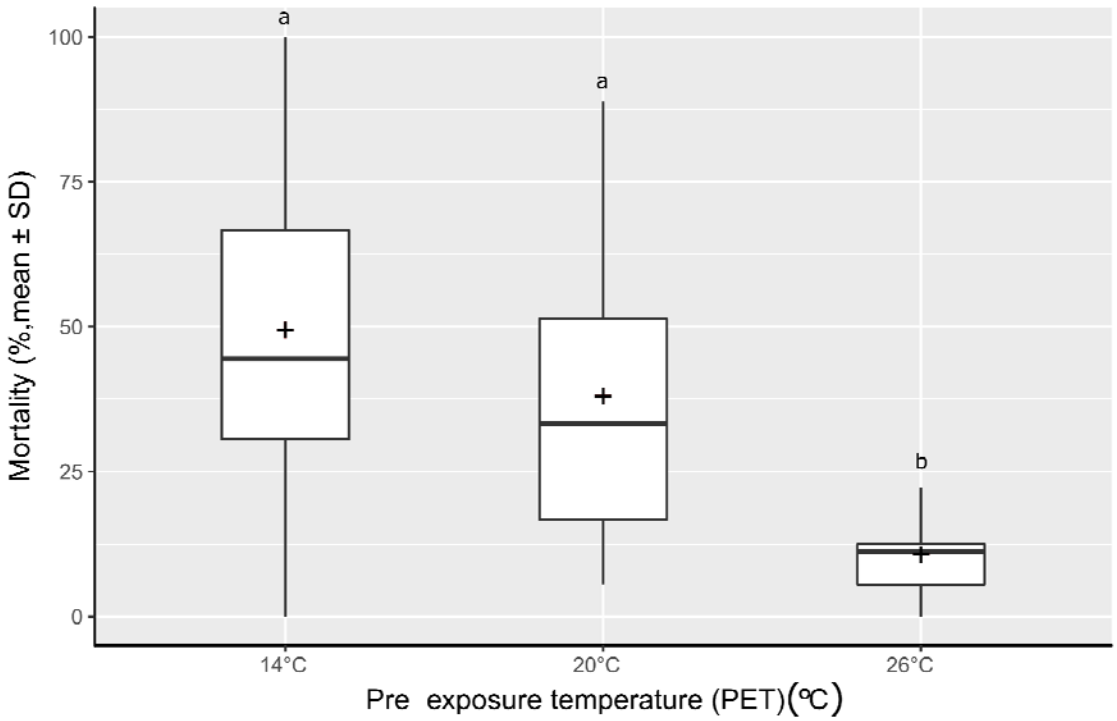
a
a
b
100
75
50
25
0
Mortality (%,mean ± SD)
14°C
20°C
26°C
Pre exposure temperature (PET)(℃)

Figure 4

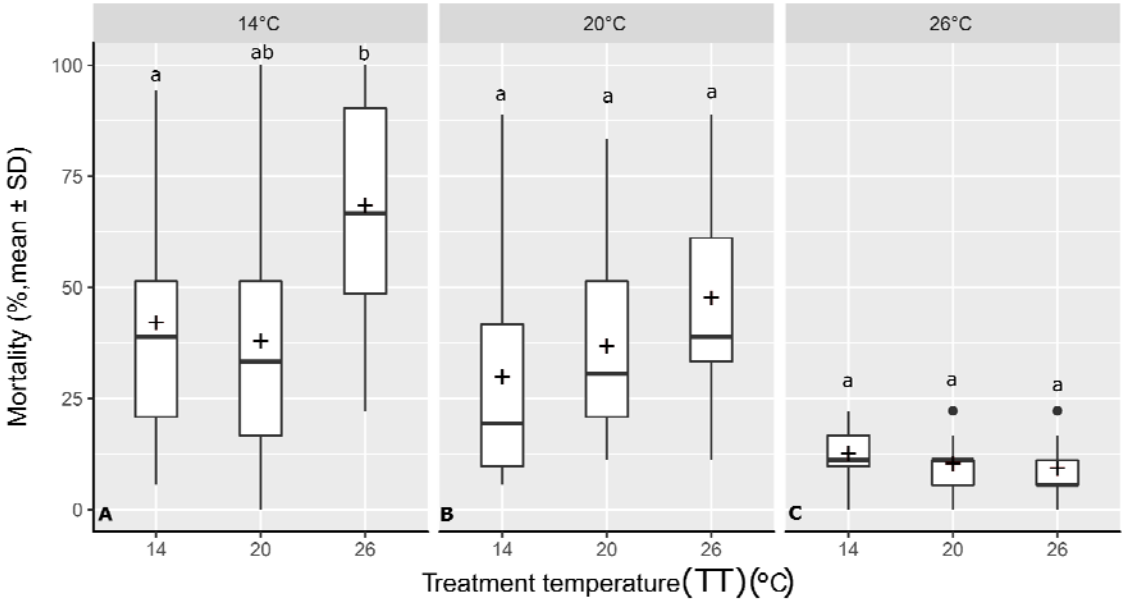
14°C
20°C
26°C
Mortality (%,mean ± SD)
100
75
50
25
0
a
ab
b
a
a
a
a
a
a
A
B
C
14
20
26
Treatment temperature(TT)(°C)

Figure 5

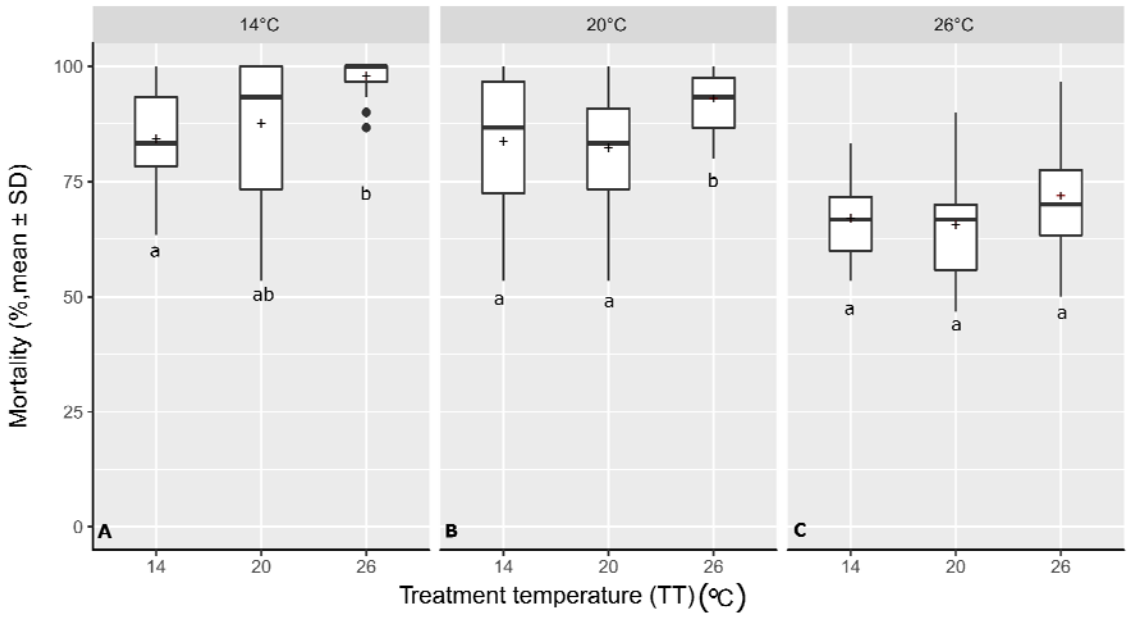
14°C
20°C
26°C
Mortality (%,mean ± SD)
100
75
50
25
0
A
B
C
a
ab
b
a
a
b
a
a
a
14
20
26
Treatment temperature (TT) (℃)

Figure 6

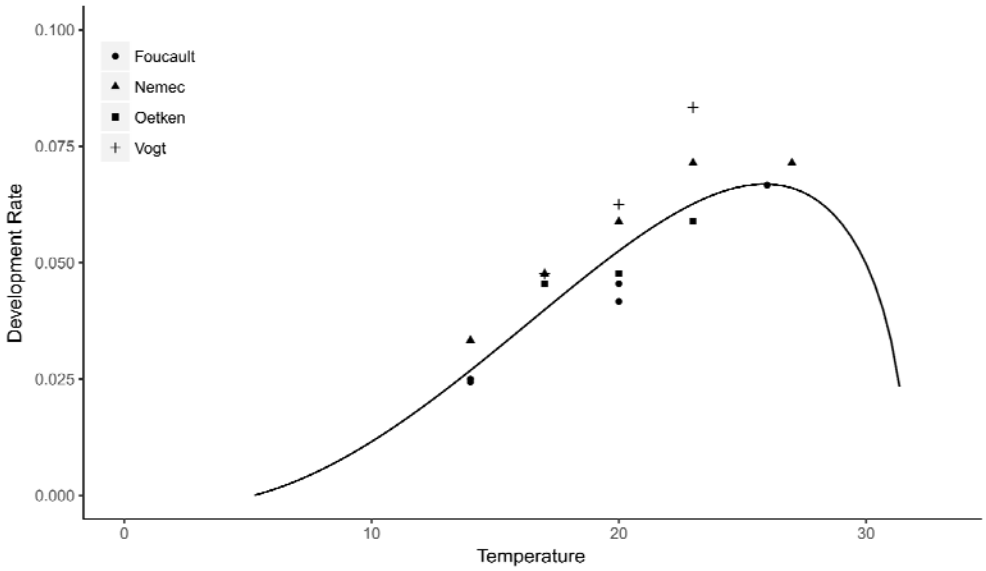
0.100
0.075
0.050
0.025
0.000
Development Rate
Foucault
Nemec
Oetken
Vogt
0
10
20
30
Temperature

Figure 7

Table 1: Summary of the general linear model executed for the larval mortality after 48h at TT

Factor	Chi-square	Pr(>Chi)	
Generation	1.2449	0.258	
PET	104.037	< 0.001	***
TT	12.8668	< 0.001	***
Generation* PET	2.7533	0.095	(*)
Generation* TT	1.3153	0.245	
PET*TT	10.2258	0.001	**
Generation*PET * TT	0.6511	0.414	

Degree of freedom = 1

Table 2: Summary of the general linear model executed for the total mortality after emergence

Factor	Chi-square	Pr(>Chi)	
Generation	0.00004	0.983	
PET	100.283	< 0.001	***
TT	18.2941	< 0.001	***
Generation* PET	0.3470	0.555	
Generation* TT	0.0007	0.979	
PET*TT	2.7639	0.096	(*)
Generation*PET * TT	0.9456	0.330	

Degree of freedom = 1