# Morph-specific and sex-specific temperature effects on morphology in the colour polymorphic damselfly *Ischnura elegans*

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## Abstract

Colour polymorphic species with extensive ranges often exhibit large-scale geographic patterns of morph frequency variation. Because colour polymorphism is associated with correlated differences in multiple traits, such as thermal performance, a likely proximate explanation for such pattern is morph-specific responses to temperature variation. The colour polymorphic Blue-tailed damselfly *Ischnura elegans* exhibits large-scale geographic variation in morph frequencies, but the possibility that temperature is a proximate explanation for the latitudinal cline in morph frequencies has only ever been tested within a single developmental stage (egg survival and hatching time), where no difference between the morphs was found. I therefore carried out a temperature manipulation on larvae of *I. elegans* which I raised to maturity in the laboratory. I found that individuals exhibited incomplete compensatory growth after being exposed to cold temperatures, and that individuals which did not emerge successfully and those that experienced cold temperatures had more juvenile morphology in the last instar. In addition, there were sex-specific and morph-specific effects of temperature on adult morphology, such that sexual size dimorphism was increased when individuals experienced warm temperatures throughout the larval stage, and that cold temperatures tended to result in larger size of androchromes and their offspring compared to the other morphs. These results are generally consistent with the large-scale geographic variation in morph frequencies found in this species.

## Introduction

Multiple morphs within a single species can only persist through evolutionary time if the relative fitness of each morph varies, so that no one morph is always favoured (Hedrick, 1986). This variation could be generated by intraspecific interactions, for example where morphs are subject to negative frequency-dependent selection (Gross, 1991; Sinervo & Lively, 1996; Takahashi et al., 2010), or result from temporal or spatial variation, for example if each morph prefers slightly different biotic and/or abiotic conditions (Ahnesjö & Forsman, 2006; Chang & Emlen, 1993; Munday et al., 2003). Colour polymorphism is usually associated with correlated differences in multiple traits (McKinnon & Pierotti, 2010), and ecological differences between morphs have been found even in species where colour polymorphism was long-thought to be neutral (e.g. Schemske & Bierzychudek, 2007). In species where morphs differ in the darkness or melanization of their colouration, temperature or light effects are often determined to be a proximate cause for geographic variation in morph frequencies (de Jong & Brakefield, 1998; Galeotti et al., 2003; Phifer-Rixey et al., 2008). However in colour morphs that do not differ in any systematic way along a "dark – light" axis, other factors may be more important in determining spatial clines, such as visibility in the water column (Terai et al., 2006) or UV resistance (Cooper, 2010). Large-scale geographic patterns of morph frequency variation may often be coupled with stochastic variation on a small scale (Barrett et al., 2004; Cook, 1998; Oxford, 2005).

Female colour polymorphism is common in damselflies (Corbet, 1999), as is large-scale geographic variation in morph frequencies within polymorphic species (e.g. Iserbyt et al., 2009; Sánchez-Guillén et al., 2011; Van Gossum et al., 2007; Wellenreuther et al., 2011). Female morphs usually differ along a "cryptic – conspicuous" axis rather than a "dark – light" axis (i.e. one female morph is often a brightly-coloured male mimic, while the other(s) are dull and brown, green, or gray in colour), suggesting that factors other than temperature may be important in such systems. Empirical evidence for a role of temperature in determining damselfly morph frequencies is conflicting; some studies have suggested a link (e.g. opposite patterns of condition in relation to weather between morphs, Bots et al., 2009; an increasing frequency of Androchrome females of with decreasing temperature, Hammers & Van Gossum, 2008; enhanced performance of Androchromes at colder temperatures, Takahashi et al., 2011), while others have found no significant morph-by-temperature interactions (e.g. no difference in thermal properties between the morphs or the sexes, Bots et al., 2008; no difference in egg survival or hatching success according to maternal morph, Bouton et al., 2011). However none of these few experimental studies which have tested for morph-specific temperature effects have considered effects across multiple life stages. I therefore carried out a temperature manipulation on larvae of *Ischnura elegans* which I raised to maturity in the laboratory, thus enabling me to look for morph-specific responses throughout ontogeny.

The Blue-tailed damselfly *Ischnura elegans* is becoming something of a model system for studying the role that sexual selection and sexual conflict can play in the maintenance of a colour polymorphism (Abbott & Svensson, 2010; Gosden & Svensson, 2008; Gosden & Svensson, 2009; Hammers & Van Gossum, 2008; Svensson et al., 2005). However this species also exhibits large-scale geographic variation in morph frequencies, specifically a latitudinal cline in the frequency of the male-mimic androchrome morph (Gosden et al., 2011). Although the ultimate causes of small-scale morph frequency dynamics are slowly becoming understood in this system and suggest a role for frequency- and density-dependent intraspecific interactions as well as precipitation (Abbott et al., 2008; Gosden & Svensson, 2007; Gosden & Svensson, 2009; Hammers & Van Gossum, 2008; Svensson & Abbott, 2005;

Svensson et al., 2005; Wellenreuther et al., 2011), the proximate explanation for the latitudinal cline in morph frequencies has not yet been determined. My aims with this study were twofold: firstly, to follow-up on a previous study of larval growth and morphology (which found that morphological differences between the morphs and the sexes are present even in the larval stage, Abbott & Svensson, 2008) by carrying out a more detailed morphological investigation using geometric morphometrics, and secondly (and more importantly), to look for morph-specific responses to the temperature manipulation. Given the large-scale geographic patterns in morph frequencies present in *I. elegans*, I predicted that androchrome females would outperform the other morphs in the cold treatment. Apart from successfully replicating previous results, I also found that sexual size dimorphism was increased when individuals experienced warm temperatures throughout the larval stage (which will have implications for the effectiveness of male mimicry), and that cold temperatures tended to result in larger size of androchromes and their offspring compared to the other morphs. These results are generally consistent with the large-scale geographic variation in morph frequencies found in this species.

## Methods

*Study species*

*Ischnura elegans* is a small damselfly which ranges from northern Spain to southern Sweden. Although it may be multivoltine at the southern edge of its range, in Sweden it is univoltine. Its preferred habitat is still ponds set in open landscapes such as agricultural fields (Askew, 1988). In Swedish populations the adult damselflies emerge from late May to early August (Abbott & Svensson, 2005). After mating, females lay eggs which hatch after several weeks and overwinter as larvae (Corbet, 1999). Females are colour polymorphic, and may belong to one of three morphs: androchrome, infuscans, or infuscans-obsoleta (colour pictures of the three morphs can be found in Svensson et al., 2008). Morph identity is controlled by a single locus with three alleles in a dominance hierarchy (androchrome > infuscans > infuscans-obsoleta, Sánchez-Guillén et al., 2005). Males are monomorphic, so this is a sex-limited polymorphism. Androchrome females have similar adult colouration to males, relatively masculinized morphology (Abbott & Gosden, 2009), and a higher intersexual genetic correlation for morphological traits (Abbott & Svensson, 2010), suggesting that they are male mimics. There is also evidence that frequency- and density-dependent selection play a role in the maintenance of the three morphs (Gosden & Svensson, 2009; Svensson et al., 2005). There is a significant latitudinal cline in morph frequencies (Gosden et al., 2011), but no evidence of genetic isolation-by-distance in a north-south direction (Wellenreuther et al., 2011).

*Rearing of larvae*

Male and female damselflies were captured in copula from a natural population outside Lund, Vombs Vattenverk, in June-July 2003, and females were taken to the laboratory to oviposit (for the location of this population, please see Abbott et al., 2008). A total of 33 clutches of eggs were obtained (one clutch per female from at least 10 females of each female morph). The same method was followed as in a previous study of larval development (Abbott & Svensson, 2008), i.e. females were kept in individual containers and allowed to lay eggs into damp filter paper for 48 hours, after which the female was removed and the container filled with water. Larvae were transferred to plastic aquaria after hatching (1 family per aquarium), and fed with brine shrimp (*Artemia sp.*) daily. After approximately two months the larvae had grown large enough to be placed into individual mesh-sided containers within the aquaria (this is done to prevent cannibalism). 20 individual larvae were randomly selected from each family, identified with a unique ID number, and assigned to one of two temperature treatments (warm vs. cold). Individuals from all families were then randomly assigned to aquaria within each temperature treatment, to avoid confounding family and block (aquarium) effects. Wooden perches were later added to the individual containers for damselflies to crawl up during emergence.

Larvae in the cold temperature treatment were kept at 12°C for 4 months, and then at 21°C until emergence. Larvae do not grow when the temperature falls below 8°C (Corbet, 1999), so 12°C was chosen as being representative of fall and spring temperatures where growth is still possible. Larvae in the warm temperature treatment were kept at 21°C under the entire larval period, for the sake of comparison with the previous study (Abbott & Svensson, 2008). All larvae experienced a constant light regime of 12:12, and were sexed and photographed once every two weeks. Mesh covers were placed over the aquaria once larvae began reaching the final instar, and they were checked daily for the presence of adult damselflies. Date of emergence was noted, and adults were measured using digital callipers for the same five morphological measures as in previous studies of morphology in this species (Abbott & Gosden, 2009; Abbott & Svensson, 2008; Abbott & Svensson, 2010), i.e. total length, abdomen length, thorax width, wing length, and width of the 4$^{th}$ segment of the abdomen. Female morph cannot be confidently identified until sexual maturity (except in the case of infuscans-obsoleta females, which have unique black patterning that is identifiable even when newly-emerged) so females were placed in small individual holding containers and manually fed *Drosophila* every day until their morph could be identified.

Larval size and shape were measured using geometric morphometrics. 14 landmarks were selected along the outline and midline of the larva (Figure 2a), then digitized and analysed using the tps suite of programs, which are freely available from http://life.bio.sunysb.edu/morph/. Only the right sides of the larvae were digitized in order to minimize non-independence of the landmarks. Centroid sizes were computed and used as a measure of overall larval size. Larval shape was described using the matrix of partial warps plus the uniform component.

*Analysis*

All analyses were carried out in JMP (SAS Institute Inc., 2007). Block (aquarium) was initially included in all analyses (nested within temperature) but was never significant (all $P >$ 0.10) and is therefore not included here. Individuals in the final instar could be identified based on the size of the wing pads, so the final measurement in the last instar was set as time = 0 (just prior to emergence). This was done to control for initial differences in size (instar) at the start of the measurement period, and should hopefully ensure that larvae were at approximately the same developmental stage at each time point, at least for the later part of the larval period. A maximum of 10 time points are included (-9 to 0) since very few individuals took more than 10 measurement periods to develop, and sample sizes for time points -10 and -11 are therefore small. Setting the first time point at the start of the measurement period, as was done in a previous analysis of larval development (Abbott & Svensson, 2008), does not affect qualitative conclusions (data not shown) but does tend to exaggerate differences at the end of the measurement period. Individuals that died before the last instar (as determined by wing pad size; Benke, 1970) were excluded from all analyses, and mortality both before the last instar and in the last instar was relatively modest (14% and 18% respectively; 32% in total), similar to the previous analysis of larval morphology (28% in total; Abbott & Svensson, 2008). Larvae that died before the last instar did so in most cases shortly after being moved into the individual enclosures, likely as a result of the change in conditions.

Differences in size over ontogeny were analysed using repeated measures MANOVAs of the following form:

Size = Family + A + B + A*B + A*Time + B*Time + A*B*Time

Where A and B are combinations of the factors maternal morph, own morph, sex, emergence success, and temperature (note that own morph can never be combined with sex or emergence success since morph cannot be determined prior to emergence and is only expressed in females). Because each family can by definition only have one value of maternal morph, family was nested within maternal morph in models where maternal morph was included.

Differences in the allometric relationship between size and shape were also tested using MANOVA models of the form:

Shape = ID(Family) + Family + A + Size + A*Size

ID (nested within family) and the family effect are to control for individual and family differences respectively. Family was also nested within maternal morph when this factor was included in the model. A is again one of the following factors: maternal morph, own morph, sex, emergence success, or temperature. A significant A*Size interaction indicates that the allometric relationship between shape and size differs between the groups. For a detailed list of the models used in the analysis of larval size and allometric effects, see the supplementary information.

For differences in size and shape in the last instar, and for development time, I included all factors that could logically be included together in the same model (i.e. maternal morph, sex, emergence success and temperature, or maternal morph, own morph, and temperature) along with all two-way interactions (see supplementary information). Sex and emergence success cannot be included in models also containing own morph since males are monomorphic and female morph can only be determined in the adult stage. Emergence success was not included in the model of development time because only individuals that emerged successfully were considered to have completed development. Differences in adult size and shape were analysed using MANOVAs of the same form as for development time, but with the five morphological traits as the dependent variables. Differences in size were analysed using the "sum" function and differences in shape using the "identity" function within the MANOVA module in JMP. See supplementary information for details.

Note that for MANOVAs in JMP an *F*-test is carried out for factors with only two levels, while for factors with three or more levels several tests (Wilks' $\lambda$, Pillai's Trace, Hotelling-Lawley, and Roy's Max Root) are carried out. Here I report *F*-values and Wilks' $\lambda$-values as appropriate.

I also tested probability of successful emergence and offspring sex ratio using generalized linear models with a binomial error distribution and logit link function (Bolker et al., 2008). For probability of successful emergence I included maternal morph, sex, and temperature as factors. For offspring sex ratio I included maternal morph and temperature as factors. All two-way interactions were included in both cases. See supplementary information for details.

*Fixed vs. random effects*

In the above models family could conceivably either be treated as a random or as a fixed effect. Family effects are usually treated as random effects (Potvin, 2001), but in this case the selection of the females producing the clutches was not random. This was in order to ensure an approximately equal sample size within each maternal morph, and such non-randomness could argue for treating family as a fixed effect (Potvin, 2001). In addition, including random effects within multivariate models and/or unbalanced models may not always be straightforward. Because of these issues I taken a pragmatic approach, and have chosen to present results from analyses which treat family as a random effect in all univariate models, but as a fixed effect in all multivariate models. Treating a factor as a fixed vs. a random effect changes the calculation of the test statistics, and may have a large impact on *P*-values. Because of this, I have used two different methods to check that including family as a fixed effect in the multivariate models has no effect on my qualitative conclusions. Firstly, for the larval size data I carried out Bonferroni-corrected univariate tests at each time point with family as a random factor. The results of these tests are presented in figure 1. Secondly, for all multivariate models (including larval size) I manually calculated *F*-values that were corrected for the inclusion of a random family effect (using data from the JMP MANOVA output). The qualitative conclusions were consistent regardless of method of analysis, so for the sake of simplicity I only report the results of the fixed effects multivariate models here.

## Results

A total of 468 individual larvae were tracked over the course of the experiment. Of these, 111 were excluded from further analysis because they died before the last instar (see Methods), leaving a total sample size of 357 individuals. The sex ratio within this sample was nearly 1:1, with 51.1% males. A total of 106 female offspring (61%) lived long enough to identify their morph in the adult stage.

*Larval size*

For temperature and maternal morph there were both significant main effects (indicating an overall difference in size in the larval stage) and significant interactions with time (indicating differences in the change in size over time) on larval size. For sex there was significant main effect only, while for emergence success there was no significant main effect, but a significant interaction with time. See supplementary information for statistical summary tables. There was no significant effect of own morph, and there were no significant two-way interactions between any of the main factors (all $P > 0.05$). Offspring of infuscans-obsoleta females were smaller than offspring of the other morphs (maternal morph: $F_{2, 108} = 25.66$, $P < 0.0001$), although this difference decreased over ontogeny (maternal morph*time: Wilks' $\lambda_{18, 200} = 0.4038$, $P < 0.0001$) and by the last instar the trend was that offspring of androchrome females were larger than offspring of both other morphs (figure 1a). Females were larger than males throughout ontogeny (sex: $F_{1, 108} = 4.536$, $P = 0.0355$), but this difference only became significant in the final instars (figure 1b). Individuals that emerged successfully were increased in size faster than individuals that did not emerge successfully (emerged*time: $F_{9, 100} = 3.958$, $P = 0.0002$), although the difference between the groups was only really apparent in the last instar (figure 1c). Individuals that had experienced warm conditions throughout development were larger than those that had experienced cold temperatures during part of development (temp: $F_{1, 108} = 6.453$, $P = 0.0125$), and this difference was not fully compensated for by the last instar (temp*time: $F_{9, 100} = 7.960$, $P < 0.0001$, figure 1d), despite the fact that individuals in the cold treatment had been moved back into warm conditions before reaching the last instar.

*Larval shape*

Unsurprisingly, shape and size were highly correlated ($P < 0.0001$ in all models). Individuals start off with large heads and relatively small abdomens, and during larval development the relative length of the abdomen increases, the eyes become larger in relation to head size, and the thorax becomes wider (figure S1a). There were also significant differences in the allometric relationship between shape and size for all factors (all $P < 0.0001$ except for own morph which had $P = 0.0234$, figure S1b-e).

*Differences in the last instar*

Because size and shape in the last instar should be relevant to adult morphology (Abbott & Svensson, 2008), I also considered data from the last instar separately. There were significant effects of maternal morph ($F_{2, 54.4} = 4.04$, $P < 0.05$) and emergence success ($F_{1, 323.9} = 22.15$, $P < 0.0001$) on size in the last instar, as well as a significant interaction between sex and temperature ($F_{2, 322.6} = 4.88$, $P < 0.05$), but no effect of own morph ($P > 0.05$). Offspring of androchromes were significantly larger than offspring of the other morphs, and individuals that emerged successfully were larger than those that did not. The degree of sexual size dimorphism was temperature-dependent; in the cold treatment there was no difference in size between the sexes, while in the warm treatment females were significantly larger than males. There were significant effects of maternal morph (Wilks' $\lambda_{48, 576} = 0.799$, $P < 0.05$), sex ($F_{24, 288} = 2.088$, $P < 0.01$), emergence success ($F_{24, 288} = 2.052$, $P < 0.01$), and temperature ($F_{24, 288} = 2.015$, $P < 0.01$) on shape in the last instar, but no significant interactions and no effect of own morph ($P > 0.05$). Offspring of androchrome females are differently shaped than offspring of the other two morphs, with smaller heads, larger eyes, and a longer abdomen (figure 2b). This pattern is similar to the pattern of sexual dimorphism in shape, since males also had larger eyes, a narrower thorax, and a longer, thinner abdomen than females in the last instar (figure 2c). Individuals that did not emerge successfully had a more immature shape than those that did emerge, with a relatively larger head and less well-developed thorax (figure 2d), and the same was true of individuals from the cold temperature treatment (figure 2e). See supplementary information for statistical summary tables.

*Development time*

There were significant effects of maternal morph ($F_{2, 31.2} = 20.83$, $P < 0.0001$), sex ($F_{1, 293} = 12.60$, $P < 0.001$), and temperature ($F_{1, 280.2} = 42.93$, $P < 0.0001$) on development time. There were no significant two-way interactions between any of the factors, and no effect of own morph on development time (all $P > 0.10$). Offspring of infuscans-obsoleta females had shorter development time than the other morphs, and females had longer development time than males. Individuals that had experienced warm conditions during development emerged sooner than those that had experienced cold conditions. See figure S2.

*Adult morphology*

Females were larger than males in the adult stage ($F_{1, 227} = 10.70$, $P < 0.01$), but there was no significant effect of maternal morph on overall size ($P > 0.2$). This is somewhat surprising given that offspring of androchrome females were significantly larger than offspring of the other morphs in the final instar. Similarly, there was no effect of temperature on overall adult size ($P > 0.6$), despite a significant difference in the last instar. Nor was there any difference in size according to own morph ($P > 0.6$), but this is consistent across stages since there was no difference in the last instar either. There was also evidence of differences in shape in the adult stage. There were significant effects of sex ($F_{5, 223}$ = 36.87, $P$ < 0.0001) and temperature ($F_{5, 223}$ = 6.51, $P$ < 0.0001) on shape. Consistent with previous results, males had relatively longer, narrower abdomens and shorter wings than females. Individuals that developed in warm conditions were larger overall but had relatively shorter wings. There were also significant interaction effects between maternal morph and temperature (Wilks' $\lambda_{10, 446}$ = 0.896, $P$ < 0.01), and between own morph and temperature (Wilks' $\lambda_{10, 120}$ = 0.739, $P$ < 0.05). In general, androchromes and their offspring were largest for most (but not all) traits in cold conditions, while infuscans or infuscans-obsoleta females and their offspring were largest for most (but not all) traits in warm conditions.  See table 1 for details.

*Successful emergence and sex ratio*

There were no significant effects on successful emergence for any of the factors included here. However infuscans-obsoleta females produced significantly more female-biased broods than the other morphs ($\chi^2_{2, 356}$ = 6.12, $P$ < 0.05, figure 3). There was no effect of temperature and no significant interaction (all $P$ > 0.40).

## Discussion

*Morphological differences between the sexes and the morphs*

Morphological results were consistent with previous data in that they suggest that (1) females are larger than males throughout most of development, and that this difference is reinforced by later emergence in females (figures 1b and S2b), (2) that a higher growth rate (i.e. steeper slope of size in relation to time) in offspring of infuscans-obsoleta females is offset by earlier emengence time (figures 1c and S2a), (3) that individuals that emerged successfully were larger than those that did not near the end of development, but smaller in the early stages of development (figure 1d), and (4) that androchromes have masculinized morphology (longer, narrower abdomens, shorter wings, and narrower thorax) in both larval and adult stages (figure 2b-c and table 1). All of this suggests that these effects are relatively robust, at least within in the source population (Vombs Vattenverk, Abbott & Svensson, 2008). The only real point of difference is in the lack of evidence of a higher growth rate in females than in males in this study, which is probably due to the differences in the methods of analysis between this study and the previous one. Growth was previously measured in terms of time since hatching of the eggs rather than relative to the last instar, which results in an exaggeration of the differences at the end of the measurement period (data not shown).

Interestingly, it seems as if males have larger eyes and a relatively wider head than females (figure 2c). Males engage in scramble competition, so the ability to detect females visually should be related to mating success. This would explain the development of larger eyes in males if visual performance is correlated with eye size. Evidence from butterflies and other insects suggests that males indeed often have larger eyes and more acute vision than females (Land, 1997; Rutowski & Warrant, 2002; Ziemba & Rutowski, 2000). Eye size and/or head size might therefore be interesting traits to measure in future morphological studies of *I. elegans*. It is reassuring, though, that the five "standard" morphological traits which have previously been measured in several studies (i.e. total length, abdomen length, thorax width, width of the 4th segment of the abdomen, and wing length, Abbott & Gosden, 2009; Abbott & Svensson, 2008; Abbott & Svensson, 2010; Gosden & Svensson, 2008) seem to capture much of the gross morphological variation in this species.

*Effects of temperature on development*

Exposure to cold temperatures during development resulted in a decrease in growth rate which was not fully compensated for by the last instar (figure 1e). This is consistent with results from some other ectothermic organisms (Ali et al., 2003; de Block et al., 2008; de Block & Stoks, 2003), but contrasts somewhat with the typical expectation that ectotherms that experience low temperatures during development should have a longer developmental period but be larger as adults (Angilletta, Jr. et al., 2004); despite a longer development time (figure S2c) individuals from the cold treatment were smaller in size. Individuals that did not emerge successfully and those who had experienced cold temperatures during development had a more juvenile morphology in the last instar (figure 2d-e). Because size and shape are highly correlated and both groups were smaller overall this is perhaps not so surprising, but is noteworthy in that it suggests that allometric effects are present even among individuals at the same developmental stage, a phenomenon known as static allometry (Cock, 1966). In the previous analysis of larval morphology shape differences were measured using principal components analysis, which made interpretation of shape differences between groups somewhat problematic. The results from this study confirm that individuals that did not emerge successfully were less well-developed than those that did, and that poor larval growth may result not only in smaller adult size but also in direct mortality costs, even in the absence of predators or competitors.

Some of the size differences seen in the last instar did not appear to carry over into the adult stage, such as the difference between individuals from the two temperature treatments. Although it’s possible that these differences really were cancelled out after emergence, it seems more likely that the lack of statistically significant differences in the adult stage is due to methodological factors. Size in the larval stage was dependent on more than five times as many parameters (i.e. the x and y locations of the 14 landmarks) than in the adult stage, and probably gives a better estimate of overall size. In addition, measurements in the adult stage were taken on live animals using calipers while data from the larval stage was obtained from photographs, which may result in a higher error variance in the adult measurements. For example, in a previous dataset repeatability of morphological measurements in *I. elegans* was >90% (Abbott & Svensson, 2010), but repeatability of landmarks in an analysis of fly wings (Abbott et al., 2010) was >99.9% (J. Abbott, unpublished data). Temperature-specific shape differences were, however, detectable in the adult stage. Individuals that experienced cold temperatures had longer wings, a phenomenon that has also been found in *Drosophila* (Frazier et al., 2008; Gilchrist & Huey, 2004; Partridge et al., 1994). There appears to be substantial evolutionary stability in the wing size-temperature reaction norm within *Drosophila* (Powell et al., 2010), so these results suggest that a general pattern of increasing wing size with decreasing developmental temperature may even be conserved across insect taxa.

*Adult offspring production*

There were also morph-specific effects on offspring production. Infuscans-obsoleta females produced more female-biased broods, at least when considering individuals that survived until the last instar (figure 3). The reason for this pattern is unknown, although male-biased early mortality is of course a possibility. Unfortunately this is impossible to test with the current dataset since many of the individuals that died early on (and were therefore excluded from the final analysis) did so when they were too small to be able to reliably determine sex. Reduced survival of male offspring of infuscans-obsoleta females could ultimately be a result of intralocus sexual conflict. Intralocus sexual conflict results when one or both sexes is displaced from its optimum trait value by counter-selection in the other sex (Rice & Chippindale, 2001). A previous study of intersexual genetic correlations for morphological traits in this species suggested that phenotypic masculinization may result in reduced intralocus sexual conflict between males and androchrome females (Abbott & Svensson, 2010). Because of small sample sizes the infuscans-obsoleta morph was not included in that study, so at present I can only speculate, but reduced survival or production of male offspring could be consistent with increased intralocus sexual conflict in this morph.

*Morph-specific and sex-specific effects of temperature on morphology*

Androchrome females grew to be larger than the other morphs when exposed to cold temperatures during development (table 1). The same was also true of the offspring of androchromes. This suggests that androchromes perform better in cold temperatures, and is consistent with results from the related species *I. senegalensis* (Takahashi et al., 2011). Any sort of advantage of large size (for example via predator avoidance in the larval stage or a fecundity advantage to large size in the adult stage) would then translate into higher androchrome frequencies in colder areas, and may account for the large-scale pattern of increasing androchrome frequencies with increasing latitude in *I. elegans* (Gosden et al., 2011).

A further interesting result from the temperature treatment was the effect on sexual size dimorphism (SSD). SSD was enhanced when larvae experienced warm conditions throughout development (table 1). This is consistent with previous research which has found an effect of temperature on SSD in another species of damselfly (de Block & Stoks, 2003), and could explain temporal and small-scale spatial variation in the degree of SSD in *I. elegans* (Abbott & Gosden, 2009; Gosden & Svensson, 2008). Increased SSD at warmer temperatures could also have an effect on androchrome frequencies by influencing how effectively they mimic males. Androchromes seem to avoid costs associated with superfluous matings via male mimicry (Gosden & Svensson, 2009; Hammers & Van Gossum, 2008; Svensson & Abbott, 2005), and although they have similar colouration and body shape to males, they (and the other female morphs) are usually larger than males overall (Abbott & Gosden, 2009; Abbott & Svensson, 2008). Increased SSD should therefore hamper this male mimicry if males can identify androchromes as females based on their larger size. However the ratio of mimics to models is also very important in determining the efficiency of mimicry (Harper, Jr. & Pfennig, 2007). When mimics are rare and models are common, mimics need not resemble the model as closely since the likelihood of encountering a mimic is relatively low. This means that when androchromes are rare relative to males selection for mimicry is relaxed (Iserbyt et al., 2011), or conversely, that androchromes should be less frequent when their ability to mimic males is reduced—for example via increased SSD. To my knowledge there has been no investigation of large-scale geographic patterns of SSD in *I. elegans*, but if SSD increases with increasing temperature (i.e. decreasing latitude) this could also contribute to the latitudinal cline in androchrome frequencies.

Although it seems likely that temperature effects explain some of the large-scale geographic variation in morph frequencies, there are of course some caveats associated with this study. For one thing, the temperature manipulation was moderate compared to the variation in temperatures experienced by natural populations of *I. elegans* in Sweden. It is therefore uncertain that similar results would be obtained under a more natural temperature regime, or whether the same results would be obtained from populations from other regions (Bouton et al., 2011). There is also good evidence that frequency- and density-dependent selection play a role in determining morph frequencies in this system (Gosden & Svensson, 2009; Svensson et al., 2005). Given the substantial temporal and within-region variation in morph-frequencies in *I. elegans* (Gosden et al., 2011; Svensson & Abbott, 2005), it seems likely that even if temperature plays a proximate role in influencing large-scale variation in morph frequencies, it may have little explanatory power on a more local scale (where local weather and especially precipitation may be more important, Wellenreuther et al., 2011). Nevertheless, the effects seen here were consistent with the latitudinal cline in morph frequencies and were obtained using a relatively modest temperature manipulation, which indeed suggests that morph-specific variation in temperature response is the cause of large-scale variation in morph frequencies, similar to the related species *I. senegalensis* (Takahashi et al., 2011).

*Future directions and conclusions*

A major limitation when trying to understand morph-specific differences in the larval stage in *I. elegans* is the inability to identify an individual's morph prior to emergence and sexual maturity. It is similarly impossible to determine a male's genotype at the morph locus since males are monomorphic. At present the only solution is to use maternal morph as a proxy for offspring genotype at the morph locus (Abbott & Svensson, 2005; Abbott & Svensson, 2008; Abbott & Svensson, 2010), but this method obviously provides rather poor resolution. Molecular markers for the morph locus are currently under development (E. I. Svensson, personal communication), so hopefully in future more detailed studies of the relationship between phenotype and genotype at the morph locus will be possible. It would, for instance, be very interesting to test the hypothesis suggested here, that androchromes perform better in cold conditions, and see whether there is a difference in performance between homozygous androchromes and heterozygous androchromes. A recent paper by Stocks and de Block (2011) examined resistance to cold shock and levels of the heat-shock protein Hsp70 in *I. elegans*. They found that more northerly populations were more resistant to cold shock, and had higher levels of Hsp70, but did not look for any differences between morphs. This is another potential avenue for further investigation. Based on the results here one might predict that androchromes would be more cold resistant and have higher levels of Hsp70.

Although frequency-dependent selection and morph-specific ecological differences are usually considered alternative explanations for the maintenance of multiple morphs with the same population, a recent paper by Takahashi and colleagues (2011) suggests that negative frequency-dependence and a genotype-by-environment (i.e. morph-by-temperature) interaction in performance actually combine to produce a latitudinal cline in morph frequencies, and that neither phenomenon is sufficient to explain the pattern in itself. Detailed investigation of the relationship between frequency-dependence and genotype-by-environment interactions should therefore be a priority in future investigations of polymorphic species of any taxon, especially since the results presented here suggest that morph-specific effects of temperature can exist even in morphs that do not exhibit obvious melanization-mediated differences in thermal properties.

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Table 1: LS mean values of the five morphological traits according to (a) sex, (b) temperature, (c) maternal morph by temperature, and (d) own morph by temperature interaction. All values are in mm, and the largest values within each treatment (or treatment combination) are highlighted in bold.

a) Sex

| | Length | Abdomen | Thorax | S4 | Wing |
|---|---|---|---|---|---|
| Female | **29.31** | 23.16 | **2.20** | **0.78** | **19.41** |
| Male | 29.28 | **23.21** | 2.12 | 0.66 | 16.59 |

b) Temperature

| | | | | | |
|---|---|---|---|---|---|
| Cold | 29.20 | 23.08 | 2.14 | 0.70 | **18.46** |
| Warm | **29.39** | **23.30** | **2.19** | **0.74** | 17.55 |

c) Maternal morph by temperature interaction

| | | | | | |
|---|---|---|---|---|---|
| A Cold | **29.90** | **23.65** | **2.16** | 0.70 | **17.58** |
| I Cold | 29.03 | 23.00 | 2.11 | **0.71** | 20.72 |
| O Cold | 28.66 | 22.60 | 2.14 | 0.70 | 17.08 |
| A Warm | 29.38 | 23.33 | **2.19** | 0.74 | **17.70** |
| I Warm | 29.27 | 23.17 | 2.21 | **0.75** | 17.54 |
| O Warm | **29.52** | **23.39** | 2.17 | 0.73 | 17.40 |

d) Own morph by temperature interaction

| | | | | | |
|---|---|---|---|---|---|
| A Cold | **29.53** | **23.40** | **2.22** | **0.75** | **21.30** |

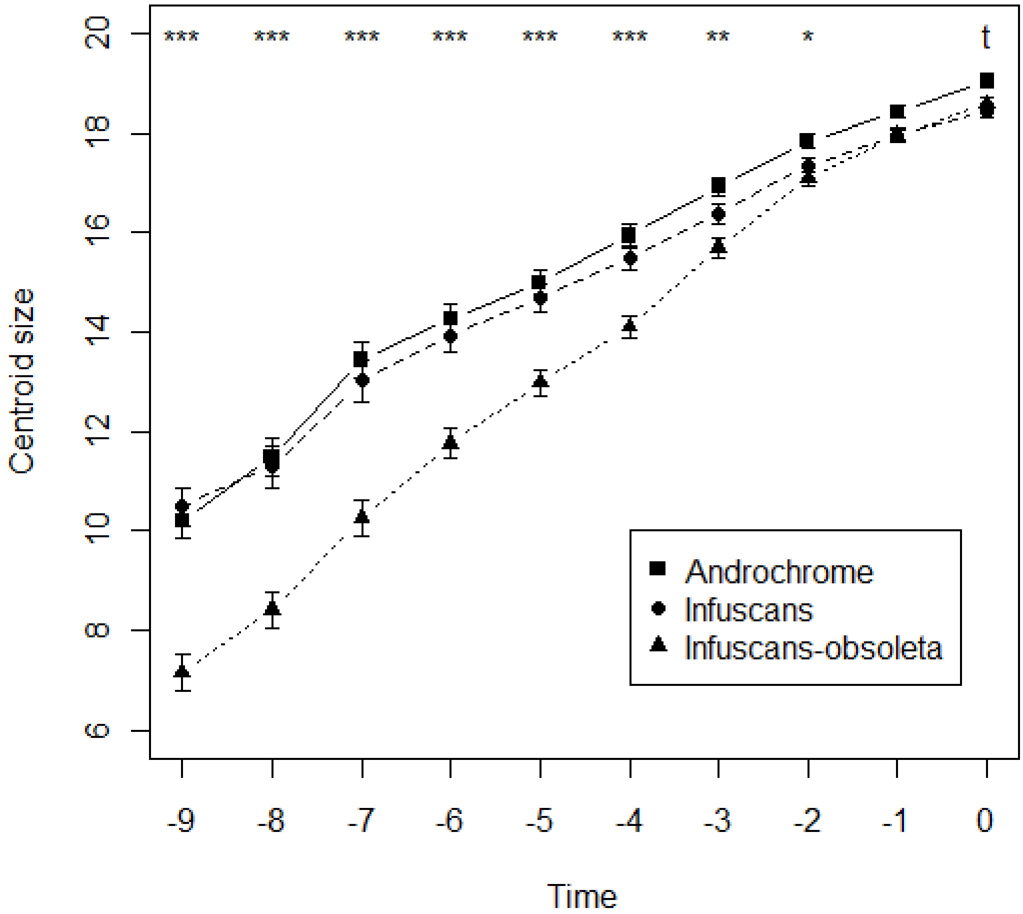
| | | | | | |
|---|---|---|---|---|---|
| I Cold | 29.03 | 22.88 | 2.09 | 0.75 | 18.80 |
| O Cold | 28.89 | 22.10 | 2.12 | 0.72 | 17.84 |
| A Warm | 29.65 | 23.39 | 2.22 | 0.79 | 16.63 |
| I Warm | **30.08** | **23.80** | **2.27** | 0.76 | **19.78** |
| O Warm | 28.94 | 23.44 | 2.21 | **0.81** | 18.98 |

Figure 1: Differences in size over development for (a) maternal morph, (b) sex, (c) emergence success, and (d) temperature. Centroid size was calculated from 11 landmarks (see Figure 2a) and time in the figure indicates two-week intervals relative to the date of emergence (or death, for individuals that died in the last instar). Offspring of infuscans-obsoleta females were initially smaller than offspring of the other morphs, but this difference decreased over ontogeny. Females were larger than males throughout ontogeny, but this difference was only significant in the final instars. Individuals that emerged successfully were significantly larger than individuals that did not emerge successfully in the last instar. Individuals that had experienced cold temperatures during part of development were smaller than those that had been in warm conditions, and although individuals in the cold treatment increased their rate of growth once they were returned to warm conditions they did not fully compensate for the difference in size. There were no significant interactions between main effects, so only main effects are shown. Bonferroni-corrected significance indicators: t = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Symbols show LS means and SEs.

Figure 2: Landmarks used in the analysis of larval shape (a) and differences in the last instar (b-e). (a) 11 landmarks were placed along the outline and midline of the larva. (b) Differences between the offspring of the three female morphs in the last instar. The grid shows how the mean infuscans or infuscans-obsoleta offspring shape must be deformed to produce the mean configuration found in androchrome offspring (note that both deformation grids are exaggerated by a factor of 5 for clarity). Offspring of androchrome females have significantly different morphology than offspring of infuscans or infuscans-obsoleta females, with smaller heads, larger eyes, and relatively longer abdomens (compare with (c)). (c) Differences between the sexes in the last instar. The grid shows how the female configuration must be deformed to produce a male configuration (exaggerated by a factor of 10 for clarity). Males

have larger eyes, a narrower thorax, and a longer, thinner abdomen than females. (d) Differences in the last instar between larvae that emerged successfully and those that did not. The grid shows deformation of the successful configuration to the unsuccessful configuration (exaggerated by a factor of 3 for clarity). Individuals that did not emerge successfully had a more juvenile configuration than those that did. (e) Differences in the last instar between individuals that experienced warm conditions or cold conditions during development. The grid shows deformation of the warm configuration to the cold configuration (exaggerated by a factor of 5 for clarity). Individuals that experienced cold conditions had a more juvenile configuration than those that experience warm conditions throughout.

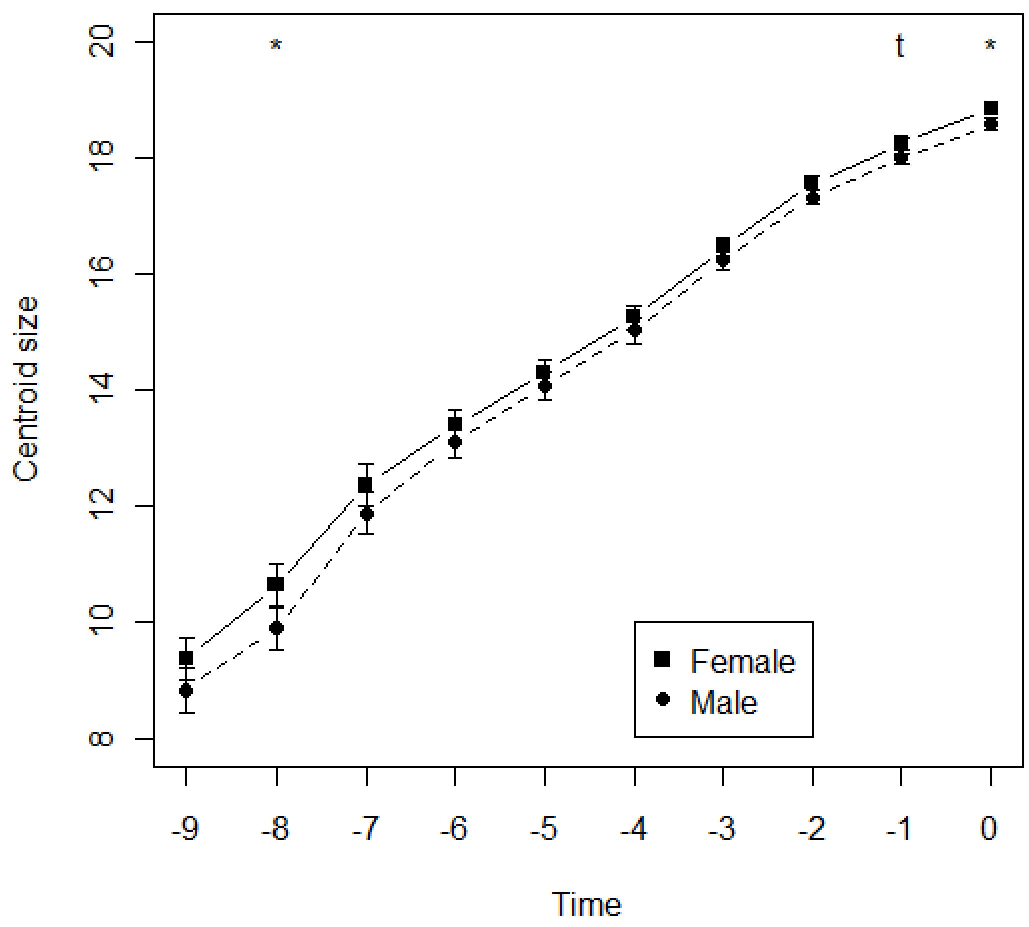
Figure 3: Effect of maternal morph on offspring sex ratio. Offspring of infuscans females had the lowest probability of successful emergence. Infuscans-obsoleta females produced the most female-biased broods.  There were no significant interactions between main effects, so only main effects are shown. Symbols show means and SEs.




812

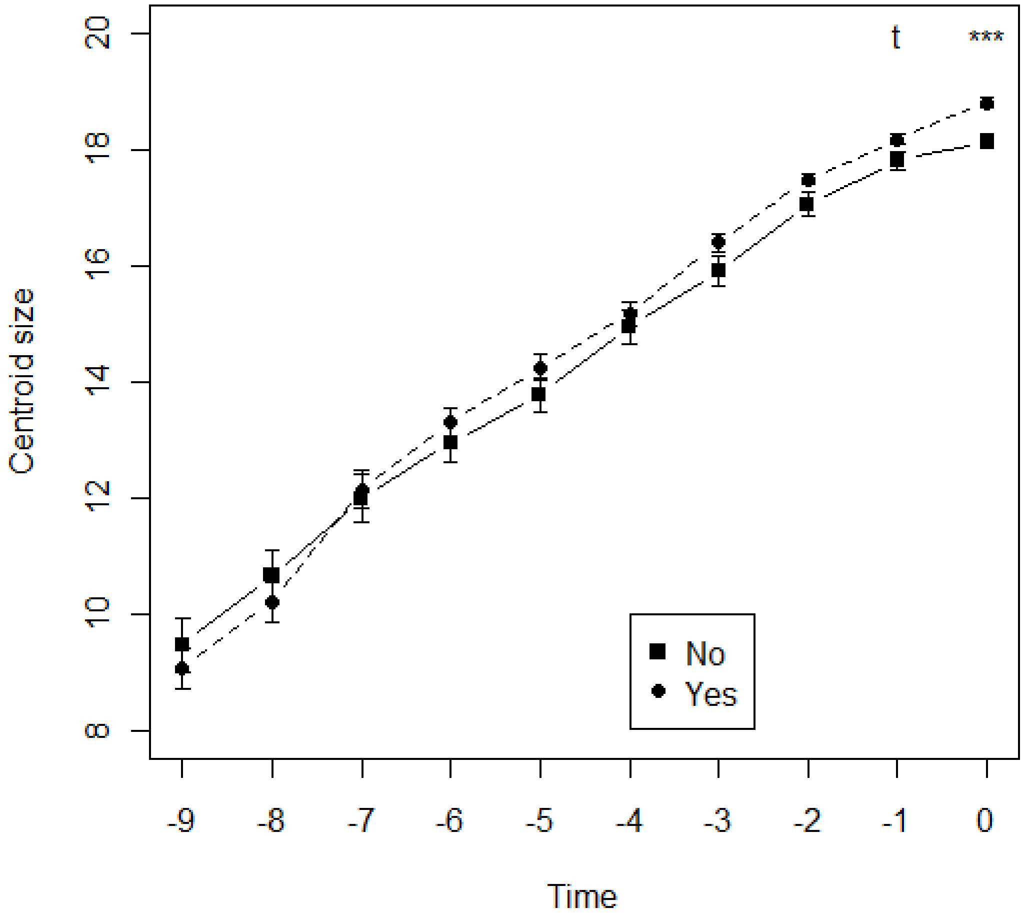
813 Figure 1A

814



Centroid size
Time
Female
Male
*
t
*
20
18
16
14
12
10
8
-9
-8
-7
-6
-5
-4
-3
-2
-1
0


Figure 1B




819 Figure 1C

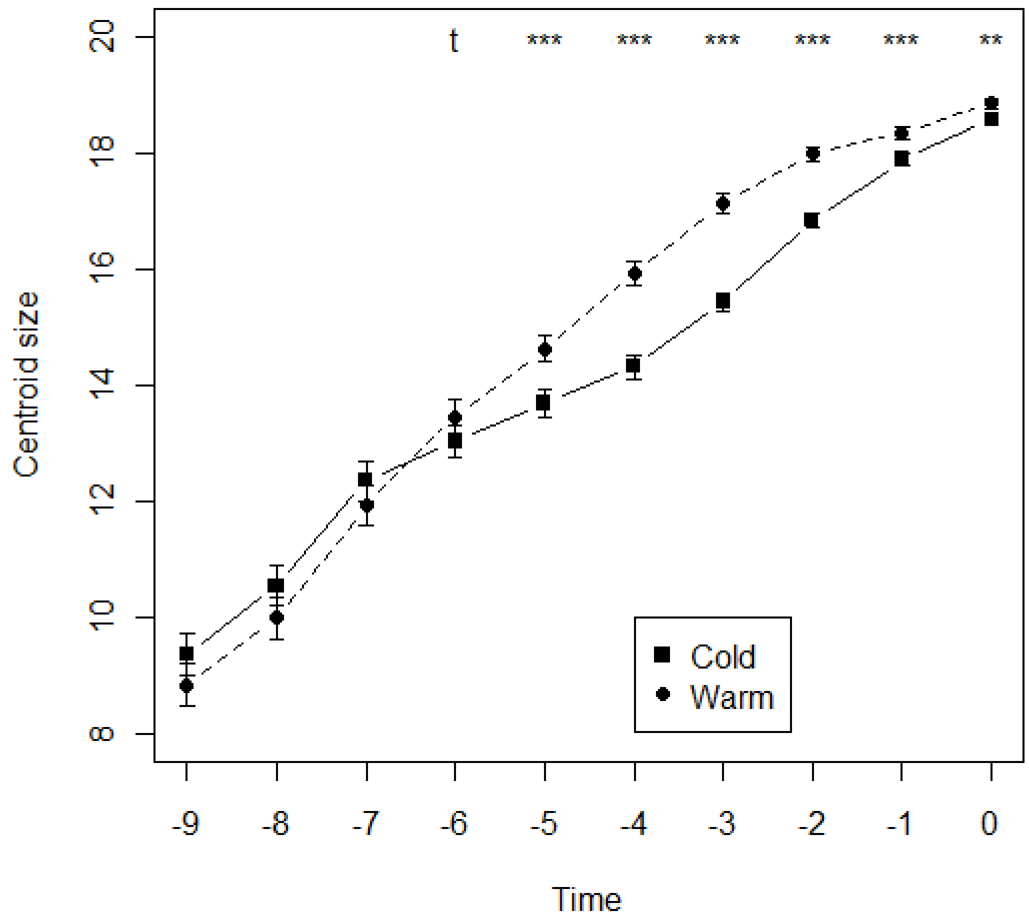



Figure 1D

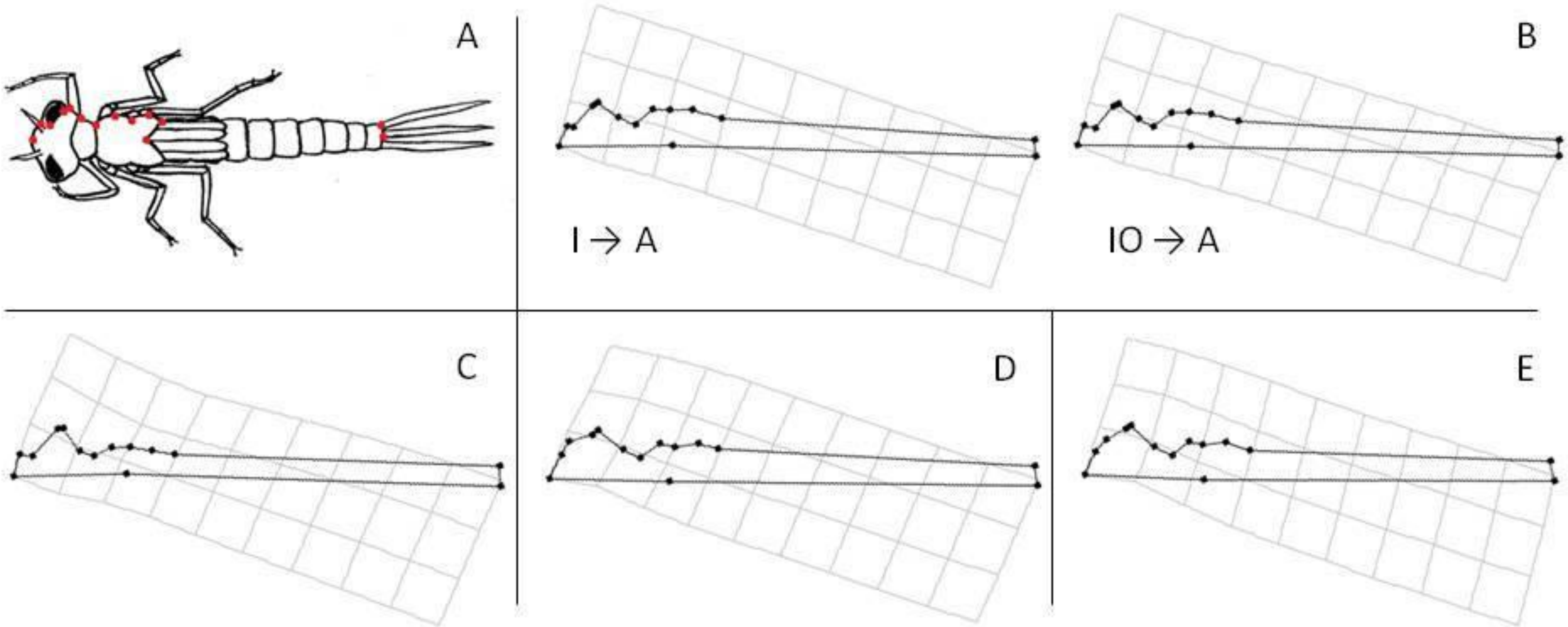



Figure 2

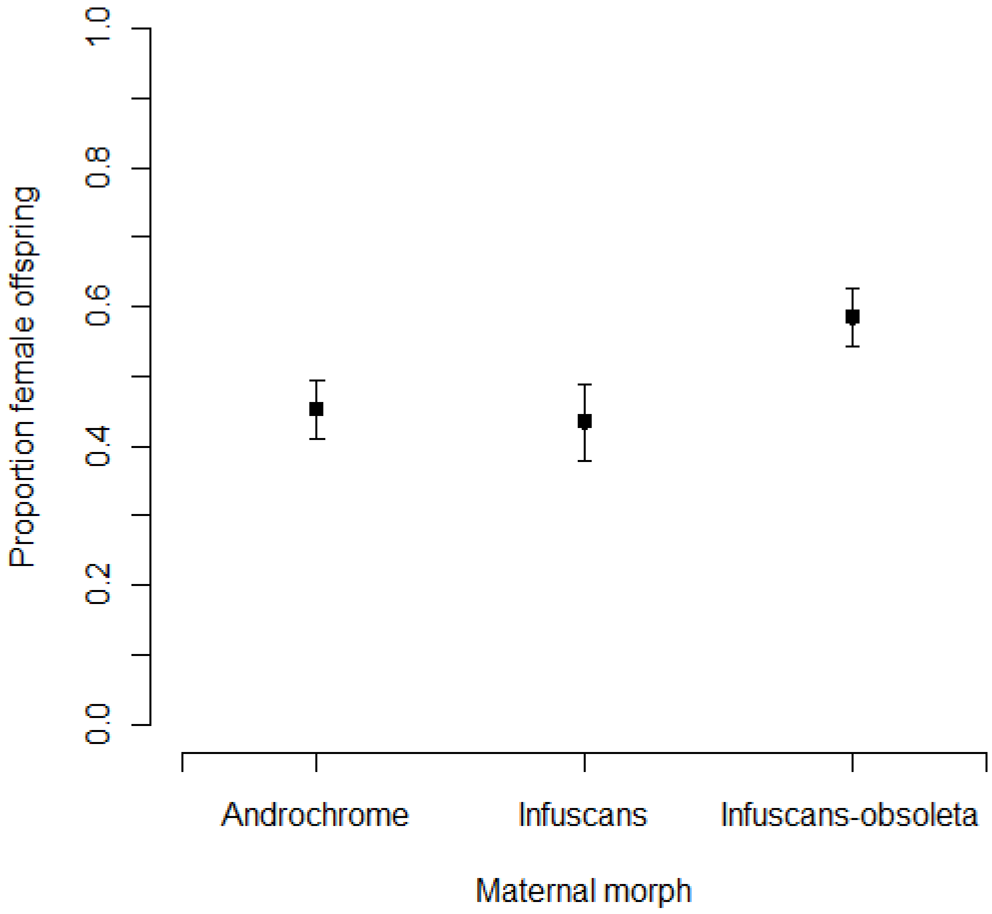

Proportion female offspring
0.0
0.2
0.4
0.6
0.8
1.0
Androchrome
Infuscans
Infuscans-obsoleta
Maternal morph

Figure 3

# Morph-specific and sex-specific temperature effects on morphology in the colour polymorphic damselfly *Ischnura elegans*: Supplementary information

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**Statistical models**

Note that only some combinations of factors are possible. For instance, own morph could only be determined for females that emerged successfully, so own morph can never be included in the same model as sex or emergence success. Similarly, each family can by definition only have one value of maternal morph, so family was nested within maternal morph for models including maternal morph.

Explanation of short forms: Fam = family, MM = maternal morph, OM = own morph in female offspring, Sex = sex of offspring, Temp = temperature, Em = emergence success in the last instar.

*Models used in the analysis of larval size over time:*

Results from different models were qualitatively similar for the same factor, so the results in the main text are from the models marked with *. For these models family was treated as a fixed effect.

Size = Fam(MM) + MM + OM + MM*OM + MM*Time + OM*Time + MM*OM*Time

*Size = Fam(MM) + MM + Sex + MM*Sex + MM*Time + Sex*Time + MM*Sex*Time

Size = Fam(MM) + MM + Em + MM*Em + MM*Time + Em*Time + MM*Em*Time

Size = Fam(MM) + MM + Temp + MM*Temp + MM*Time + Temp*Time + MM*Temp*Time

Size = Fam + OM + Temp + OM*Temp + OM*Time + Temp*Time + OM*Temp*Time

Size = Fam + Sex + Em + Sex*Em + Sex*Time + Em*Time + Sex*Em*Time

Size = Fam + Sex + Temp + Sex*Temp + Sex*Time + Temp*Time + Sex*Temp*Time

*Size = Fam + Em + Temp + Em*Temp + Em*Time + Temp*Time + Em*Temp*Time

*Models used in the analysis of differences in the allometric relationship between size and shape:*

For these models family was treated as a fixed effect. Differences in the shape matrix were tested using the “identity” funtion.

Shape = ID(Family) + Family(MM) + MM + Size + MM*Size

Shape = ID(Family) + Family + OM + Size + OM*Size

Shape = ID(Family) + Family + Sex + Size + Sex*Size

Shape = ID(Family) + Family + Em + Size + Em*Size

Shape = ID(Family) + Family + Temp + Size + Temp*Size

*Models used in the analysis of size and shape in the last instar:*

Size = Fam(MM) + MM + OM + Temp + MM*OM + MM*Temp + OM*Temp

Size = Fam(MM) + MM + Sex + Em + Temp + MM*Sex + MM*Em + MM*Temp + Sex*Em + Sex*Temp + Em*Temp

Models of the same form as these were also used to test shape differences in the last instar, with the shape matrix as the dependent variable and using the "identity" funtion. For the size models family was treated as a random effect, while for the shape models it was treated as a fixed effect.

*Models used in the analysis of development time, and size and shape in the adult stage:*

Development time = Fam(MM) + MM + OM + Temp + MM*OM + MM*Temp + OM*Temp

Development time = Fam(MM) + MM + Sex + Temp + MM*Sex + MM*Temp + Sex*Temp

For these models family was treated as a random effect. Multivariate models of the same form as these were also used to test differences in shape and size in the adult stage (with the five morphological traits as the dependent variables). For adult size differences the "sum" function was used, while for shape differences the "identity" function was used. For the multivariate models family was treated as a fixed effect.

*Generalized linear models:*

For the analysis of probability of successful emergence I used the following model with family as a fixed effect:

Prob(Em) = Fam(MM) + MM + Sex + Temp + MM*Sex + MM*Temp + Sex*Temp

For offspring sex ratio I used the following model with family as a fixed effect:

SR = Fam(MM) + MM + Temp + MM*Temp

## Tables of statistical results

*Larval size over time: MM*Sex model*

| Effect | Test statistic | | Num DF | Den DF | *P*-value |
|---|---|---|---|---|---|
| | ***F*-value** | **Wilks' *λ*** | | | |
| **Family(MM)** | 2.8045 | | 28 | 108 | **<0.0001** |
| **Maternal Morph** | 25.661 | | 2 | 108 | **<0.0001** |
| **Sex** | 4.5356 | | 1 | 108 | **0.0355** |
| **Time** | 391.67 | | 9 | 100 | **<0.0001** |
| **MM*Sex** | 0.9413 | | 2 | 108 | 0.3933 |
| **Family(MM)*Time** | | 0.0392 | 252 | 880.36 | **<0.0001** |
| **Sex*Time** | 0.0746 | | 9 | 100 | 0.5915 |
| **MM*Time** | | 0.4038 | 18 | 200 | **<0.0001** |
| **MM*Sex*Time** | | 0.8174 | 18 | 200 | 0.2814 |

*Larval size over time: Em*Temp model*

| Effect | Test statistic | | Num DF | Den DF | *P*-value |
|---|---|---|---|---|---|
| | ***F*-value** | **Wilks' *λ*** | | | |
| **Family** | 4.6042 | | 30 | 108 | **<0.0001** |
| **Emergence** | 2.8302 | | 1 | 108 | 0.0954 |
| **Temperature** | 6.4528 | | 1 | 108 | **0.0125** |
| **Time** | 228.68 | | 9 | 100 | **<0.0001** |
| **Em*Temp** | 0.7069 | | 1 | 108 | 0.4023 |
| **Family*Time** | | 0.0195 | 270 | 885.79 | **<0.0001** |
| **Em*Time** | 3.9576 | | 9 | 100 | **0.0002** |
| **Temp*Time** | 7.9569 | | 9 | 100 | **<0.0001** |
| **Em*Temp*Time** | 0.8961 | | 9 | 100 | 0.5318 |

*Larval size in the last instar:*

| Effect | Test statistic | | Num DF | Den DF | *P*-value |
|---|---|---|---|---|---|
| | ***F*-value** | **% Variance** | | | |
| **Family(MM)** | | 17.368 | -- | -- | -- |
| **Maternal Morph** | 4.0367 | | 2 | 54.42 | **0.0232** |
| **Sex** | 1.9220 | | 1 | 329.6 | 0.1666 |
| **Emergence** | 22.147 | | 1 | 323.9 | **<0.0001** |
| **Temperature** | 3.6526 | | 1 | 322.4 | 0.0569 |
| **MM*Sex** | 0.3850 | | 2 | 328 | 0.6808 |
| **MM*Em** | 0.8456 | | 2 | 324.1 | 0.4303 |
| **MM*Temp** | 0.5376 | | 2 | 314.1 | 0.5847 |
| **Sex*Em** | 1.6334 | | 1 | 330.8 | 0.2021 |
| **Sex*Temp** | 4.8788 | | 1 | 322.6 | **0.0279** |
| **Em*Temp** | 0.7579 | | 1 | 326.7 | 0.3846 |

*Larval shape in the last instar:*

| **Effect** | **Test statistic** | | **Num DF** | **Den DF** | ***P*-value** |
|---|---|---|---|---|---|
| | ***F*-value** | **Wilks' λ** | | | |
| **Family(MM)** | | 0.0594 | 696 | 5450 | **<0.0001** |
| **Maternal Morph** | | 0.7986 | 48 | 576 | **0.0342** |
| **Sex** | 2.0880 | | 24 | 288 | **0.0026** |
| **Emergence** | 2.0518 | | 24 | 288 | **0.0032** |
| **Temperature** | 2.0149 | | 24 | 288 | **0.0040** |
| **MM*Sex** | | 0.8864 | 48 | 576 | 0.8966 |
| **MM*Em** | | 0.8664 | 48 | 576 | 0.6804 |
| **MM*Temp** | | 0.8470 | 48 | 576 | 0.4057 |
| **Sex*Em** | 0.8505 | | 24 | 288 | 0.6700 |
| **Sex*Temp** | 1.2075 | | 24 | 288 | 0.2335 |
| **Em*Temp** | 0.5103 | | 24 | 288 | 0.9744 |

*Larval development time:*

| **Effect** | **Test statistic** | | **Num DF** | **Den DF** | ***P*-value** |
|---|---|---|---|---|---|
| | ***F*-value** | **% Variance** | | | |
| **Family(MM)** | | 9.621 | -- | -- | -- |
| **Maternal Morph** | 20.833 | | 2 | 31.203 | **<0.0001** |
| **Sex** | 12.603 | | 1 | 293.03 | **0.0004** |
| **Temperature** | 42.932 | | 1 | 280.15 | **<0.0001** |
| **MM*Sex** | 1.9817 | | 2 | 292.23 | 0.1397 |
| **MM*Temp** | 0.0888 | | 2 | 280.11 | 0.9150 |
| **Sex*Temp** | 0.3688 | | 1 | 288.32 | 0.5441 |

*Adult size:*

| **Effect** | **Test statistic** | **Num DF** | **Den DF** | ***P*-value** |
|---|---|---|---|---|
| | ***F*-value** | | | |
| **Family(MM)** | 1.2909 | 28 | 227 | 0.1580 |
| **Maternal Morph** | 1.5151 | 2 | 227 | 0.2220 |
| **Sex** | 10.701 | 1 | 227 | **0.0012** |
| **Temperature** | 0.2260 | 1 | 227 | 0.6350 |
| **MM*Sex** | 1.4208 | 2 | 227 | 0.2437 |
| **MM*Temp** | 2.2232 | 2 | 227 | 0.1106 |
| **Sex*Temp** | 1.3356 | 1 | 227 | 0.2490 |

*Adult shape:*

| **Effect** | **Test statistic** | | **Num DF** | **Den DF** | ***P*-value** |
|---|---|---|---|---|---|
| | ***F*-value** | **Wilks' λ** | | | |
| **Family(MM)** | | 0.4869 | 140 | 1106.0 | **0.0381** |
| **Maternal Morph** | | 0.9482 | 10 | 446 | 0.2868 |
| **Sex** | 36.870 | | 5 | 223 | **<0.0001** |
| **Temperature** | 6.5089 | | 5 | 223 | **<0.0001** |
| **MM*Sex** | | 0.9525 | 10 | 446 | 0.3606 |
| **MM*Temp** | | 0.8957 | 10 | 446 | **0.0058** |
| **Sex*Temp** | 0.3818 | | 5 | 223 | 0.8610 |

*Adult shape, own morph effect included:*

| **Effect** | **Test statistic** | | **Num DF** | **Den DF** | ***P*-value** |
|---|---|---|---|---|---|
| | ***F*-value** | **Wilks' λ** | | | |
| **Family(MM)** | | 0.1608 | 130 | 300.60 | 0.3926 |
| **Maternal Morph** | 0.1768 | | 5 | 60 | 0.9703 |
| **Own Morph** | 0.4130 | | 5 | 60 | 0.8379 |
| **Temperature** | 2.6633 | | 5 | 60 | **0.0307** |
| **MM*OM** | | 0.8752 | 15 | 166.03 | 0.9100 |
| **MM*Temp** | | 0.7997 | 10 | 120 | 0.1800 |
| **OM*Temp** | | 0.7393 | 10 | 120 | **0.0441** |

*Offspring sex ratio:*

| **Effect** | **Test statistic** | **DF** | ***P*-value** |
|---|---|---|---|
| | **$\chi^2$-value** | | |
| **Family(MM)** | 32.729 | 29 | 0.2888 |
| **Maternal Morph** | 8.5048 | 2 | **0.0142** |
| **Temperature** | 0.3494 | 1 | 0.5544 |
| **MM*Temp** | 1.3811 | 2 | 0.5013 |

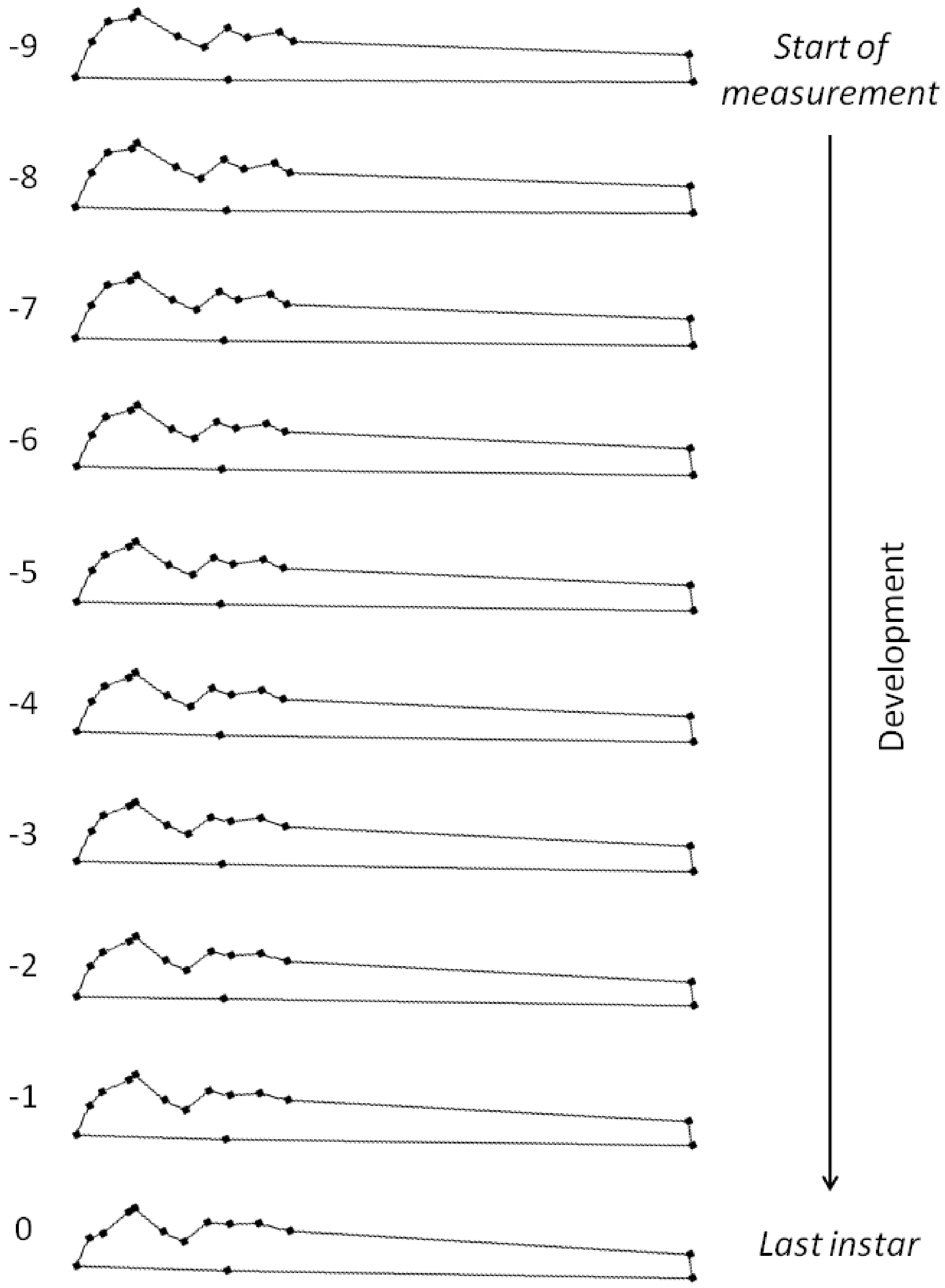



Figure S1a: Larval shape change over time. Individuals start out with relatively large heads and small abdomens, and over the course of development relative head size decreases, while relative eye size and relative abdomen length increase.

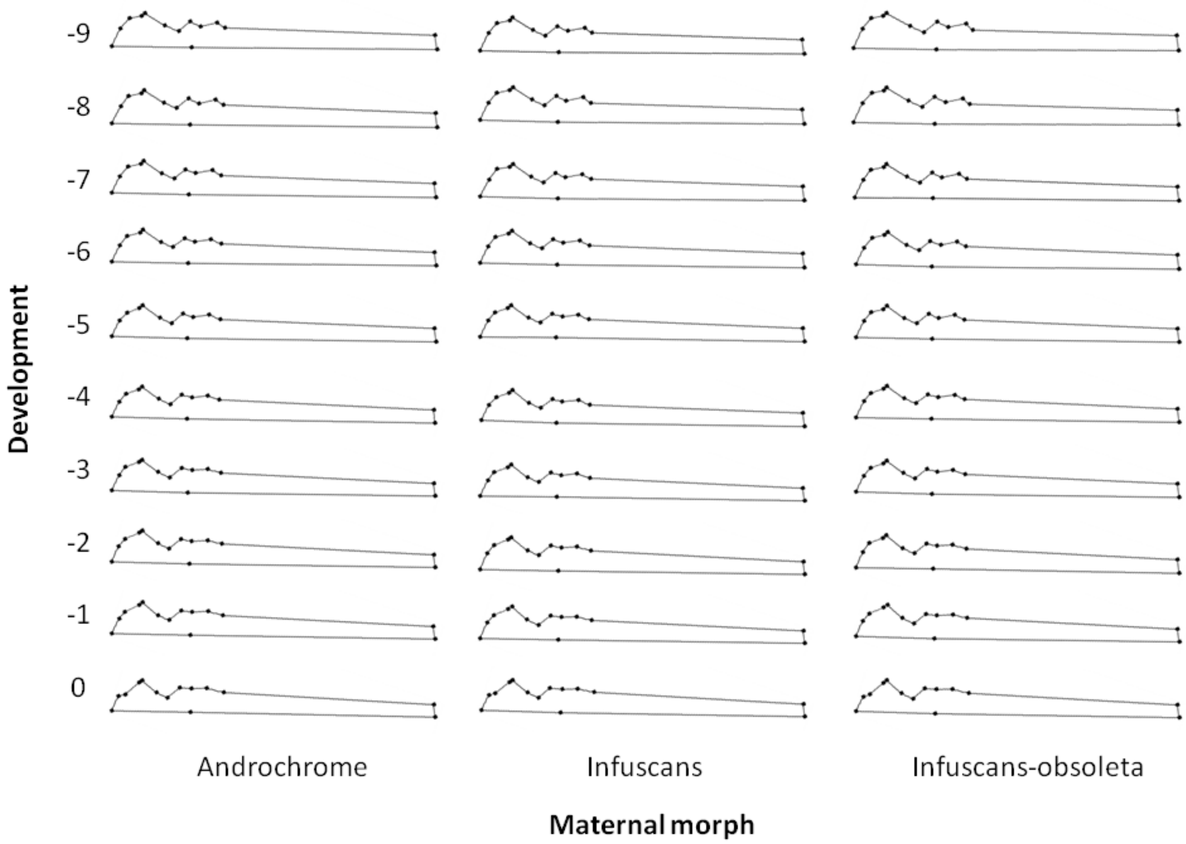
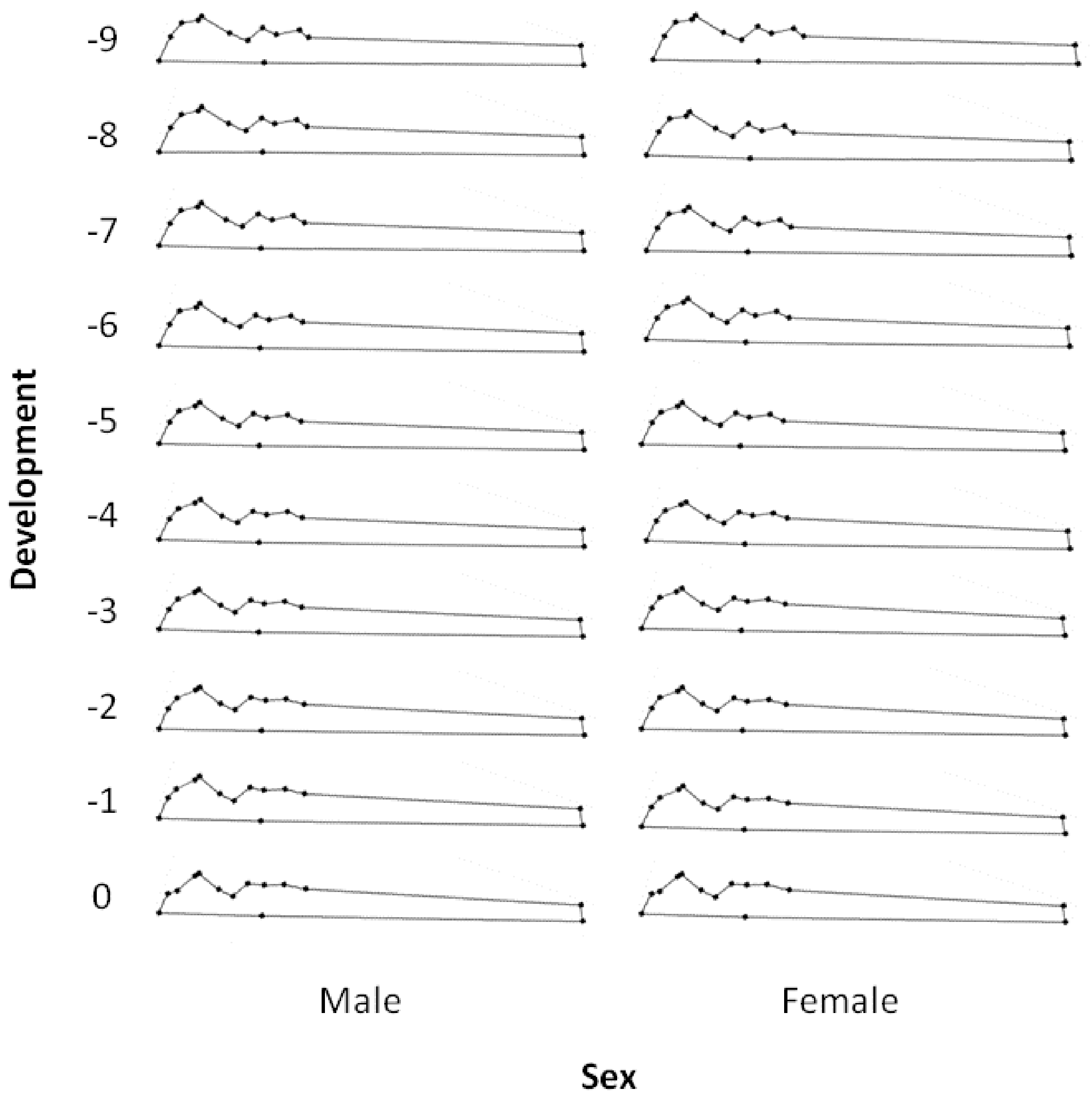


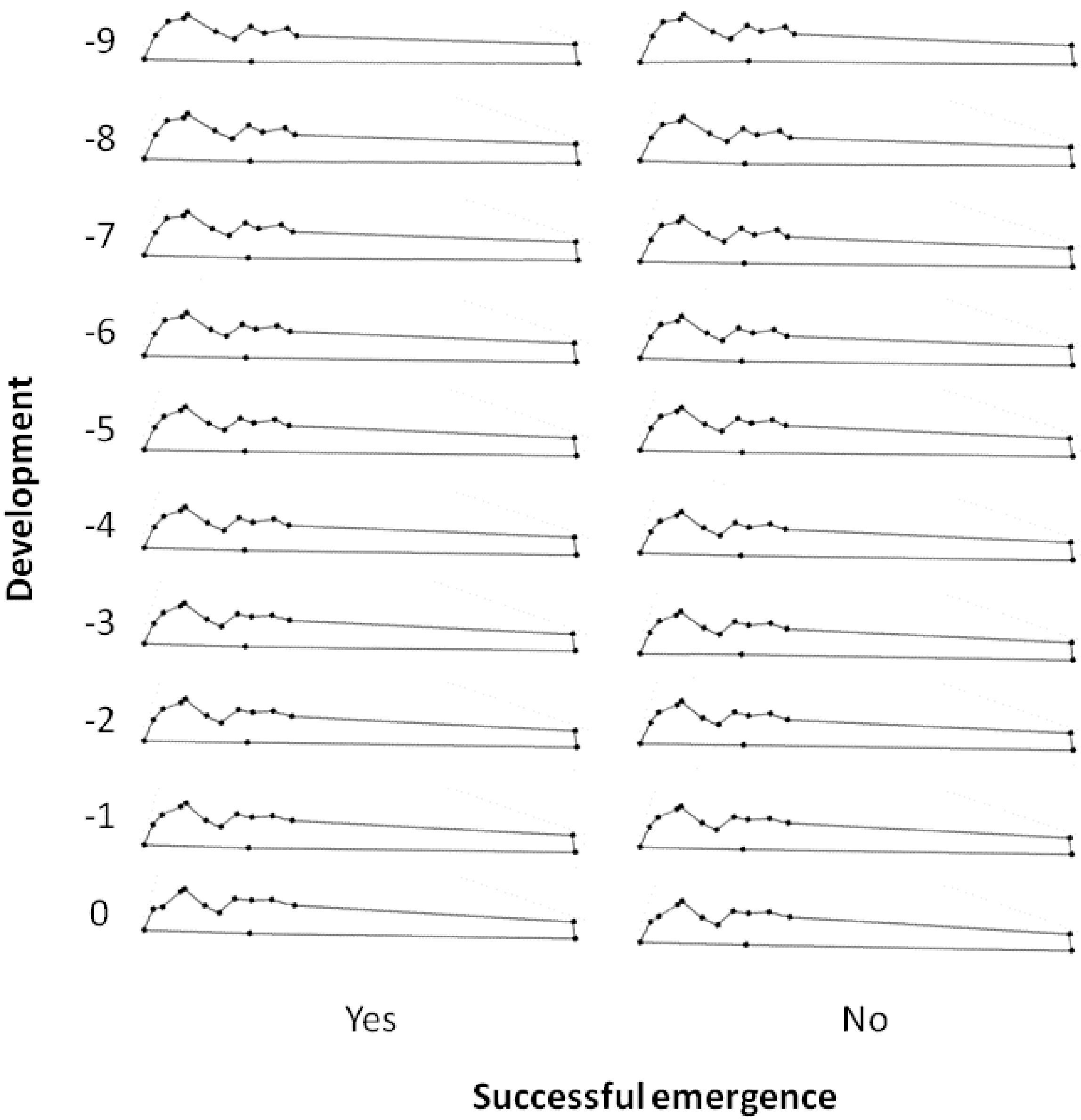

Figure S1b: Differences in the pattern of ontogenetic allometry in relation to maternal morph.




935

936 Figure S1c: Sex differences in the pattern of ontogenetic allometry.

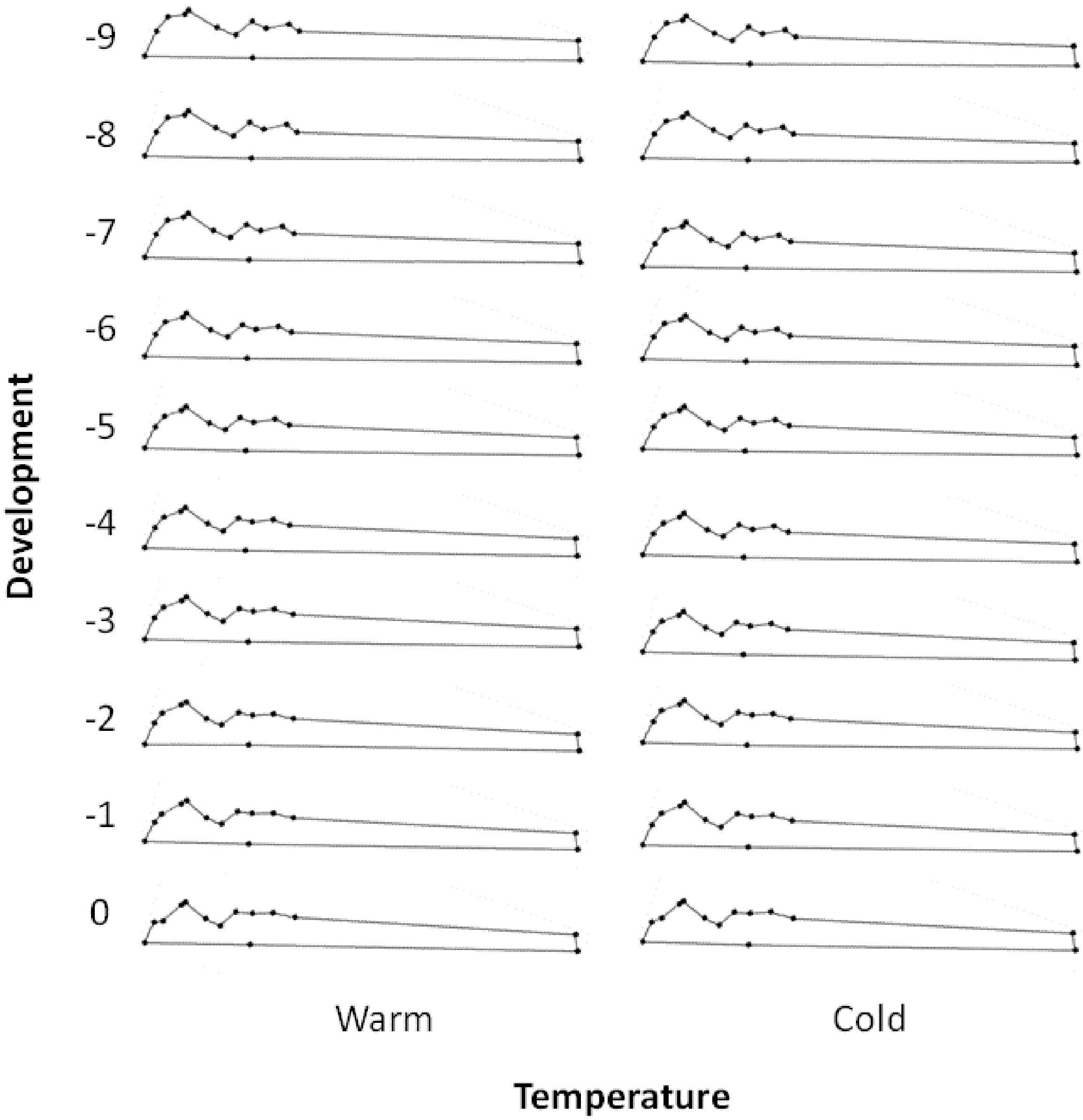
937




938

939 Figure S1d: Differences in the pattern of ontogenetic allometry between individuals that
940 emerged successfully, and those that died in the last instar.

941




942

943 Figure S1e: Differences in the pattern of ontogenetic allometry according to the temperature
944 experienced during development.

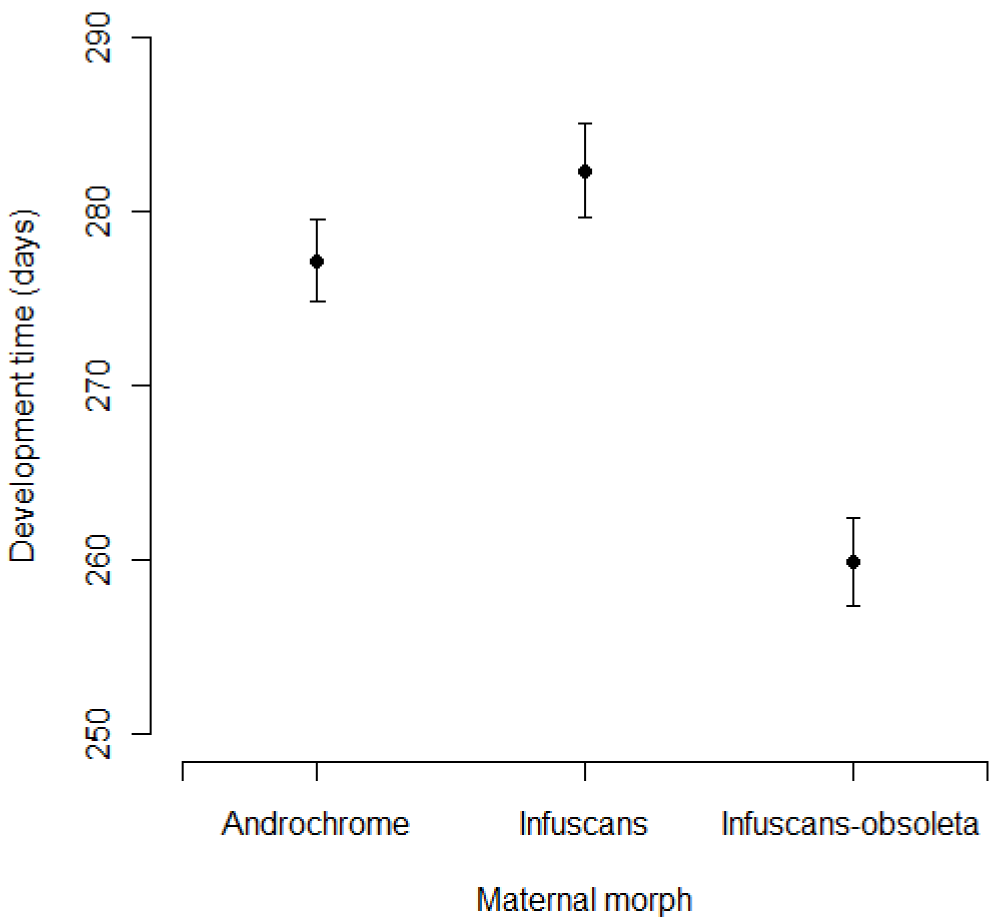



Figure S2a: Differences in development time in relation to maternal morph. Offspring of infuscans-obsoleta females had significantly shorter development time than offspring of the other morphs. Symbols show LS means and SEs.

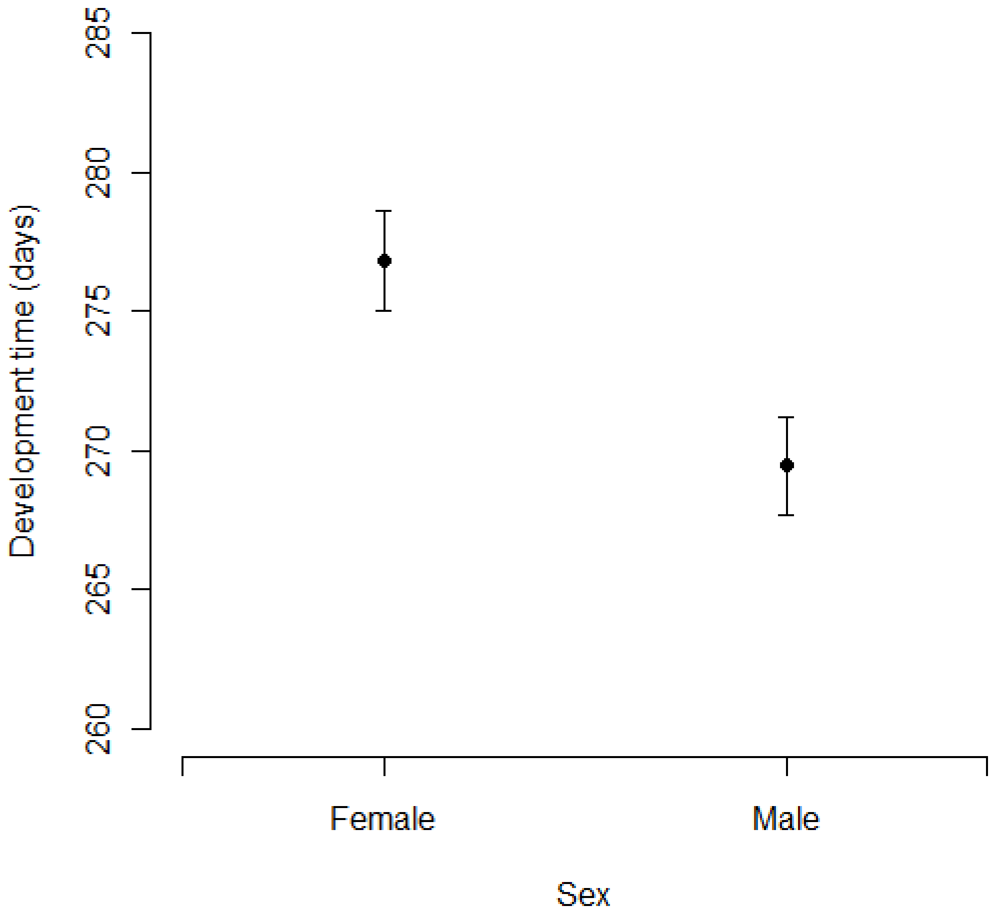



Figure S2b: Differences in development time in relation to sex. Males have significantly shorter development time than females. Symbols show LS means and SEs.

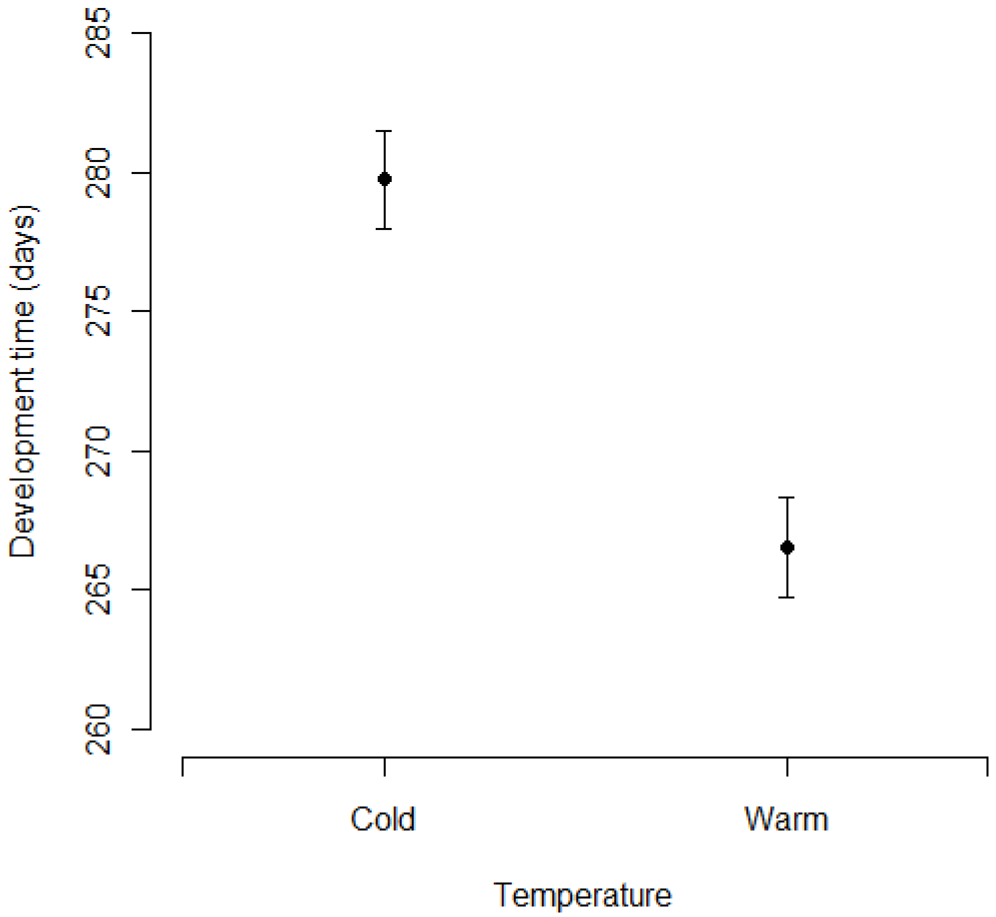



Figure S2c: Differences in development time in relation to temperature. Individuals that experienced warm conditions throughout development had shorter development time than individuals that experienced cold conditions. Symbols show LS means and SEs.