

*Adult size in insects: developmental responses to
temperature of the german cockroach Blatella germanica
and the large milkweed bug Oncopeltus fasciatus*

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1. ABSTRACT

The temperature-size rule, apparent in the majority of ectotherms, states that individuals respond to cooler temperatures with larger size at maturity, but slower development. This contradicts major life history models, which predict a smaller body size in conditions that retard growth. A number of hypotheses propose that this thermal plasticity of body size may be mere consequence of physiological constraints at the cellular level. In the present study, two unrelated insect species were examined for their response to different rearing temperatures. The effect of temperature was studied on the organismal (body size) and cellular (cell size and number) level, as well as on developmental time and mortality. In the german cockroach, *Blatella germanica*, the temperature-size rule holds for organ and cell size but not for cell number. Thus, body size increase was entirely accounted for by increase in cell size. The decrease in cell number was explained by compensation between cell number and cell size. As expected, developmental time was longest in individuals reared at cooler temperatures and mortality was highest in individuals reared in warmer temperatures. Where *B. germanica* holds to the rules, the large milk weed bug *Oncopeltus fasciatus* responds differently. The reaction norm of *O. fasciatus* for body size to growth temperature was non-linear: body size was largest at intermediate rearing temperature. This suggests that individuals were tested at the extremes of their viable thermal range which was further supported by higher mortality at very high and lower temperatures. Yet, when considering that cell size increases from low to intermediate temperatures and if the temperature-size rule holds for cell size, then *O. fasciatus* might be one of the exceptional species that exhibit the converse temperature-size rule. Sex differences are evident in both species. In *B. germanica*, temperature affects males more than females.

2. INTRODUCTION

2.1 Plasticity of body size and developmental time, an effect of temperature

An organism with a given genotype, producing several distinctly different alternative phenotypes in response to the ever-changing environment, is described as being phenotypically plastic. During development the reaction norm is the individual's response to the changing environment so that it emerges as an adult that seems to be adapted to its surroundings (Nijhout 1999). Many species of insects exhibit phenotypic plasticity. The western white butterfly (*Pontia occidentalis*), for example, develops a summer form when grown during the summer and a spring morph when grown in the spring. Seasonal polyphenism in this case is triggered by environmental temperature and photoperiod (Brakefield, 1996).

Many traits can be plastic. Body size variation in response to temperature in the wild is very common among animals. Bergmann (1847) noted that within the same species, individuals inhabiting higher latitudes are larger and develop slower than those from lower latitudes. So-called 'Bergmann size clines in natural environments' were originally observed in endotherms and later on they were also discovered in many ectotherms (Atkinson, 1994).

Size clines were shown by natural selection experiments to be at least partly due to genetic variation, indicating that populations from different geographic climates are differentially adapted to their local temperature. In many insects size cline variation in populations persists for a few generations when reared under standard conditions (*Drosophila melanogaster*, Partridge *et al*, 1994; *Stator limbatus* Stillwell & Fox, 2005).

An individual's reaction norm results from the direct effect of its environment. Organisms achieve a larger body size and grow slower when reared in lower temperatures in the laboratory (Atkinson, 1994). This is referred to as the temperature-size rule which describes reaction norms relating environmental temperature to body size (Angilletta & Dunham, 2003) and thus body size is regarded

as phenotypically plastic in response to temperature (thermal plasticity). Although there are exceptions, the cause of an increase in adult size by a reduction in environmental temperature is observed in over 80% of various studied ectothermic species (Atkinson, 1994). It has to be noted, however, that these studies included only species that were tested within their viable temperature range. When exposed to extremely high or very low temperatures, drosophilid species often exhibit decrease in body size, possibly due to heat and cold stress (Pétavy *et al*, 2001, David *et al*, 2006).

Curiously, developmental time, another plastic trait along with body size, increases with decreasing environmental temperature. According to the temperature-size rule organisms grow slower but larger at colder temperatures. This contradicts classic theories of life-history evolution that predict smaller sizes at maturity in environments that retard growth. Considering factors that affect growth (e.g. food availability) favouring environments normally result in faster growth to larger body size (Angilletta & Dunham, 2003). Several hypotheses have been proposed to explain the life history puzzle of the temperature reaction norm. Some describe a possible adaptive advantage of larger adult body size in colder environments and a trade-off in developmental time and others consider the effect of temperature on growth as a non-adaptive physiological consequence (reviewed in Atkinson, 1994, Angilletta *et al*, 2004).

2.2 Growth and size – basic mechanisms that control body size in insects

Adult body size is determined by the length of growth period and the amount of mass gained during this period (growth rate) (Dadivowitz *et al*, 2004). In insects, growth occurs during the larval or nymphal stages and involves moulting events. Stages between moults are called instars and every species has a genetically determined but environmental sensitive number of instars (Edgar, 2006). Once an instar emerges as an adult, its final size is determined, because adult insects do not grow. Therefore, the size an individual has at maturity is already determined in the final instar stage at the time of metamorphosis (Dadivowitz, 2005). Growth is exponential in insects and thus, most growth occurs during the last larval instar (D'Amico, 2001).

In most insects, an individual moults when a critical weight is achieved. In *Oncopeltus fasciatus* so-called stretch receptors sense the critical weight. Abdominal stretch reception induces prothoracicotropic hormone (PTTH) and ecdysteroid secretion, which then causes moulting (Nijhout, 2003). In *Manduca sexta* juvenile hormone (JH) levels drop when the critical weight is achieved. JH prevents PTTH and ecdysteroid release. When JH is fully cleared from the hemolymph, PTTH and ecdysteroid secretion becomes disinhibited. PTTH can only be released during a specific time window during the daily light-dark cycle (photoperiodic gate). This stimulates secretion of ecdysteroid and induces a behavioural response of the larvae: it stops feeding and starts to moult. The point when larva stops growing is when the moulting starts (reviewed by Davidowitz *et al*, 2005, Figure 1). The time interval between the critical weight and the secretion of the moulting hormone ecdysteroid is called the interval to cessation of growth (ICG). Critical weight, determined primarily by genotype, determines the onset of cessation of JH secretion. Critical weight together with the interval of cessation of growth, are the major timing events during the growth period (Figure 1, Davidowitz *et al*, 2005).

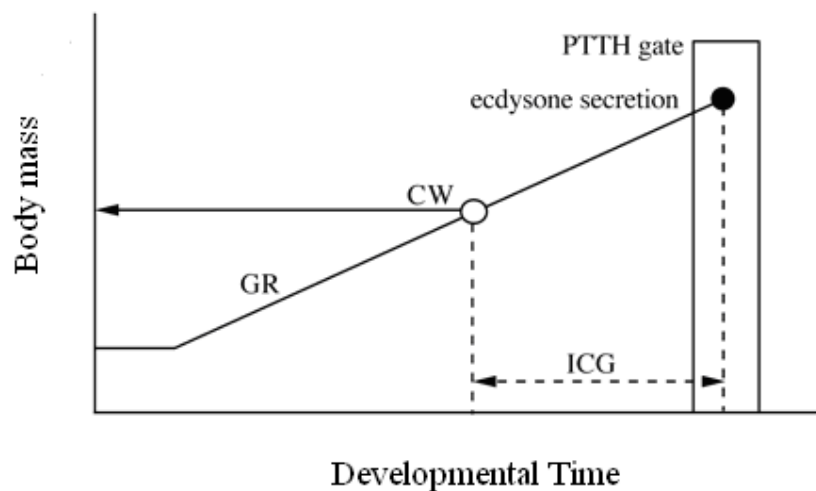


Figure 1: The physiological control of body size and developmental time in *M. sexta*. The vertical column represents the photoperiodic gate for PTTH release. GR, growth; CW critical weight; ICG, interval of cessation for growth (adapted from Davidowitz *et al.*, 2005).

It is widely distinguished that body or organ size is the result of size and number of component cells (Partridge *et al.* 1994; French 1998; Azevedo, 2002; Blanckenhorn, 2005). Growth is usually accompanied with an increase in cell number (Johnston & Gallant, 2002). Nevertheless, it can be uncoupled from cell division. It was shown that when cell division is blocked or inhibited, cells increase in size by cell growth. Equally, when cell division is increased, they divide at a smaller size (Johnston & Gallant, 2002). These observations suggest independent genetic variation for cell size and cell number. The environment can have similar effects. The effect of developmental temperature on size is often only mediated by change in cell size (Partridge *et al.* 1994), whilst the effect of crowding results in a change of both cell size and number (Robertson, 1959). Moreover, temperature effects can be more complicated in that strains that evolved at different temperatures exhibit body size variation mainly due to changes in cell number (James *et al.*, 1995; Zwaan *et al.*, 2000). Cell size and cell number might regulated by different mechanisms (Nijhout, 2003) or at least in part, jointly regulated because there is evidence that cell division compensates for cell size or vice versa (cellular compensation, McCabe *et al.*, 1997; Zwaan *et al.*, 2000).

A major genetic constituent of the control of growth rate is the insulin receptor signalling pathway. Its function is involved in the control of protein translation. Mutations in any components of the pathway cause reduction in cellular and organismal growth (Johnston & Gallant, 2002). Many genes are involved in the regulation of growth. The FOXO (forkhead box, sub-group "O") transcription factors are important targets of insulin signalling. In mammals, activation of FOXO factors result in cell death and DNA replication arrest. In addition, FOXO factors have been implicated in stress resistance and longevity (*reviewed by* Edgar, 2006).

2.3 A biophysical model to explain reaction norms

Among the explanations for the life-history puzzle it was suggested that general cellular mechanisms cause thermal plasticity in body size due to physiological constraints. Van der Have and de Jong (1996) proposed a biophysical model based on enzymatic activity connected to biological rates. They argue that growth rate is mainly affected by protein translation. Translation, itself largely dependent on

diffusion, is less affected by temperature than the rate of cell division, which is highly dependent on temperature. Diffusion is thus expected to be more rate-limiting for protein synthesis (cell growth) than for DNA replication (cell division). Accordingly, if the enzyme activation energy constant of the rate of cell division (differentiation, $\Delta H_{A,d}$) is higher than for growth ($\Delta H_{A,g}$), leading to a higher temperature threshold for differentiation, then cells will be smaller after dividing at high temperatures ($\Delta H_{A,d} - \Delta H_{A,g} > 0$; Figure. 2). Briefly, at high temperatures, cells divide faster but cellular growth remains constant and the emerging adult will be smaller due to decreased cell size. The mechanism explains why organisms at higher temperatures reach maturity more quickly, but grow less rapidly and result in smaller body size.

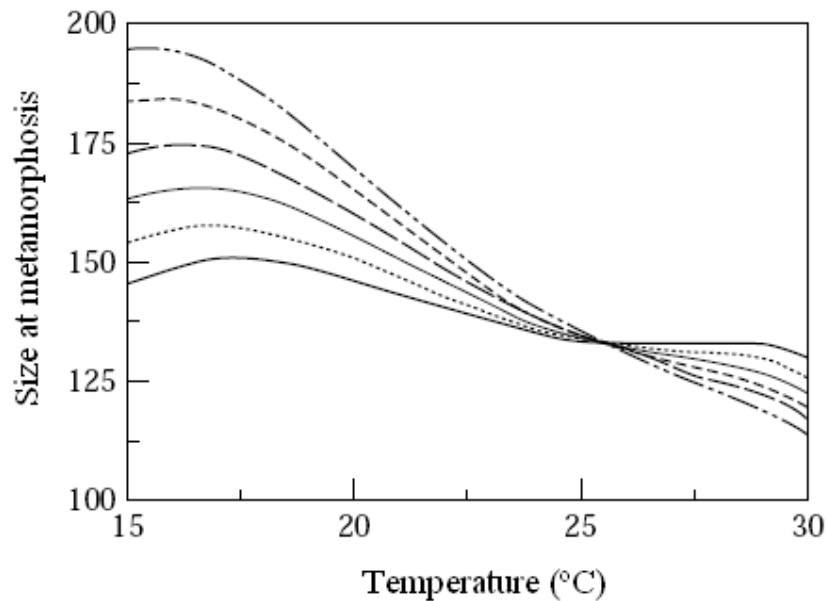


Figure 2: If the slope of adult size with temperature is negative ($\Delta H_{A,d} - \Delta H_{A,g} > 0$), the ectotherm should obey the temperature-size rule (adapted from van der Have and de Jong, 1996).

The validity of the biophysical model in natural conditions has been demonstrated on *D. melanogaster* (van der Have & de Jong, 1996). Furthermore, depending on the different temperature thresholds for growth and differentiation the model can also explain how species follow the converse temperature-size rule (Walters & Hassal, 2006).

2.4 Insect species investigated in this study

The german cockroach *Blatella germanica* (Dictyoptera: Blatellidae) is omnivorous and common throughout the world, often inhabiting human settlements. It is a multivoltine species producing more than two generations per year. Females produce egg capsules that protrude from the abdomen and usually contain 30 to 48 eggs. Egg capsules are carried until hatching, when they are deposited in sheltered places. Cockroaches exhibit hemimetabolous growth patterns and usually have six larval instars (Encyclopedia Encarta, 2006). There is a negative correlation of body size and overall developmental time to temperature (Gent, unpublished).

The large milkweed bug *Oncopeltus fasciatus* (Hemiptera: Heteroptera: Lygaeida) is originally found from southern Canada to Brazil. It usually lives on several species of its host plant the milkweed which is a member of the family Asclepiadaceae. The species is univoltine; population numbers rise during late summer, usually peak by mid-September and subsequently decrease. *Oncopeltus fasciatus* responds to low temperature by migrating to warmer areas (Dingle, 1980). Dingle (1974) found that late summer adults enter reproductive diapause, induced by photoperiod, enables them to migrate longer distances to warmer areas. When the temperature is raised from 23 to 27°C, only 10% of females were found to migrate (*reviewed by* Dingle, 1980). Mature females oviposit on their host plant with egg clutches of 30 or more. Hatching of all eggs occurs simultaneously. It was found that reproductive activity is earlier at higher temperature and longer photoperiods but death rate is increased (Dingle, 1968). This true bug exhibits hemimetabolous development through five instars to a winged adult. Nijhout (1979, 1981) demonstrated that moulting is caused when a critical size is reached in each instar. A critical amount of body mass results in an abdominal stretch which causes secretion of moulting hormones. Thus moulting is determined by the quality and quantity of food consumed by the milkweed bug (*reviewed by* Nijhout, 2003). In *O. fasciatus*, the length of various life stages and overall developmental time was observed to depend on temperature and food condition, where developmental time was defined as the time required completing growth until adult. Decreased temperature increased developmental time (minimum temperature tested: 24°C, Niswander, 1951). Temperature also has an effect on *Oncopeltus* grouping behaviour with an increase in lower temperatures (Sauer & Feir, 1979).

2.5 Aims of this study

The present study focused on the plasticity of body size and growth in response to developmental temperature rather than examining the adaptive theories of costs and benefits by studying genetically distinct strains showing different responses to temperature. Given that the temperature-size rule is evident in the majority of studied ectotherms this study investigated whether two unrelated species exhibit larger body size and slower developmental time at lower temperatures. It was intended to confirm the negative linear relationship between adult body size and temperature that has been observed previously for *B. germanica* (Gent, unpublished). The temperature-size rule for *O. fasciatus* is not established yet and therefore was investigated in the present study. Additionally, overall developmental time was investigated.

Altering developmental temperature affects cell size but not cell number as found in previous studies on *Drosophila* (Partridge *et al.*, 1994; French *et al.*, 1998; Azevedo *et al.*, 2002). In the present study, the third aim was to study the effect of temperature on cell size and cell number in the wing of *B. germanica* and *O. fasciatus*.

Individuals reared at high or extreme temperatures often result in a heat stress response (Pétavy *et al.*, 2001, David *et al.*, 2006). Therefore the effect of temperature on mortality of the two species was considered.

Preliminary visual observations suggest that females are larger than males. This was further investigated in the present study.

3. METHODS

3.1 Experimental Design

Parental animals were reared at constant temperature (27 °C) and light-dark cycle (12:12) conditions. F2 generation eggs of *B. germanica* and *O. fasciatus* laboratory-reared females were kept at 27 °C until hatching. To initiate growth conditions for all nymphs simultaneously, no food was provided until they were set up in containers. In *B. germanica* unavailable food prevents first instars from moulting (Kunkel, 1966). Containers were cylindrical (600ml) and consisted of water (cotton-stopped 20ml scintillation vials) and a small pot of food (ground up cat food for *B. germanica* and sunflower seeds for *O. fasciatus*). Containers were closed with a tightly fitting lid with a net covered breathing hole.

Experimental growth conditions and procedures were the same for both *B. germanica* and *O. fasciatus*. Thirty newly hatched nymphs were transferred into each replicate container. Nymphs were collected randomly and of mixed sex, since it is unfeasible to distinguish sex at that stage. Fifteen replicates were set up in constant darkness for each rearing temperature condition (24°, 27° and 33°C). *Oncopeltus fasciatus* instars were handled using a paint brush. *Blatella germanica* nymphs are fast-moving and had to be anaesthetised with carbon dioxide beforehand. Food and water provided *ad libitum* and checked every two days for 30 minutes for each species and temperature condition. Containers were randomised to reduce effects of local temperature variations.

3.2 Data Collection

As soon as adults emerged they were collected daily, sexed and their developmental time was recorded. Individuals in larval instars were left in the container to complete their development. Collected adults were anaesthetised, frozen for about 20 minutes, pierced in the abdomen and preserved in 70% alcohol. *Blatella germanica* was sexed on the basis of their size dimorphism (Figure 3 B). Abdominal patterns in *O. fasciatus* fade in higher rearing temperatures (Feir, 1974), thus sex was distinguished by

genitalia in accordance with abdominal pattern at 24 °C rearing temperature (Figure 3 A).

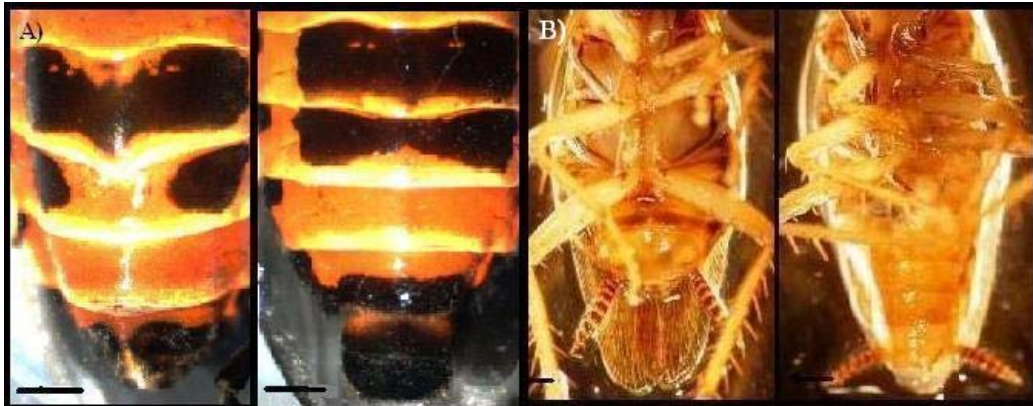


Figure 3: Difference between females (left) and males (right) under dissecting microscope magnification x5 in A) *O. fasciatus* and B) *B. germanica*

3.2.1 Measurements of body size

Body size for *B. germanica* and *O. fasciatus* was deduced from body part measurements of femur, head and wing. All body parts were cut and measured in 70% alcohol. Measurements were taken to the nearest 0.02mm with an eyepiece graticule under a dissecting microscope as shown in Figure 4. One millimetre corresponds to 1 graticule unit at x10 magnification (wing length), to 1.2 graticule units at x12 magnification (femur length) and to 5 graticule units at x50 magnification (head width or distance between eyes). All measurements were converted to millimetres.

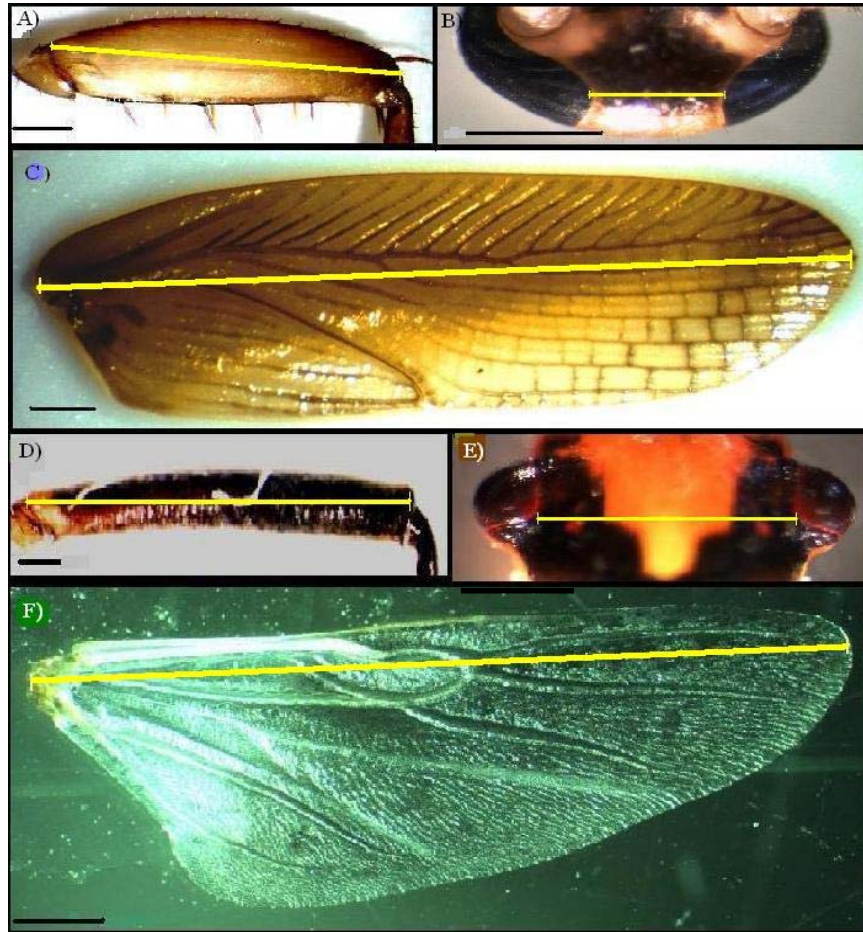


Figure 4: Yellow line represents measurements taken of given body parts of *B. germanica* A) third right femur, B) head width, C) right forewing; and *O. fasciatus* D) third right femur E) head width F) right hindwing.

3.2.2 Measurements of cell size and number

Once wings were measured, only those of 50 randomly sampled females were preserved in 70% alcohol for slide preparation to deduce cell size and number. Slide preparation and cell counts were adapted from James *et al* (1997). A standard section of the right wing was determined for each species (Figure 5 A, C), cut under ethanol and fixed in propan-2-ol before being mounted on a microscope slide in Aquamount. The slide was covered with a coverslip and left overnight to dry. Cuticular petrusions on the surface of the forewing in *B. germanica* and of the hindwing in *O. fasciatus* were similar to trichomes seen on the *D. melanogaster* wing (Figure 5 B, D). One trichome in *D. melanogaster* corresponds to one cell (Robertson, 1959). Therefore,

petrusions found on the wings in *B. germanica* and *O. fasciatus* were assumed to correspond to cells (Figure 5 B, D).

Prepared slides were placed under a compound microscope under appropriate magnification (x400 for *B. germanica* and x250 for *O. fasciatus*) and cells were individually marked on a piece of paper and counted on the dorsal side of the wing within a standard area (0.0225mm^2 in *B. germanica* and 0.0529mm^2 in *O. fasciatus*) using a *camera lucida* attached to the compound microscope. Cell size was deduced by dividing the standard area by the number of counted cells (*see James et al 1997*). An index of the total cell number of the wing was calculated by dividing the standard wing area by cell area ($[\text{wing length}]^2 / \text{cell area}$, as done on *D. suboscuro* by Calboli *et al*, 2003). The length of a wing was squared; it was thus assumed that wing shape remains the same during any change of size with temperature and according to Moed *et al*. (1997) wing shape differences in *Drosophila* are generally small. Furthermore, uniform cell density across the wing was assumed. However, variation between individuals should be concordant for different measurement regions on the wing (Zwaan *et al*, 2000, Partridge *et al*, 1994).

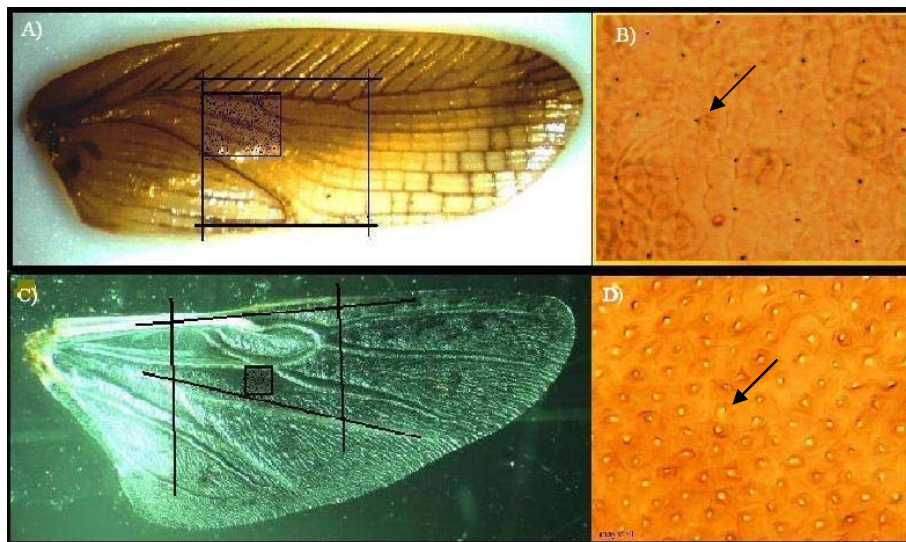


Figure 5: Right wings were cut as shown and cell density (one petrusion pointed out by black arrow) was obtained from the enclosed box within the cut area. A) Cut area shown of forewing and B) petrusions (arrow) seen on wing cuticle of *B. germanica* under x400 magnification. C) Cut area shown of hindwing and D) petrusions seen on wing cuticle of *O. fasciatus* under x250 magnification.

3.2.3 Developmental time and Mortality

In *B. germanica* egg-to-adult developmental time was noted as soon as individuals started emerging as adults. However, due to difficulties in catching adults individually, they were only collected when there were more than 50% adults in one container. Thus it was not possible to deduce exact developmental time for each adult and for data analysis an average of developmental time was taken for each temperature condition. *O. fasciatus* adults are slow-moving and thus developmental time for *O. fasciatus* was recorded for each collected adult.

Juvenile mortality for both species was inferred as the percentage of individuals that died before reaching adult age and was determined for each container.

3.3 Data Analysis

All data analysis was carried out with MINITAB package version 14 and Microsoft Excel. Data points that violated the assumptions of normality even when transformed were omitted in the analysis.

Data for body size was analysed using General Linear Models (GLM). In this study temperature was not treated as a covariate (as in many previous studies, e.g. Blanckenhorn & Llaurens, 2005; Azevedo *et al*, 2002; French *et al*, 1998), because its effect on body size could be masked by the GLM regression line which assumes linearity. It was decided that regression analysis was not accurate enough with data points of only three temperature conditions. Thus both temperature and sex were treated as fixed factors. Response variables included femur length, head width and wing length for body size analysis.

Analysis on cellular responses was similar with the exception of GLM regression for *B. germanica*, since the relationship between temperature and body size turned out to be linear. Regression analysis was adapted from Stevenson (1995) and Zwaan (2000) using linear regression on log-transformed wing size and wing length.

Developmental time was treated separately in the statistic analysis because data did not meet the assumptions of normality, even when transformed. The nonparametric version of the Student t-test (the Mann-Whitney test) was used to compare developmental times between two temperature treatments at a time. One-way ANOVA revealed levels of significance about the percentage of mortality per container between temperature conditions.

For all data significance was accepted at the 0.05 confidence level.

4. RESULTS

4.1 Effects of rearing temperature on body size and its association with sex

4.1.1 In *Blatella germanica*

Data of organ sizes of *B. germanica* met assumptions of normality. Basic statistics of femur length, head width and wing length presenting mean, standard error and standard deviation are summarised in Table A1 in *Appendix A*.

In order to observe an effect of sex and temperature on body size, the length of three different organs was measured. As expected, femur length, head width and wing length were correlated ($r_{\text{femur, head}} = 0.661$, $p < 0.001$; $r_{\text{femur, wing}} = 0.788$, $p < 0.001$; $r_{\text{head, wing}} = 0.841$, $p < 0.001$). Body size was significantly larger in females than in males across all rearing temperatures (Table 2, Figure 6 A). Magnitudes of effects of sex were stronger than those of temperature in all organs (Table 2). It can be concluded that *B. germanica* follows the temperature-size rule which implies that organisms mature at larger body size in colder environments (Angilletta *et al*, 2004).

Table 2: General linear models on femur length, head width and wing length including temperature and sex as fixed factors. Significant levels are shown in bold.

Source of Variation	df	F	p
Femur length			
Temperature	2	101.04	<0.001
Sex	1	258	<0.001
Sex x Temperature	2	6.71	0.001
Head width			
Temperature	2	41.14	<0.001
Sex	1	1693.7	<0.001
Sex x Temperature	2	4.71	0.01
Wing length			
Temperature	2	138.82	<0.001
Sex	1	1361.12	<0.001
Sex x Temperature	2	12.23	<0.001

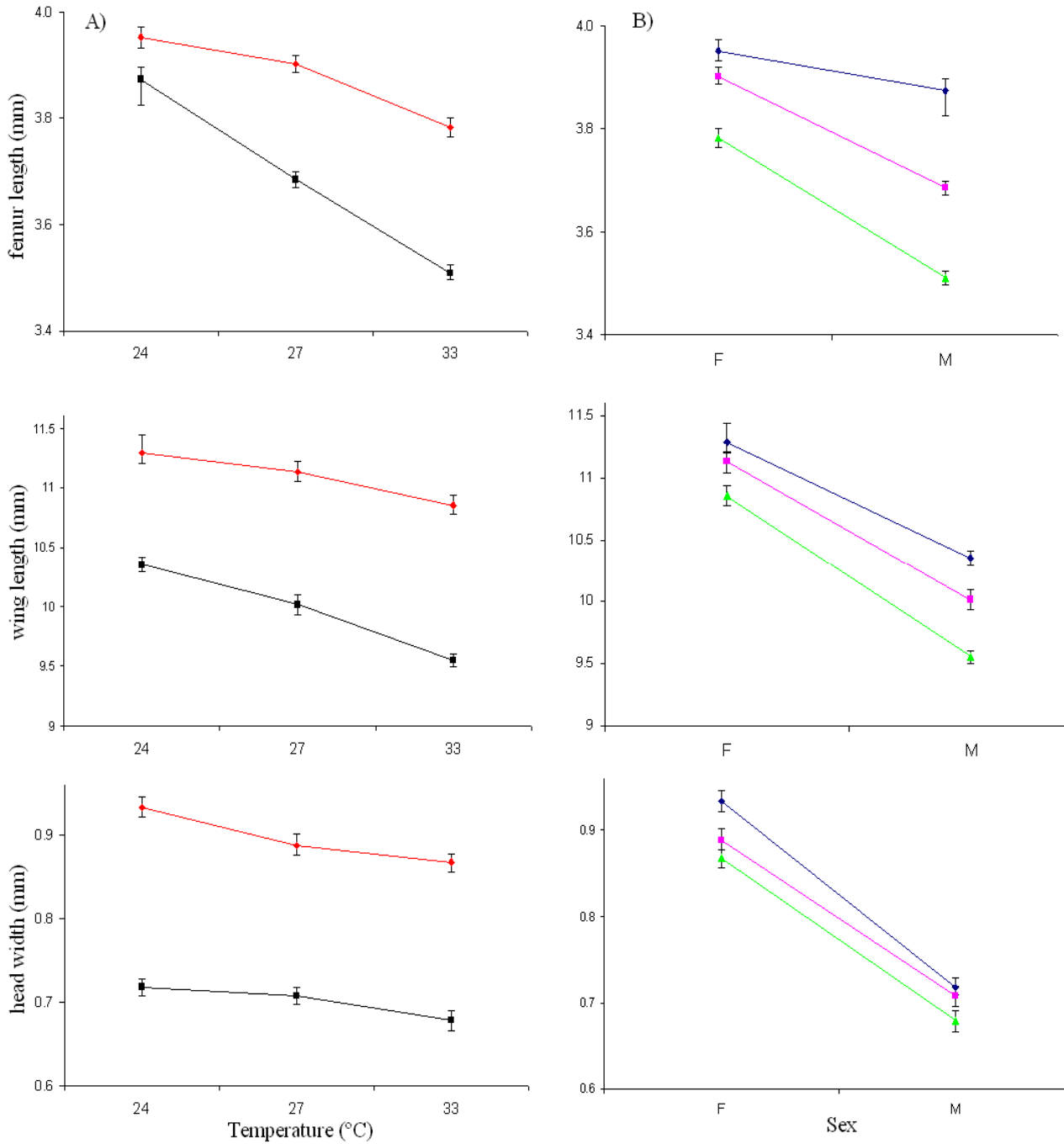


Figure 6: A) The effect of temperature (°C) on organ size (mm) (means \pm 95% confidence intervals) in *B. germanica*. Organs measured are femur length, hind wing length and head width. Females: diamonds and red lines; Males: squares and black lines. B) Temperature x sex interaction plots (means \pm 95 % confidence intervals) of femur length, hind wing length and head width. 24°C: diamonds and blue lines; 27°C: squares and pink lines; 33°C: circles and green lines.

There was a significant interaction between temperature and sex in all organs (Table 2, Figure 6 B). The interactions between sex and temperature are expected to be consistent across organ sizes, meaning that body size increases with only one sex. However, the effects of temperature on head width showed no clear pattern, because 95% confidence intervals (CI) overlap for two temperatures in each sex (Figure 6 B). Yet, a clearer interaction was observed for femur and wing length, whereby 95% CI are clearly distinct for each sex and temperature. In this case temperature had a greater effect on femur and wing size in males than in females (Figure 6 B).

4.1.2 In *Oncopeltus fasciatus*

Data points that violated the assumptions of normality were omitted in the analysis. An overview of the basic statistics on the data obtained from organ sizes of *O. fasciatus* is given in Table A2 in Appendix A.

Femur length, head width and hindwing length were correlated ($r_{\text{femur, head}} = 0.623$, $p < 0.001$; $r_{\text{femur, wing}} = 0.762$, $p < 0.001$; $r_{\text{head, wing}} = 0.750$, $p < 0.001$). All traits are larger in females than in males (Table 4, Figure 7 A). As in *B. germanica* effects of sex were stronger than effects of temperature in all organs (Table 4).

Table 4: General linear models on femur length, head width and wing length including temperature and sex as fixed factors. Significant levels are shown in bold.

Source of Variation	df	F	p
Femur length			
Temperature	2	95.83	<0.001
Sex	1	886.98	<0.001
Sex x Temperature	2	1.98	0.139
Head width			
Temperature	2	77.39	<0.001
Sex	1	1342.34	<0.001
Sex x Temperature	2	5.23	0.006
Wing length			
Temperature	2	208.16	<0.001
Sex	1	2073.48	<0.001
Sex x Temperature	2	4.33	0.013

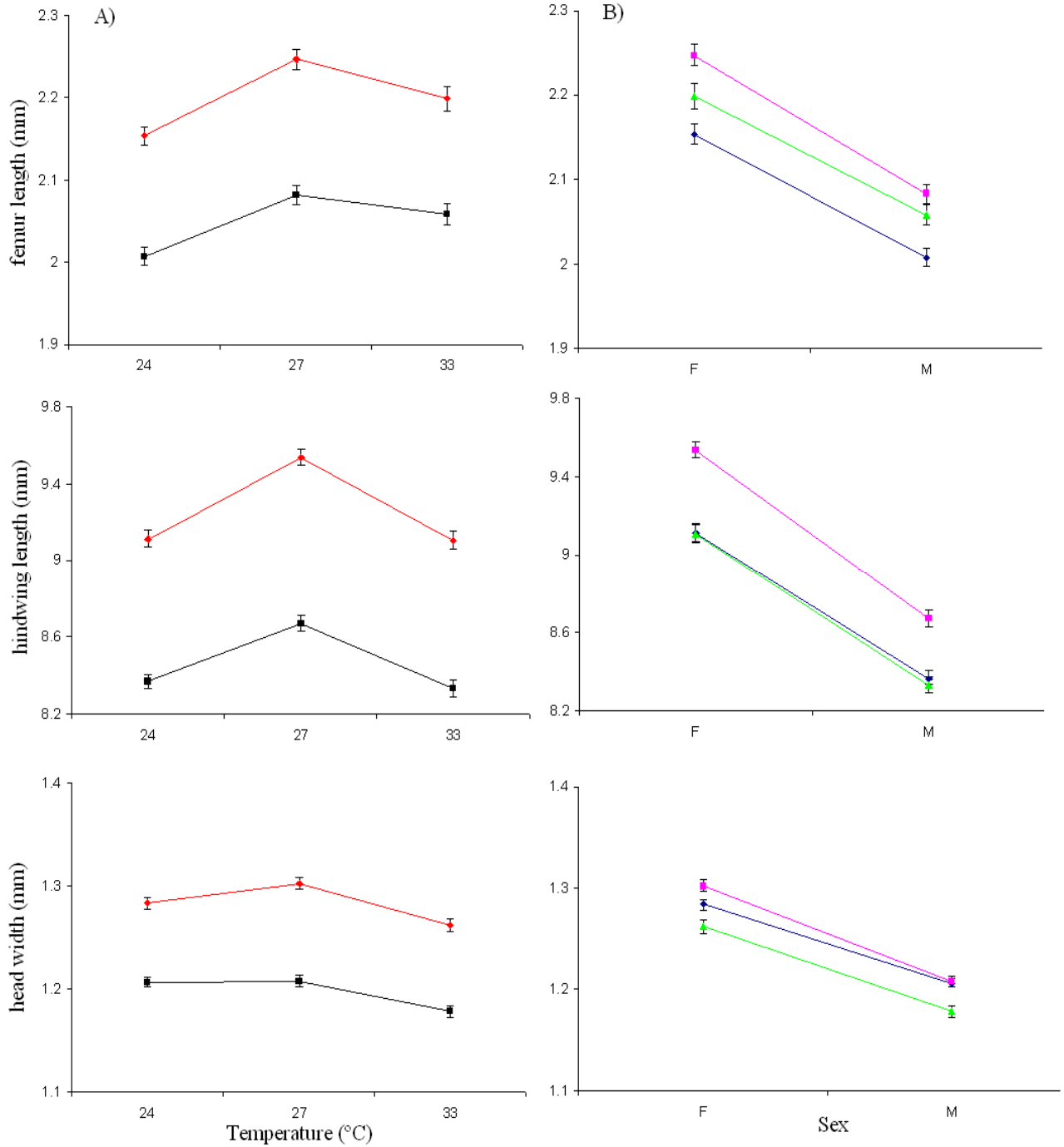


Figure 7: A) The effect of temperature (°C) on organ size (mm) (means \pm 95% confidence intervals) in *O. fasciatus*. Organs measured are femur length, hind wing length and head width. Females: diamonds and red lines; Males: squares and black lines. B) Temperature x sex interaction plots (means \pm 95 % confidence intervals) of femur length, hind wing length and head width. 24°C: diamonds and blue lines; 27°C: squares and pink lines; 33°C: circles and green lines.

Trait size significantly differs across all temperature conditions (Table 4; Figure 7 A). However, unlike in *B. germanica*, the relationship between body size and temperature is not linear in that 27°C reared adults are larger than those reared at both 24°C and 33°C (Figure 7 A). Furthermore, there were inconsistencies between interactions of sex and temperature across different organs. There was a significant interaction for wing length and head width, but not for femur length (Table 4). Unlike in *B. germanica*, where the interaction between sex and temperature for wing length comes from males, the interaction for hindwing length in *O. fasciatus* was hard to interpret. Although the CI did not overlap between certain temperatures, it is not clear whether the difference is bigger in males or in females (Figure 7 B). The interaction for head width was more similar to the interaction seen in *B. germanica*, where no clear trend can be observed. Possible explanations are given in the discussion section.

4.2 The cellular basis of the effects of rearing temperature on body size

4.2.1 In *Blatella germanica*

For statistic analysis, all data met the assumptions of normality. Means, standard errors and standard deviations are given in Table B1 in *Appendix B*.

Mean cell size in females was significantly increased at low temperature ($F_{2, 146} = 162.05$, $p < 0.001$, Figure 8 A) and there was a significant positive correlation between wing area and cell area ($r = 0.492$, $p < 0.001$). There was also a considerable effect of temperature on wing cell number ($F_{2, 146} = 154.16$, $p < 0.001$, Figure 8 B).

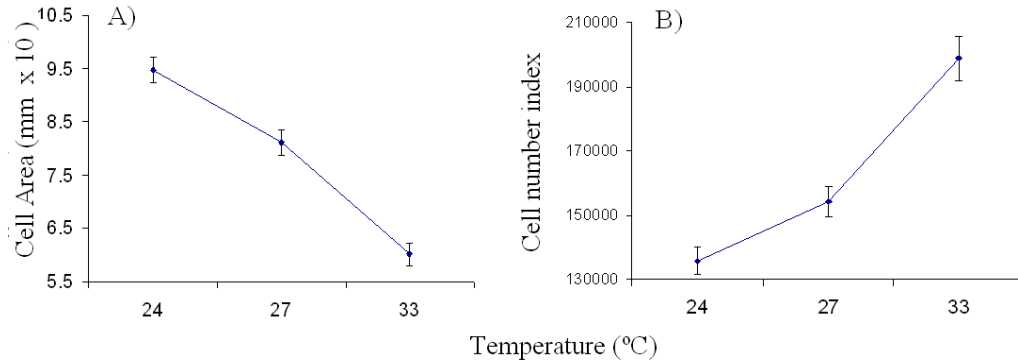


Figure 8: Mean \pm 95 % confidence intervals of A) wing cell size and B) cell number index of *B. germanica* females at different temperatures.

Cell number, however, decreased with decreasing temperature. Therefore, larger body size in individuals reared at colder temperature is attributable to an increase in cell size only. Furthermore, regression analysis for allometric cell and wing area (log-transformed) as done by Stevenson (1995) and Zwaan (2000) revealed a positive regression slope greater than 1, which indicates that all wing area differences can be accounted for by cell size (regression analysis: $\log(\text{cell area}) = 3.62 \log(\text{wing area}) - 6.89$). Additionally, correlations between $\log(\text{cell size})$ and $\log(\text{cell number})$ were negative and highly significant for all temperature conditions ($r = -0.962$, $p < 0.001$). This indicates changes in compensation between cell size and cell number in the establishment of total organ size. Besides, since the slope was greater than 1, cell number overcompensates for increases in cell size (wing area increases more slowly than cell size because numbers are decreasing; Zwaan, 2000).

To ensure the above conclusions were not misconstrued, percentage of cell area change was compared to percentage of wing area change (Table 5, Figure. 9). It is evident that wing area increased much less than cell size. This is in agreement with the conclusions above.

Table 5: Percentage increase of different temperature conditions for *Blatella germanica*.

Temp conditions (°C)	% of cell area increase	% of wing area increase
33 - 27	35	5.110
27 - 24	16.75	2.850

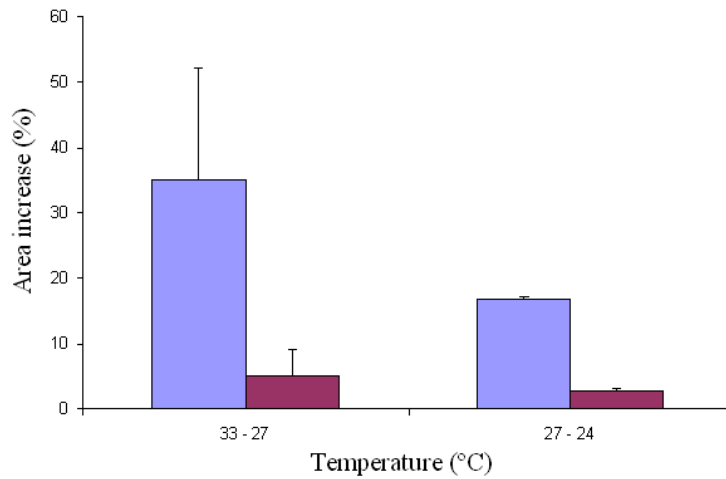


Figure 9: Mean and 95% confidence levels of the percentage increase of cell area and wing area between two temperature conditions in *B. germanica*. Cell area is highlighted in grey and wing area in black.

At colder temperatures individuals with a larger body size have fewer but larger cells than in warmer temperatures, where individuals are smaller. It can be concluded that larger body size in *B. germanica* is due to an increase in cell size rather than number.

4.2.2 In *Oncopeltus fasciatus*

All data were normally distributed and showed homogeneity of variances. Descriptive statistics are shown in Table B2 in *Appendix B*.

There is a negative association between area and number of cells in the wing ($r = -0.220$, $p = 0.008$), which is an indication of compensation of cell size and number (Zwaan, 2000). However, no regression analysis was possible because of non-linear trend in body size variation. Unlike in *B. germanica*, in hindwings of female *O. fasciatus*, cell area and wing area did not correlate ($r = -0.127$, $p = 0.124$). However, this should be expected, given that the response in wing area is not concordant with the response in cell area. (see Figure 7 A and Figure 10 A).

Although temperature has an overall significant effect on cell size ($F_{2, 144} = 21.41$, $p < 0.001$), confidence intervals of cell area overlap between temperatures 27°C and

33°C (Figure 10 A). Therefore, there is no difference in cell size on wings among individuals reared at 27°C and 33°C; although 27°C raised adults had substantially larger wings than those reared in 33°C (Figure 7 A).

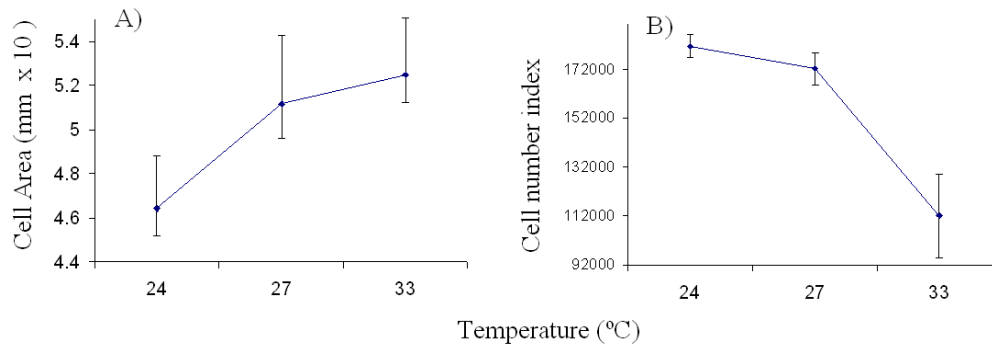


Figure 10: Mean \pm 95 % confidence intervals of A) wing cell size and B) wing cell number index of *O. fasciatus* females at different temperatures.

Decrease in wing size (Figure 7 A) can be explained when looking at cell number index. There is a decrease of 53.6% in cell numbers on the wing surface from adults reared at 27°C to 33°C (overall temperature effect on index: $F_{2,144}=22.46$, $p<0.001$, Figure 10 B). Therefore, decrease in wing size of adults reared at 33°C is more due to cell number rather than size.

To the contrary, cell size in wings of smaller females, reared at 24°C was notably smaller than cell size in wings of the larger females, reared at 27°C (decrease of 10.34%, Figure 10 A). Furthermore, unlike the difference in cell number index between females reared at 27°C and at 33°C, there is no difference in cell number in wings between females reared at 24°C and at 27°C. Thus, it can be concluded that increase in wing size between females reared at 24°C to at 27°C is due to cell size increase rather than cell number. Conversely, decrease in body size at 33°C is due to cell number decrease only.

4.3 Effects of rearing temperature on developmental time and mortality

In *B. germanica*, as expected, the nonparametric Mann-Whitney test revealed that the average developmental time was longest in individuals reared at 24 °C and shortest at 33°C (Table 6, Figure 10 A).

Table 6: Mann-Whitney test for comparisons of mean average developmental times of individuals reared at 24, 27 and 33°C in *B. germanica*. Significant levels are shown in bold.

Temperature conditions compared (°C)	N	w	p
24 & 27	95	72.5	0.012
24 & 33	89	77	0.002
27 & 33	85	69	0.04

In *O. fasciatus* the nonparametric Mann-Whitney test was used to compare developmental times between temperature conditions. Similarly to the pattern observed in *B. germanica*, individuals reared at cooler temperature took longer to eclose to adults (Table 7, Figure 10 B).

Table 7: Mann-Whitney test for comparison for mean developmental time of each individual reared at 24, 27 and 33 C in *O. fasciatus*. Significant levels are shown in bold.

Temperature conditions compared (°C)	N	w	p
24 & 27	538	112086	<0.001
24 & 33	535	112086	<0.001
27 & 33	561	39903	<0.001

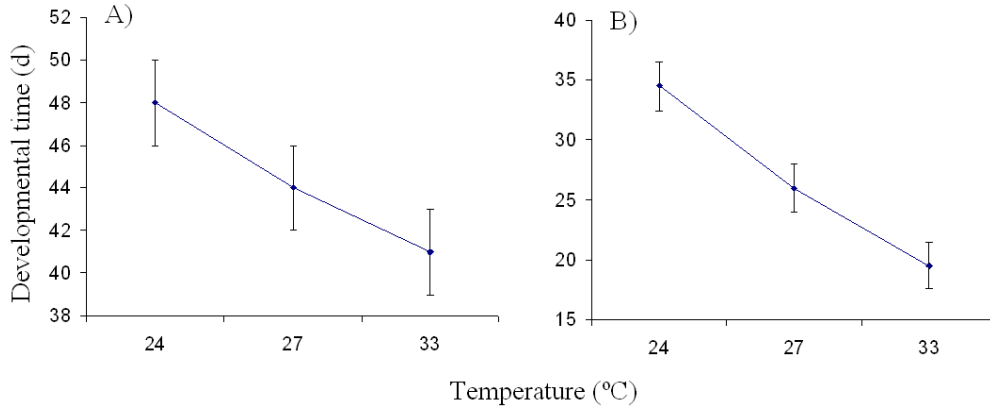


Figure 10: Mean \pm 95% confidence intervals for developmental time of individuals reared at 24, 27 and 33 °C A) average developmental time of *B. germanica*; B) developmental time for *O. fasciatus*.

Rearing temperature had a significant effect on mortality in *B. germanica* ($F_{2, 82} = 9.52$, $p < 0.001$). Juvenile mortality was lowest in individuals reared at 24°C and highest in individuals reared in containers at 33°C (Figure 11 A). Furthermore, variation in mortality was notably higher at 33°C reared individuals.

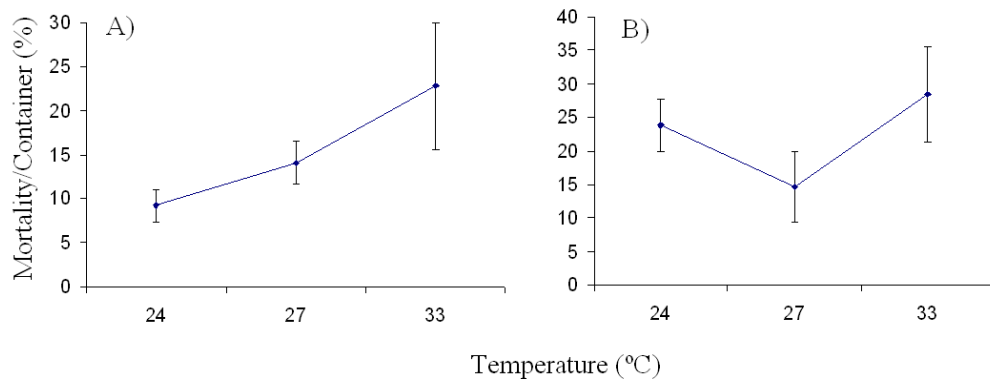


Figure 11: Mean \pm 95% confidence interval of juvenile mortality per container of A) *B. germanica* and B) *O. fasciatus* reared at different temperatures.

Rearing temperature had a significant effect on mortality in *O. fasciatus* ($F_{2, 42} = 7.26$, $p = 0.002$). Mortality per container was lowest in individuals reared at intermediate 27°C (Figure 11 B). This trend concurs with the response to temperature of organ size. Individuals grow largest and survive best at 27 °C compared to lower 24°C or higher 33°C. Additionally individuals reared at 33°C have higher mortality and larger variation in mortality than those reared at 24°C (Figure 11 B).

5. DISCUSSION

5.1 The temperature-size rule for body size and cell size

Roaches raised under cold temperature conditions developed slower and had larger body size at eclosion. The response of *B. germanica* adds to the generality of previous findings of the temperature-size rule in many ectotherms. Body size is significantly reduced with an increase in developmental temperature (Atkinson, 1994). In the present study, body size was determined by studying three organs independently and all organs were highly correlated and responded to temperature equally. Therefore, head width, femur and wing length are good indicators for body size. However, as discussed later for the temperature x sex interaction, measurement of head width has its caveats.

Studies on *Drosophila* show that increased wing size at lower temperatures is due to increased cell size with no or little effect on cell number (Partridge *et al*, 1994; French *et al*, 1998; Azevedo *et al*, 2002,). However, other studies have shown that temperature has an effect on both cell size and number (*Scathophaga stercoraria*, Blanckenhorn & Llaurens, 2005; *see also review by* Atkinson, 1994). Therefore, Atkinson (1994) argues that an explanation of the temperature-size rule should not be sought at the level of cell physiology, because it is not well supported. However, the temperature-size rule can still be entirely explained by direct changes in cell size and number in response to temperature, which is supported by the present study. It was shown that an increase in organ size is proximately mediated due to the direct effects of temperature on increased cell size. Van de Have and de Jong (1996) argue that if the activation energy (in the form of temperature) for cell division is lower than for cell growth, cells will be larger after dividing at lower temperatures and this leads to increased body size. This is a non-adaptive explanation of the temperature-size rule, which remains feasible in this study.

5.2 Cellular compensation - Cell size increase and cell number decrease

The negative association between cell size and number in wings of *B. germanica* indicates compensation between cell size and number. To date, regulation of cell size and whether cell size and number are independently controlled is not well understood (McCabe *et al* 1997, Nijhout, 2003). However, both phenotypic and genetic negative correlations of cell number and cell size have been reported (de Moed *et al*, 1997, McCabe *et al*, 1997). Furthermore, McCabe *et al* (1997) showed that artificial selection on one trait in the *Drosophila* wing results in a correlated negative response in the other trait. This provided strong evidence for compensatory control of cell size and number. In the present study the decrease in cell number and the increase in cell size at lower temperatures could be due to compensatory action in the *B. germanica* wing. Additionally, Zwaan *et al* (2000) found a tight negative interdependence between cell size and number in the *Drosophila* wing, because both size and number changed in the same way across latitudes. It was suggested that body size differs as a result of changes in cell size and number or both, but no change in joint regulation. The level of compensatory action was not investigated in this study, but it would be interesting to see if this is applicable to *B. germanica*. Furthermore, joint regulation of cell size and number would indicate that overall wing size is controlled, rather than its cellular components independently.

5.3 Stress conditions and/or the converse temperature- size rule

Most insects respond to heat stress conditions with decreased body size and high mortality. Furthermore, in *D. melanogaster*, it was found that variation in mortality significantly increased under stressful temperatures (Imasheva *et al*, 1998). In both *O. fasciatus* and *B. germanica*, mortality and variation in mortality was highest at 33°C. Therefore, 33°C was probably a stressful temperature for both species, with a larger effect in *O. fasciatus*, because size as well as mortality was decreased. However, *O. fasciatus* responds with decreased body size also at lower 24°C; whereas optimum growth was at 27°C. Therefore the temperature-size rule cannot be clearly deduced for *O. fasciatus*. It can be argued that individuals at 24°C and 33°C were under heat stress conditions: juvenile mortality was increased and size was reduced. However,

increased mortality might only have been due to bad food conditions at 24°C. In *D. melangoster*, adults eclose at smaller body size, when they developed at low quality food (Crill, 1996). Although food was changed regularly for *O. fasciatus*, fungus grew quickly under 24°C conditions, which probably caused increased mortality. Furthermore, variation in mortality at 24°C was not much different than variation at 27°C.

Inevitably, 33°C temperature conditions are at the upper extreme of the viable thermal range in *O. fasciatus*. This is also supported by a study from Baldwin & Dingle (1986), who described individuals reared at 35°C where unable to emerge as adults and if they did, there was a substantially higher incidence of malformed adults. Dingle (1986) found that individuals of *O. fasciatus* reared at 23°C, however, had highest survival rates compared to 27°C and 31°C. In the present study, it is likely that *O. fasciatus* was not heat stressed but food stressed at 24°C and therefore might exhibit the converse temperature-size rule. Some species follow the opposite trend to the temperature-size rule where body size increases with increasing temperature and developmental time stays prolonged at colder rearing temperatures (e.g. *Allonemobius fasciatus*, Mousseau, 1999).

Investigations on cell size and number in *O. fasciatus* revealed that there is an increase in cell size from 24°C to 27°C. If the temperature-size rule really holds for cell size, as seen in *B. germanica*, then the trend observed would indicate that *O. fasciatus* follows the converse temperature-size rule on the cellular and organismal level and decreased body size at high temperatures is explained by heat stress conditions. Subsequently this would suggest that environmental stress conditions results in cell number decrease, since cell number index decreased from 27°C to 33°C where cell area remained constant. Indeed, recent findings on the genetics of stress response in *Drosophila* indicate that starvation results in a decrease in cell number (Jünger *et al*, 2003). The *Drosophila* homologue of mammalian FOXO, dFOXO was proposed to be an important downstream effector of *Drosophila* insulin signalling and regulator of stress response (starvation). dFOXO caused a reduction in cell number but not in cell size in response to reduced insulin signalling and it was suggested that under starvation conditions, nuclear dFOXO activates target genes that reduce cell division rates (Jünger *et al*, 2003).

The striped ground cricket, *Allonemobius fasciatus* and the field grasshopper *Chorthippus brunneus*, univoltine species like *O. fasciatus*, are examples of the group of ectotherms that follow the converse temperature-size rule. Hereby, animals exhibit larger body size with shorter developmental time at higher environmental temperatures (Mousseau, 1999). Mousseau (1999) claims that *A. fasciatus*, cells do not respond to temperature by increasing in size. Instead, cell size decreased at higher rearing temperatures and increased body size is explained by increased cell number. Therefore, the inverse temperature-size rule on body size would not apply on cell size. This contradicts what was discussed above. However, Mousseau never specified how he counted cells on wing, femur and eye (pers. comm, Mousseau 2004). Thus experiments on the effect of temperature on cell size and number should be replicated before final conclusions can be made.

It was shown by Walters and Hassal (2006) that the biophysical model proposed by van der Have and de Jong (1996) can also explain the inverse of the temperature-size rule. Plasticity in adult body size of *C. brunneus* was determined for the relative difference between the minimum temperature thresholds (relative activation energy) for rates of growth and development. Rate of differentiation, determined by cell division and its duration, is defined as developmental rate. A lower temperature threshold (lower activation energy) for developmental rate than for growth rate (cell size increase) should be found in animals that exhibit the converse temperature-size rule like *C. brunneus*, where the opposite is true for animals that grow larger at lower rearing temperatures (Walters & Hassal, 2006).

Blanckenhorn and Demont (2004) argued that in their natural environment larger univoltine species, such as *C. brunneus* or *O. fasciatus* in this study, with longer development in relation to season length are more likely to experience seasonal time constraints and thus exhibit converse Bergmann's clines. An increase in temperature can be considered as an increase in season length. Certainly in most insects a larger body size is favoured by natural selection (e.g. larger females have a larger clutch size, Walters & Hassal, 2006). In univoltine species, populations benefit by maturing at larger body size with temperature because fitness is directly related to female fecundity and females have to reproduce before a season ends. Multivoltine species, such as *D. melanogaster* or *B. germanica*, have many generations per year. Here,

fitness is maximised by increasing the potential number of generations. Therefore, as proposed by Walters and Hassal (2006) selection pressures for lower temperature thresholds for growth rates in univoltine species are expected to be greater than for multivoltine species. However, this hypothesis is hard to test experimentally and thus remains speculative.

5.4 Interaction of temperature and sex – plasticity of sexual size dimorphism

At last, for all temperatures in *B. germanica* and *O. fasciatus* females were larger than males. This is concurrent with what is found in most insects (Atkinson, 1994). Additionally, in *B. germanica*, sexual size dimorphism was plastic in response to rearing temperature. Environmental temperature had a larger effect on body size in males than in females, so the slope of the response curve of males was steeper than in females. In an analysis of 158 insect species where females were the larger sex, 30% showed increasing sexual differences with decreasing body size (Teder & Tammaru, 2005). It was suggested that natural selection favours earlier emergence in males than in females, constraining final body size by developmental time limitations (Teder & Tammaru, 2005). In the current study, sex differences in emergence rate were not quantified, but an interesting extension to this would be to investigate if a trend were apparent in *B. germanica*.

Rensch's rule implies that male body size varies more than female body size among related species (reviewed by Blanckenhorn *et al*, 2006). Blanckenhorn *et al* (2006) found that there is an association of Rensch's rule with Bergmann's rule in that within species male body size varied more with latitude than female body size in about two thirds of species studied. Hereby, males showed a steeper slope of body size-latitude relationship than females (Blanckenhorn *et al*, 2006). Latitude is often associated with temperature differences. In *B. germanica* the slope of femur and wing length with temperature was steeper in males than in females. This implies that there is also an association of Rensch's rule with the temperature-size rule whereby sex-specific variation in body size results from environmental temperature. Variation in head width was not of clear trend. This discrepancy could be because head width was

measured between the eyes and might not be a reliable indicator for body size, because of variation in width of ommatidia growth. Blanckenhorn *et al.* (2006) also found that the decrease in male size (relative to female size) with increasing latitude in species exhibiting the converse Bergmann clines was more pronounced than in Bergmann clines. Unfortunately, a sex difference in body size variation for *O. fasciatus* was hard to elucidate. The results might have been masked by the stress response to higher rearing temperature and males might respond differently to stress than females. An investigation of mortality in *O. fasciatus* in crowded (13.5 bugs/10cm) versus uncrowded (3.8 bugs/10cm) populations revealed a greater number of dead females than males (Dingle, 1966). A study by Gwynne (2004) showed that males of food stressed katydids, *Kawanaphila nartee* (Orthoptera), respond with decreased body size, whereas females showed little loss in mass.

5.6 Limitations of the present study and implications for the future

Several assumptions were made which might affect the validity of the conclusions drawn from this study.

Nymphs were assumed to start development simultaneously. *Blatella germanica* nymphs only start moulting upon food intake (Kunkel, 1966). In contrast, there is evidence that newly hatched nymphs cannibalise unhatched clutch mates in species of *Oncopeltus* (Root & Chaplin, 1976) and therefore, in this study, have started their development earlier than thought. This might have had influence on developmental times of individuals, but should not have altered results substantially.

The greatest assumption that has been made was that one cuticular petrusion on the dorsal surface of the forewing in *B. germanica* and of the hindwing in *O. fasciatus* was assumed to correspond to one cell. This however has not been established yet and for future studies it would be of importance before any definite conclusions can be drawn.

Cell size was deduced from a determined area of the wing. Cell size might be variable throughout the wing and thus measurements might not have been representative for the entire wing surface area. However, the same determined area was measured in each individual; thus obtained data should be suitable. Similarly, when cell number index was calculated, wing length was simply squared. Therefore, cell number might not represent the real number of cells on an entire wing since a wing is not square. Nevertheless, since calculations for number index were consistent for each individual, it should not have impacted on the results.

Due to time constraints only a small sample size of organ measurements (N=100) were taken in *B. germanica*. The study on cockroaches could be improved by taking a larger sample size for both wings for cell count, and organ measurements, since this might make a difference to significant levels of the effects. Small sample size is also evident in that confidence levels for interaction terms were very low. If more data were collected, a clearer trend might become apparent.

One aim of the study was to find out whether the temperature-size rule holds for *O. fasciatus*. However, no definite conclusions on that could be made since the shape of the response curve in the temperature gradient was concave. If the study were to be repeated it would be sensible to rear bugs in their viable temperature range. This might also reveal whether there is an interaction between sex and temperature, as seen in *B. germanica*. Furthermore, for the experimental design, three or more experimental temperatures should be used in order to do a reliable regression analysis, if a linear trend were to be observed.

It would be interesting to establish the cellular basis of the effects of temperature stress in insects. Likewise, the cellular basis of ectotherms that exhibit the converse temperature-size rule is not well established yet and would add to the understanding of the cellular basis of growth control. Furthermore, a closer examination of the joint regulation of cell size and number in *B. germanica*, as done in *D. melanogaster*, would reveal further understanding of growth control at the cellular and organismal level.

Sex differences in developmental time in response to temperature could add to the understanding of sex differences in body size response.

5.5 Main conclusions of the present study

The multivoltine *B. germanica* follows the temperature-size rule on the cellular (cell size) and organismal level (organ size). Cell number decrease in larger wings is probably due to compensatory action of cell number with cell size and thus provides evidence for joint regulation.

- In both species, *B. germanica* and *O. fasciatus*, individuals reared at 33°C were heat stressed.

- It is likely that the univoltine species *O. fasciatus* follows the converse temperature-size rule on the cellular and organismal level with a positive correlation of body size and temperature. Therefore this study would indicate that heat stress results in cell number decrease. However, no explicit conclusion could be drawn.

- Rensch's rule cannot only be associated with Bergmann's size clines but also with the temperature-size rule.

- Finally, it can be concluded that unrelated species with different life histories are likely to respond differently to their environment. Blanckenhorn (1999) found that different species respond differently even when sharing similar life histories (Blanckenhorn 1999). Therefore, even if there is a biological law for ectothermic species, differences in reaction norms between species would be expected.

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8. APPENDICES

A. Effects of rearing temperature on body size and its association with sex

Table A1: Descriptive statistics (sample size N, mean \pm SE and standard deviation StDev) for *B. germanica* organ sizes. All sizes are transformed from microscopic lens graticule units into mm. Temp denotes temperature condition; F denotes females and M males.

Temp (°C)	Sex	N	Femur length (mm)			Head width (mm)			Wing length (mm)		
			Mean	\pm SE	StDev	Mean	\pm SE	StDev	Mean	\pm SE	StDev
24	F	50	1.89	0.0098	0.069	0.93	0.0061	0.043	11.29	0.041	0.29
	M	50	1.82	0.0065	0.046	0.72	0.0048	0.034	10.35	0.030	0.21
27	F	50	1.87	0.0079	0.056	0.89	0.0064	0.046	11.13	0.041	0.29
	M	50	1.77	0.0070	0.049	0.71	0.0055	0.039	10.01	0.041	0.29
33	F	50	1.82	0.0091	0.064	0.87	0.0056	0.040	10.86	0.041	0.29
	M	50	1.68	0.0064	0.045	0.68	0.0061	0.043	9.55	0.027	0.19

Table A2: Descriptive statistics (sample size N, mean \pm SE and standard deviation StDev) for *O. fasciatus* organ sizes. All sizes are transformed from microscopic lens graticule units into mm. Temp denotes temperature condition; F denotes females and M males.

Temp (°C)	Sex	N	Femur length (mm)			Head width (mm)			Wing length (mm)		
			Mean	\pm SE	StDev	Mean	\pm SE	StDev	Mean	\pm SE	StDev
24	F	148	2.57	0.0067	0.077	1.08	0.0032	0.041	9.13	0.024	0.29
	M	151	2.41	0.0052	0.068	1.00	0.0027	0.032	8.37	0.018	0.23
27	F	151	2.61	0.0063	0.077	1.13	0.0031	0.039	9.55	0.019	0.23
	M	150	2.42	0.0067	0.070	1.04	0.0029	0.041	8.66	0.021	0.26
33	F	140	2.54	0.0089	0.079	1.10	0.0033	0.053	9.12	0.023	0.27
	M	160	2.33	0.0075	0.081	1.03	0.0032	0.048	8.32	0.021	0.26

B. The cellular basis of the effects of rearing temperature on body size

Table B1: Mean, standard error (SE) and standard deviation (StDev) of cell size and cell number index in forewing of *B. germanica*

Temp	N	Cell size (mm x10²)			Cell number index		
		<i>Mean</i>	$\pm SE$	<i>StDev</i>	<i>Mean</i>	$\pm SE$	<i>StDev</i>
24	50	0.094	0.00120	0.0085	135718	2112	14937
27	50	0.081	0.00121	0.0085	154176	2258	15969
33	50	0.060	0.00103	0.0073	198716	3471	24547

Table B2: Mean, standard error (SE) and standard deviation (StDev) of cell size and cell number index in hindwing of *O. fasciatus*.

Temp	N	Cell size (mm x10²)			Cell number		
		<i>Mean</i>	$\pm SE$	<i>StDev</i>	<i>Mean</i>	$\pm SE$	<i>StDev</i>
24	47	4.65	0.058	0.40	181205	2381	16323
27	50	5.12	0.078	0.55	172101	3299	23329
33	50	5.25	0.066	0.46	112015	8538	60371