Complex response in size-related traits of the bulb mites (*Rhizoglyphus robini*) in elevated thermal conditions – an experimental evolution approach.

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SUMMARY

Temperature is a key environmental factor affecting almost all aspects of life histories in ectotherms. The theory predicts that they grow faster, reach smaller sizes and produce smaller offspring when temperature increases. In addition, temperature changes, through their effects on metabolism, may also influence the expression of alternative reproductive phenotypes (ARP) in ectotherms. Although many studies investigated phenotypic plasticity of life history traits in relation to temperature change, little is known about how those traits and phenotypic plasticity may evolve together. In our study we subjected the bulb mites (non-model, soil organisms that normally experience rather stable thermal conditions) to experimental evolution in two temperature treatments: control (24° C) and elevated (28° C). After 18 generations we measured adult body size, eggs size and development time of both treatments at control as well as at elevated temperature (test temperatures). Thus, we were able to detect genetic changes (the effect of selection temperature) and environmental effects (the effects of test temperature). We also observed the ARP expression throughout the experimental evolution. Our results revealed quite complex patterns of life history in traits response to temperature. Mites developed faster and reached smaller sizes at increased temperature, but evolutionary responses to increased temperature were not always parallel to the observed phenotypic plasticity. Additionally, despite smaller body sizes females laid larger eggs at higher temperature. This effect was more pronounced in animals evolving at elevated temperature. Evolution at increased temperature affected also ARP expression with the proportion of armored fighters decreasing from generation to generation. We propose that this could be the consequence of temperature sensitivity of cost to benefits ratio of the expressing ARPs.

INTRODUCTION

Ambient temperature, particularly in ectotherms is a key environmental factor affecting nearly every aspect of their life history (e.g. Blackenhorn, 2000) and shaping both evolutionary adaptation and phenotypic plasticity. A well-known example of a phenotypically plastic response to thermal conditions is the temperature size-rule (TSR; Atkinson, 1994): the increase of body size with decreasing temperature. Similarly, because the egg size is related to body size of a mother and optimal egg size depends on temperature (Perrin 1988), females at

lower temperature are expected to lay fewer but bigger eggs (Blanckenhorn, 2001). However, the TSR in arthropods is not universal as some species exhibit reverse or even more complex temperature-size patterns (e.g. David et al. 2006, Kammenga et al. 2007) and, more importantly, species geographical clines seem to contrast those of thermal plasticity observed in laboratory studies (e.g. *Drosophila* spp.; Angilletta, 2009).

Adult size is a product of growth rate and development duration (Davidovitz et al. 2004) and, although body size and development time are strongly related to fitness, it is still debated what is the extent to which their responses to thermal conditions are mediated by phenotypic plasticity and evolutionary change. Experimental evolution approach is potentially a very powerful tool in solving this problem. However, only a few experimental evolution studies have explored adaptive response of body size (Partridge et al., 1994; Santos et al., 2006) or development time (Santos et al., 2006; Huey et al., 1991; James and Partridge, 1995) to selection at changed temperature. In addition, the majority of the studies were carried out on *Drosophila*, questioning the generality of observed patterns. Even less data are available on evolutionary changes in parental investments (egg or offspring size and number). However, it has been observed that changes in egg size with ambient temperature may occur within a single generation, indicating the effect of phenotypic plasticity (Blanckenhorn, 2001). Despite a growing number of studies have investigating the selective benefits of the egg size at different temperatures (Blackenhorn, 2000, Fischer et al., 2003, Bownds et al., 2010, Burgess and Marshall 2011), to our knowledge, only one experiment attempted to determine if egg size and phenotypic plasticity for this trait can evolve (Azevedo et al., 1996).

Furthermore, growth rate and development duration differ between the sexes triggering sexual size dimorphism (SSD). Larger body size increases female fecundity, male attractiveness and competitiveness, but when associated with prolonged development, it can expose an individual to external sources of mortality. Males also experience a conflict between selection for rapid development (protandry), often leading to smaller size, and selection for large body size. Interestingly, males and females have been shown to differ in their phenotypic plasticity for body size (Stillwell et al., 2010). Although females are often more plastic than males, the differences in plasticity may vary between environmental factors as well as the range of specific environmental variables (Stillwel and Fox, 2009). Therefore, sexes may handle temperature variation differently and the patterns of these differences might not be consistent, depending on a species, a population and temperature ranges (Stillwell et al., 2010).

Besides SSD, in some species sexes can differ in expression of discrete morphological variants - so called alternative reproductive phenotypes (ARP), often differing in both growth rate and development time and thus in adult body size. These forms of adaptive phenotypic variants, usually males, are manifested as morphological differentiation in structures used in male-male competition, such as horns in scarab beetles (Emlen 1994, Kotiaho et al. 2003) or male leg dimorphism in acarid mites (Woodring, 1969; Radwan, 1995, 2001). The problem of shifts in proportion of ARP under different thermal conditions is very poorly studied, despite the fact that it may be of great importance as such shifts may influence the strength of sexual selection acting in a population at different temperatures as ARP differ in their competitiveness. Our study mite species, *Rizoglyphus robini*, has two male morphs with different strategies of reproduction. Fighters, possessing a thickened third pair of legs that is used in fights with other males, are more aggressive and able to kill rivals and thus achieve higher reproductive success in mixed populations (Radwan and Klimas, 2001). Scramblers, with "normal" legs, are less aggressive and harmless to other males (Radwan, 2009). Morph expression in *R. robini* is genetically controlled (Radwan, 1995) but seems to be also condition dependent. It has been shown that fighters develop from larger trythonymps (last larval stage of the bulb mite) and that poor diet suppresses fighter expression (Smallegange, 2011; Radwan, 1995). Hence, environmental conditions through their effects on body size and/or development time can change the proportion of morphs (Radwan, 1995; Smallegange, 2011; Simpson et al., 2011). Furthermore, a study by Shepherd et al. (2008) implies that weapon structure can change body surface and thus may affect fighters' thermoregulatory behavior and costs of weapon possession.

As a consequence of the above mechanisms, changes in thermal conditions may modify morph ratio in several ways. First, as fighters develop from larger trythonymps (Smallegange, 2011), increasing temperature may decrease the ratio of fighters by its effect on body size. What is more, fighting structures can change energy dissipation as a consequence of changes in body surface as suggested for horn-beetles (Shepherd et al., 2008), even though adult fighters have smaller abdomen than scramblers. Second, temperature is likely to affect cost of both production of thickened legs and performing aggressive strategy (e.g. Nicieza and Metcalfe, 1999). Due to its energetic costs, it is likely that the fighter strategy may become more costly at high temperature, so only males in particularly good condition would be able to benefit from it. In other words the switch point between the two morphs may be moved up by a temperature increase.

In the current study we applied an experimental evolution approach where populations of bulb mites *Rhizoglyphus robini* were allowed to evolve in two thermal treatments. After 18 generations the size-related life history traits were measured at both experimental temperatures (elevated and control), which enabled us to distinguish between genetic changes (the effect of selection temperature) and environmental effects (the effect of test temperature). The proportion of fighters was measured throughout 35 generations of the experimental evolution. Thus, the objective of the study was threefold. First, we compared thermal phenotypic plasticity and evolutionary changes in eggs size, development rate and size at maturity. Second, we compared thermal reaction norms for females and males to estimate sex-specific response to temperature. Third, we assessed how expression of ARP had changed during evolution in elevated temperature conditions.

MATERIALS AND METHODS

General procedures

Base populations and large mite groups were maintained in plastic containers (2 cm high, 2.5 cm in diameter), whereas individuals and small groups of mites were kept in 0.8 cm diameter glass tubes (2 cm high) with plaster of Paris bases soaked with water. Mites were maintained under humidity of >90% and fed powdered yeast *ad libitum*.

Base population

The mites used in the experiment originated from a stock culture combined of two populations derived from colonies of ca. 200 individuals found on onions in a garden near Kraków, Poland in 1998 and 2008. Since then, each population had been maintained in the lab at large numbers (>1000 individuals). The two populations had been mixed about ten generations prior to the beginning of the experimental evolution so as to increase genetic variation, which is crucial in the limited time span of laboratory experiments on multicellular organisms where adaptations arise from standing genetic variation rather than *de novo* mutations (see Barret and Schulter, 2007 for review).

Experimental lines

To test the impact of increased temperature on the evolution of life history traits in the bulb mite, we established five lines which were maintained at 28° C (high temperature; HT lines)

and five lines that were kept at 24° C, the temperature to which our base population had been adapted to (control temperature; C lines).

Each generation, 20 virgin males and 20 virgin females were placed into one container and left to interact freely for 5 days. After this time, all females within each line were transferred to a common container to lay eggs. Densities of developing larvae and ovipositing females were low in all the lines thanks to the size of containers. When tritonymphs (the last larval stage in bulb mites) emerged, ca. 80 - 90 of them were isolated to individual glass tubes. Emerging adults were then sexed and each male morph was noted. Twenty individuals of each sex from each line were used to start a new generation.

Experimental procedures

After 18 generations of experimental evolution the following life history traits were measured: eggs size, adult body size and development time. We established a full two by two factorial experimental design so that each line was tested at both temperatures. As female fecundity (the number of eggs) was measured four generations earlier and reported in a previous study (Plesnar-Bielak et al., 2012) we decided not to replicate these measurements. Instead, in the current study we discuss changes in egg and body size in the context of fecundity results obtained earlier.

Life history traits were measured in the next generation after transferring mites to experimental thermal environment to avoid maternal effects caused by developing, mating and ovipositing of females at the selection temperatures. Twenty previously mated females from each line were placed together for two days to lay eggs. After this time half of the eggs were put at 24° C and the other half at 28° C to develop. When the eggs developed to the tritonymph stage they were isolated to individual tubes. After reaching adulthood ca. 40 individuals per line per test temperature treatment were put into a common mating vial for five days. Then, mated females were transferred to individual tubes and left to lay eggs. Two eggs laid by each female were taken for egg size measurements and some of the others were left to develop and were later isolated to individual tubes (at the tritonymph stage) and left to mature. Emerged adults were used later to measure adult body size and in the development time assay.

a) Egg size measures

Two eggs laid by each female (total of ca. 500 eggs) were photographed using a stereomicroscope (30 times enlargement). The length and width of each egg were measured

using ImageJ software and the mean values for the female were taken to calculate egg volume as $1/(6\pi w^2 l)$ (Smallegange, 2011), where *w* is egg width and *l* is egg length. Egg size was analyzed using General Linear Model in Statistica 10 with log-transformed mean egg volume for a female as a response variable, selection temperature and test temperature as fixed factors and line ID nested in selection temperature as random factor.

b) Adult body size measures

Two male and two female offspring of each female (see *Experimental procedures*) were photographed using the stereomicroscope and their body length (with absence of mouthparts) was measured using the ImageJ software. In the case of males, only scramblers were measured because of very low frequencies or even no fighters in some of the treatments (see Results: changes of fighter proportion). Due to technical reasons (small number of individuals available) males from two lines were not included in the analyses, male and female body sizes were analyzed separately as there were different numbers of male and female lines in the analysis. The General Linear Model was applied with mean body length as a response variable, selection temperature and test temperature as fixed factors and line ID nested in selection temperature as random factor.

c) Development time measures

Fifteen males and fifteen females per each line per test temperature (not more than one from each female) were placed together into one container. After two days during which multiple mating took place, females were transferred to fresh containers in which they were allowed to lay eggs. Twenty-four hours after the first eggs were laid, the females were discarded. The containers were checked daily for emerging adults. Number of adults and their sex were verified and noted each day. Data were analyzed using a General Linear Model with sex, selection temperature and test temperature as fixed factors and line ID nested in selection temperature as random factor.

Changes of fighter proportion

Numbers of fighters and scramblers were noted each generation while sexing individuals. The proportion of fighters to total number of males was recorded throughout 35 generations of selection. We performed a repeated measure ANOVA on square root transformed fighter proportions with selection temperature as a predictor and generation as the repeated factor. In this analysis we included the data collected in generations 5, 10, 15, 20, 25, 30 and 35.

RESULTS

Egg size measures

Egg size was affected by test temperature with larger eggs laid at increased temperature (Tab.1, Fig. 1). Although the effect of selection temperature was non-significant, its interaction with test temperature significantly influenced egg size (Tab.1). At both temperatures HT females laid smaller eggs than C females, with eggs smaller at 24° C than at 28° C (the temperature at which they have evolved; Fig. 1). There was also a significant effect of line ID (Tab. 1).

Adult body size measures

Body size of both sexes was affected by test temperature (Tab. 1, Fig. 2). Note also that the decrease in body size with temperature was greater for females (19% decrease) than for males (7%). Neither selection temperature nor its interaction with test temperature had a significant effect on body size. There were significant differences in body size of both sexes between the lines (Tab. 1).

Development time measures

Males developed significantly slower than females (Tab.2, Fig.3). Both test temperature and selection temperature as well as their interaction affected development time. Development was faster at increased temperature, but HT lines developed slower than C lines, especially at control temperature. The effect of line ID was also significant (Tab. 2). No interaction with sex was significant (Tab. 2). Removing those interactions from the model did not influence the results quantitatively.

Changes of fighter proportion

The proportion of fighters decreased from generation to generation in HT lines (Fig. 4). Repeated measure ANOVA revealed significant effect of selection temperature, generation and, most importantly, their interaction on the proportion of fighters (Tab. 3).

DISCUSSION

We demonstrated differences between evolutionary and plastic responses (the effects of selection vs. test temperature) of the studied traits to ambient temperature increase. One of the most striking of our outcomes is the response of egg size to temperature change – eggs laid by HT females were smaller than those laid by C females at both test temperatures. In a previous study (Plesnar-Bielak at al., 2012), it has been shown that both C and HT females laid fewer eggs at increased temperature. Hence, the difference in egg size between HT and C lines can be explained by a trade off between egg number and egg size. This kind of compensation is also supported by the steeper increase of egg size with test temperature in HT lines. Together with HT lines having a fecundity advantage over control lines at the increased temperature (Plesnar-Bielak at al., 2012), our results indicate higher lifetime fitness of HT populations and thus their adaptation to elevated thermal conditions.

A trade off between the size of eggs and their number is quite common in populations exposed to changing thermal conditions. Similarly to our outcomes, a study by Seko et al. (2006) has showed that butterfly, *Parnara guttata guttata,* laid fewer but bigger eggs at higher temperature. Furthermore, the authors have also found, that both females and males were smaller at increased temperature. An opposite pattern (more smaller eggs in elevated thermal conditions) has been shown in amphipod *Gammarus lacustris* (Wilhelm and Schindler, 2000) as well as in tropical butterfly, *Bicyclus anynana*, (Geister et al, 2008), however females investment in progeny was higher at elevated (more beneficial) thermal conditions (Geister et al, 2008).

We did not observe the evolution of neither adult body size nor phenotypic plasticity of this trait. The relationship between temperature and body size of both males and females was straightforward and followed the TSR (Atkinson, 1994) as mites reached smaller sizes at increased temperature, irrespective of their selection regime. This outcome seems to correspond to development time as all of the lines developed faster when kept at higher temperature (as, in accordance with TSR, temperature negatively affects development and leads to smaller body size). According to Karl and Fischer (2008), energy assimilation and its conversion to biomass may increase with temperature, which is followed by faster growth. However, irrespective of thermal conditions, C lines developed faster than HT lines, again supporting the possible evolution of HT populations. An open question remains, why the direction of an evolutionary response in our populations to development time was opposite to the direction of phenotypic plasticity. If longer development of HT lines was a side-effect of selective pressure to increase body size, one would expect selection temperature to significantly affect size, which was not the case. Probably, their prolonged development may

have been associated with investment into some physiological traits increasing their performance under thermally stressful conditions (Plesnar-Bielak 2012), which might have led to the fecundity advantage demonstrated before (Plesnar-Bielak et al., 2012), perhaps through higher maternal investment as observed in *B. anynana* (Geister et al, 2008).

In our study, we observed that irrespective of temperate and despite being smaller, males took longer to develop relative to females, which would suggest slower growth rate of males than females. Our results seem to contradict the wildly accepted hypothesis explaining SSD - natural selection should favor either protandry (then males should be smaller and develop faster) or synchronized emergence of both sexes (then males should be the size of females). At the same time, although production of sperm is cheaper than eggs production (Darwin, 1871; Andersson, 1994; Simmons, 2001, Blanckenhorn et al. 2007), the production of gonads may be more costly for males than for females (Reed and Beckage 1997, Dixon 2000), which would elongate male development time. In addition, selection pressure for increased body size should be stronger for females than for males (Andersson, 1994, Blanckenhorn et al. 2007), because female fitness depends presumably on fecundity which in turn depends on size (Honek, 1993, Blanckenhorn et al. 2007). This would explain the observed pattern of sex related differences in development time. Furthermore, larger individuals may suffer greater heat stress (Blackenhorn, 2000) because an excess of heat (originating either from metabolic processes or from environment) in elevated temperature is harder to dissipate (Shepherd et al., 2008). Smaller individuals also need less energy for maintenance, thus they have more energy for any other activities including searching for partners and food, mating and investment in their sperm supply (Blackenhorn 2000).

At the same time, one should keep in mind that for males we examined only scramblers because fighter morphs almost disappeared in HT lines during the course of the experiment – what is in fact, one of the most interesting results of our study. The proportion of alternative phenotypic forms can be induced by changes in environmental conditions affecting developmental trajectories (see Fusco and Minelli, 2010 for review). Indeed, thermal environment affected morph expression in our lines – at increased temperature the proportion of fighters decreased in subsequent generations, whereas it did not change in control lines. As fighters have been shown to develop from larger trythonimphs (Smallegange, 2011), the decrease in the proportion of fighters may be simply an effect of the decrease in body size with temperature. However, if that would be the case, the decrease in fighter proportion should be instantaneous, because body size was affected only by the temperature during development (test temperature) and did not evolve during experimental evolution. Another

possible explanation is that increased temperature changed the costs-to-benefits ratio of inducing fighter and scrambler morphs or changed the relationship of this ratio with body size and condition. If fighter strategy had become more costly, it might have been beneficial only to males in very high condition. In fact, the costs of expressing fighter strategy are likely to be affected by temperature as aggressive behavior and long fights consume lots of energy (Nicieza and Metcalfe, 1999) and the energetic costs of behavioral and physiological traits increase with temperature (Elliot et al., 2005). Indeed, among genes with sex-biased expression, there are more genes expressed only in fighters than in scramblers and, importantly, many of them are metabolically related (Stuglik et al., unpublished). The increase in costs of fighter expression may have moved the switch point between the two morphs (the size at which a trythonimph is equally likely to develop into either of the two morphs) towards a bigger size. Estimating the switch point between the morphs in populations evolving at different temperatures as well as measuring relative costs and benefits of expressing both morphs at different temperatures would be needed to further verify this hypothesis.

Overall, our study shows that reaction norms for body size and development time are in line with the TSR predictions as mites develop faster and reach smaller sizes at increased temperature. At the same time, we have found that genetic effects of thermal adaptation were not always parallel to the reaction norms. Together with differences between males and females in their phenotypic plasticity in body size, life history traits revealed quite complex patterns of response to the changes in temperatures. We have also demonstrated that evolution at increased temperature affects the expression of alternative reproductive phenotypes in the bulb mites. Although we propose an explanation of the evolutionary mechanism behind this effect (which is the change of cost/benefits ratio of expressing a given morph with temperature), testing it would certainly need further investigations.

Two outcomes of our study are rather puzzling. First - despite smaller body sizes, the bulb mite females laid larger eggs at a higher temperature, and the pattern was more striking for lines evolving in elevated thermal conditions (HT lines). Second – males took longer to develop than females which is in contrast with the commonly accepted hypothesis that natural selection favors either protandry or synchronized emergence of both sexes. Further investigation into egg dry mass and their associated nutritional values, embryonic and larval viability as well as fitness of different male morphs at different test and selection temperatures could help solve these questions.

Our study may also help answer the question of adaptability of populations in the face of climate change. Together with temperature rise observed during the last few decades, climate warming has already caused shifts in latitudinal ranges and in phenology of many species (see Kingsolver, 2009 for review). At the same time, experimental evolution studies have showed that at least in case of microbes populations are able to adapt to novel thermal conditions but fitness improvement depends on temperature range (see Hoffmann and Sgrò 2011 for discussion). The bulb mites inhabit rather stable thermal environment as they live in soil (Diaz et al. 2000) and we may predict that *R. robini* would be highly thermosensitive (Angilletta 2009, Kingsolver 2009). We exposed our populations to quite substantial temperature increase (4°C), but it remained within their adaptive capacities as indicated by the evolution of trade-offs between development time and number and size of eggs. We showed that populations can respond to temperature increase by both phenotypic plasticity and complex evolutionary changes depending on a trait under consideration. What is more, we found that adaptation may occur rapidly, allowing populations to survive sudden environmental changes. Interestingly, elevated thermal conditions have also influenced male alternative reproductive phenotypes implying that climate warming may affect male competitiveness and sexual selection and these have been show to influence the adaptive potential of populations (Plesnar-Bielak et al., 2012).

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AUTHOR CONTRIBUTION:

A. P-B developed the concept, A. P-B and A.J performed experiments and data analysis, A. P-B and P.K. prepared and edited the manuscript prior to submission. The authors declare no competing interests.

TABLES:

Table 1. Effects of selection temperature (selection) and test temperature (test) on egg size and female and male body size analyzed using a nested ANOVA (selection temperature and test temperature are fixed factors, line ID is a random factor in selection temperature).

	Egg size			Female body size			Male body size		
	df	F	p	df	F	p	df	F	p
selection	1, 8	2.070	0.188	1, 8	0.047	0.833	1, 6	0.086	0.779
test	1, 267	43.802	<0.001	1, 178	76.273	<0.001	1, 98	18.924	<0.001
selection x test	1, 267	8.022	0.005	1, 178	0.233	0.630	1, 98	0.574	0.451
line ID (selection)	8, 267	50.518	<0.001	8, 178	6.476	<0.001	6, 98	2.995	0.010
error	267	-	-	178	-	-	98	-	-

Table 2. Effects of selection temperature (selection) and test temperature (test) and sex on development time analyzed using a nested ANOVA (selection temperature, test temperature and sex are fixed factors, line ID is a random factor in selection temperature).

	Development time		
	df	F	p
selection	1, 8	9.672	0.014
test	1, 2939	377.605	<0.001
sex	1, 2939	11.598	<0.001
selection x test	1, 2939	11.88	<0.001
selection x sex	1, 2939	0.985	0.321
test x sex	1, 2939	0.506	0.477
selection x test x sex	1, 2939	1.576	0.209
line ID (selection)	8, 2939	22.616	<0.001
error	2939	-	-

Table 3. Effects of generation number and selection temperature (selection) on the proportion of fighters analyzed with repeated measure ANOVA.

effect	df	F	p
selection	1, 7	23.766	0.002
generation	6, 42	6.553	<0.001
selection x generation	6, 42	4.750	<0.001

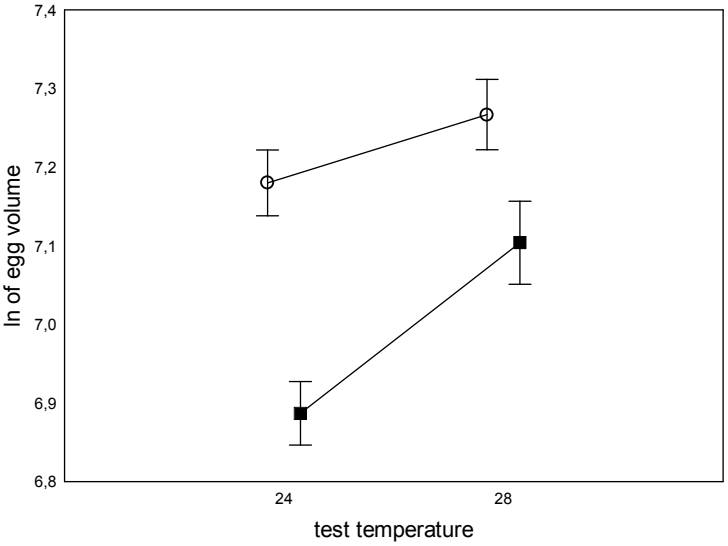
FIGURE LEGENDS:

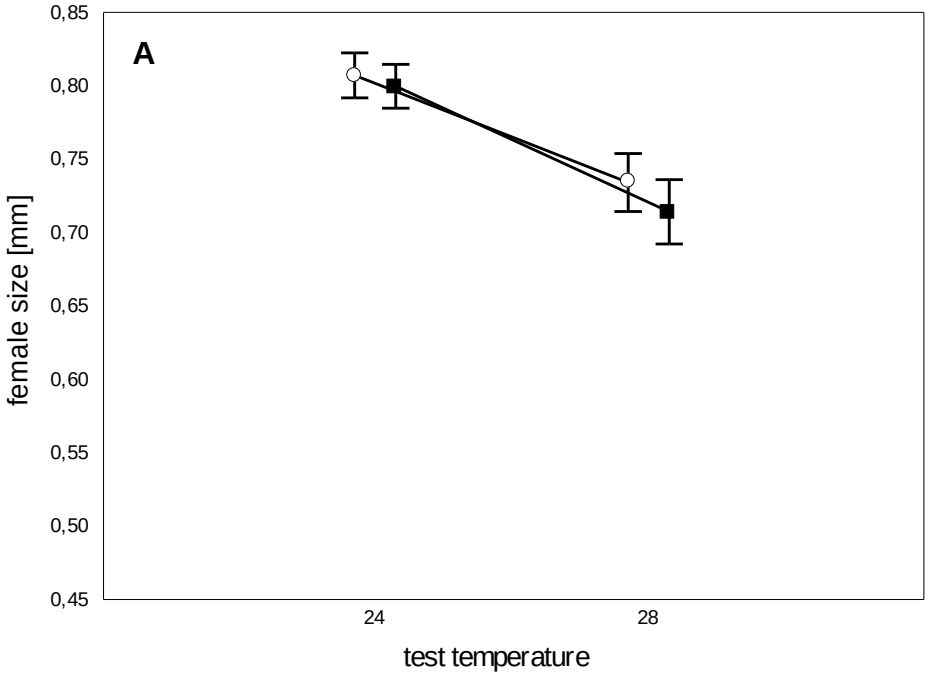
Figure 1. **Mean (transformed to *ln*) volume of eggs at 24°C and 28°C in HT (black squares) and C (white circles) lines.** Bars donate 95% confidence intervals.

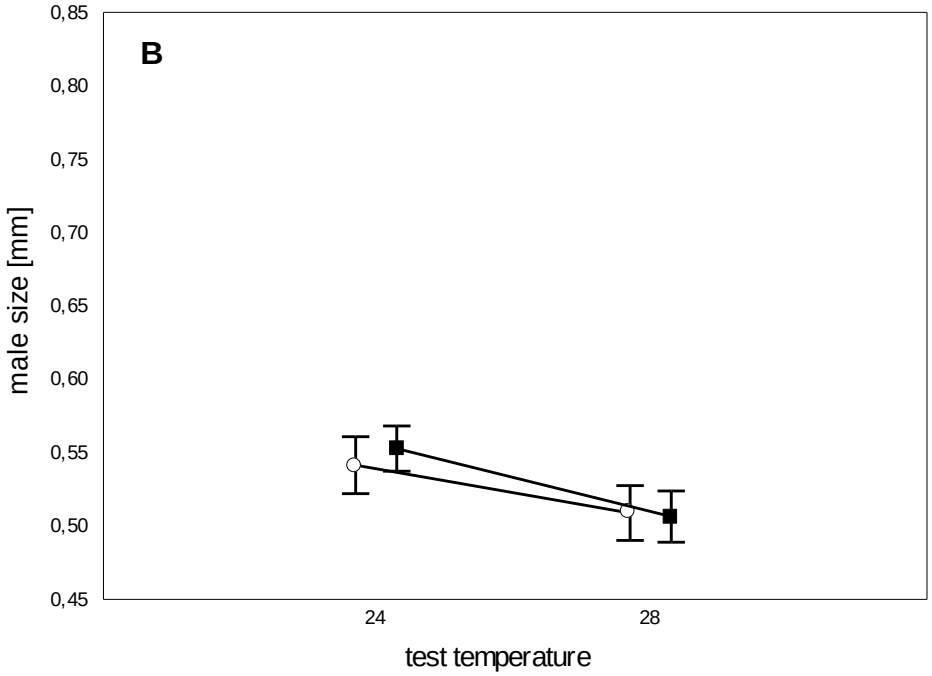
Figure 2. **Mean body size of females (A) and males (B) at 24°C and 28°C in HT (black squares) and C (white circles) lines.** Bars donate 95% confidence intervals.

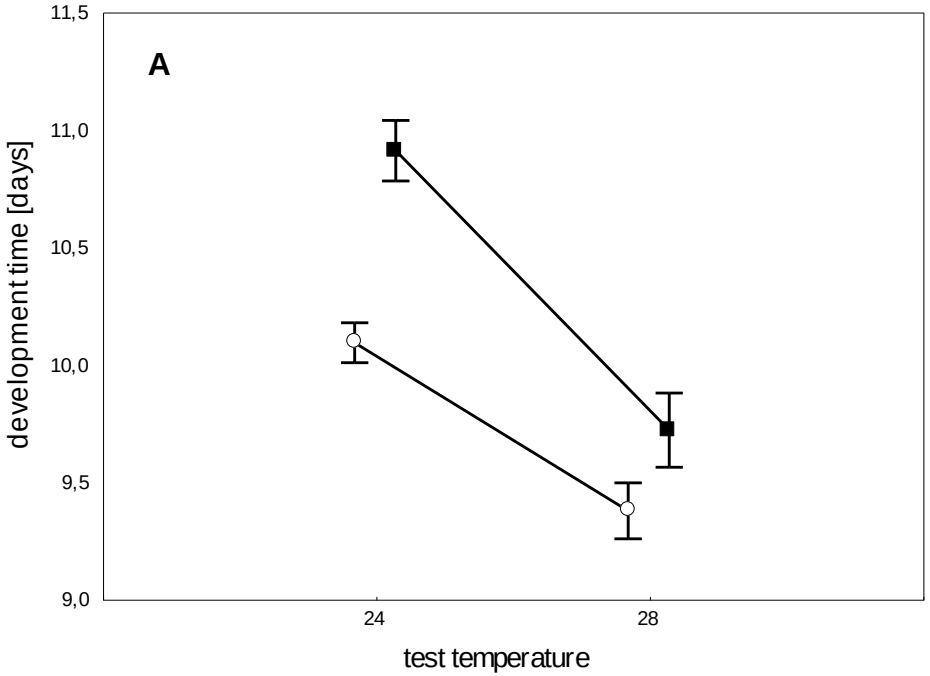
Figure 3**. Mean development time of females (A) and males (B) at 24°C and 28°C in HT (black squares) C (white circles) lines.** Bars donate 95% confidence intervals.

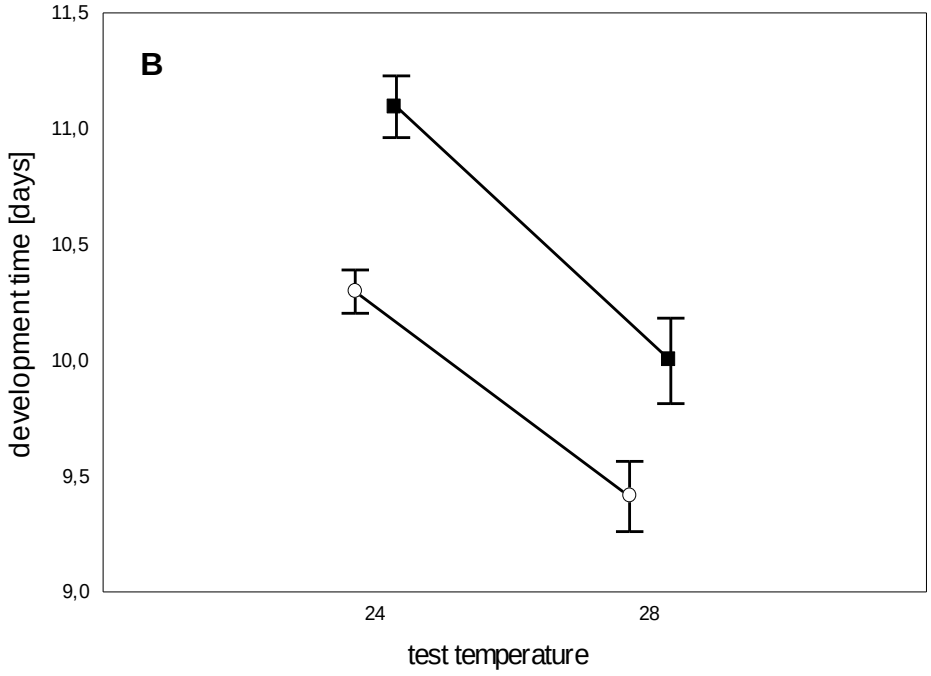
Figure 4. **Proportion on fighters measured in generation 5, 10, 15, 20, 25, 30 and 35 in HT (black squares) and C (white circles) lines.** Bars donate 95% confidence intervals for means.




A
0,85
0,80
0,75
0,70
0,65
0,60
0,55
0,50
0,45
female size [mm]
24
28
test temperature


B
0,85
0,80
0,75
0,70
0,65
0,60
0,55
0,50
0,45
male size [mm]
24
28
test temperature


A
11,5
11,0
10,5
10,0
9,5
9,0
development time [days]
24
28
test temperature


B
11,5
11,0
10,5
10,0
9,5
9,0
development time [days]
24
28
test temperature