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Effects of temperature on cell size and number in the yellow dung fly *Scathophaga stercoraria*

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Abstract

(1) A number of hypotheses suggest that the temperature-size rule (larger at cooler temperatures), and consequently Bergmann clines in whole-organism body size (larger at higher latitudes), may be a mere consequence of processes at the cellular level, i.e., a physiological constraint.

(2) We show that in the yellow dung fly, *Scathophaga stercoraria* (Diptera: Scathophagidae), the temperature-size rule holds for wing cell and ommatidia size. Increases in cell number made up two-thirds (eye) to three-quarters (wing) of the increase in organ size. Temperature effects on body size can be fully explained by its effects on cell size and number.

(3) Our study adds to the generality of previous results in *Drosophila spp*. The physiological constraint hypothesis remains viable as a proximate, non-adaptive explanation for the temperature-size rule in ectotherms.
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Keywords: Allometry; Bergmann's rule; Body size; Cell size and number; Gene regulation; Physiological constraint; Temperature

1. Introduction

Bergmann's rule, the phenomenon that organisms tend to grow bigger in colder climates, is a common large-scale geographic pattern in animals (Blackburn et al., 1999). Referring exclusively to endothermic (warm-blooded) animals, the adaptive explanation originally suggested by Bergmann (1847) was that larger individuals have smaller surface-to-volume ratios more conducive to conserving heat. However, 150 years later the evidence supporting Bergmann's rule in birds and mammals is inconsistent at best, so the generality of this supposed cause, and in fact Bergmann's rule itself, continues to be contended (Geist, 1987, 1990; Paterson, 1990; Blackburn et al., 1999; Ashton et al., 2000; Ashton, 2002). Nevertheless, about one hundred years after Bergmann it transpired that Bergmann's rule also applies to ectothermic (cold-blooded) organisms (Ray, 1960; Atkinson 1994; Atkinson and Sibly 1997). Here the cause must be different, because especially small ectotherms such as insects adapt to ambient temperatures almost instantly (Stevenson, 1985). Ectothermic Bergmann size clines are also common in nature (e.g. Conover and Present, 1990; James et al., 1997; Huey et al., 2000), and there is ample experimental evidence that ectotherms grow larger at lower temperatures (also known as the temperature-size rule: reviewed by

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Atkinson, 1994; Angilletta and Dunham, 2003). A unifying explanation for this phenomenon is still lacking, although there is agreement in that Bergmann's rule seems to be effected by temperature per se (Atkinson and Sibly, 1997). In contrast, the opposite trend, the so-called 'converse' Bergmann rule, which also exists (e.g., Blanckenhorn & Fairbairn 1995; Mousseau 1997), is largely mediated by season length rather than temperature (reviewed in Blanckenhorn and Demont, 2004).

There is much debate about whether ectothermic Bergmann clines are adaptive. In nature, such clines have been shown to evolve repeatedly and predictably, suggesting an adaptation (Partridge and Coyne, 1997; Huey et al., 2000). In contrast, physiologists and developmental biologists emphasize mechanisms to explain Bergmann's rule. Bertalanffy (1960) argued that physical processes affecting energy assimilation, such as foraging activity at the whole-organism level and nutrient absorption or diffusion at the cellular level, are less affected by temperature than chemical processes driving energy dissimilation (i.e., metabolism). This implies relatively less energy available for somatic growth at higher temperatures, and consequently smaller size (formalized by Perrin, 1995). Similarly, van der Have and de Jong (1996) argued that the rate of growth is primarily affected by protein synthesis, which largely depends on diffusion and is thus less limited by temperature, whereas the rate of cell differentiation and cell division (i.e., development) is highly temperature dependent. This implies that at higher temperatures organisms reach maturity much more rapidly while at the same time growth increases less rapidly, resulting in smaller size. Both arguments can be understood as non-adaptive hypotheses due to physiological constraints, although both may ultimately still be grounded in (adaptive) energetic trade-offs at the physiological (e.g. ATP) level (Atkinson and Sibly, 1997).

These mechanistic arguments apply generally to all parts of the body such as eggs, sperm or individual cells, so that Bergmann clines in whole-organism body size may be a mere consequence of processes at the cellular level (Partridge et al., 1994; James et al., 1995, 1997; Stevenson et al., 1995; van der Have and de Jong, 1996; Van Voorhies, 1996). Bergmann clines in egg size have been shown in *Drosophila melanogaster* (Azevedo et al., 1996) and the pitcher-plant mosquito (Armbruster et al., 2001), and smaller egg sizes at higher temperatures have been experimentally demonstrated in a number of insects (Ernsting and Isaaks, 1997, 2000; Blanckenhorn, 2000; Fox and Czesak, 2000; Fischer et al., 2003). In this context, Bradford (1990) and Woods (1999) provided a third physiological mechanism possibly explaining why eggs and cells should be smaller at higher temperatures: while oxygen diffusion depends only weakly on temperature, oxygen consumption depends strongly on it, so large cells may suffer from hypoxia at high temperatures.

Similar effects of temperature on body, organ, cell and gamete size suggest a unifying physiological mechanism, or constraint, underlying Bergmann's rule extended to ectotherms (van der Have and de Jong, 1996; Van Voorhies, 1996). Direct evidence for the adaptive nature of Bergmann clines or the temperature-size rule typically requires demonstration of temperature-dependent life history trade-offs at the whole-organism level. There is essentially no such empirical evidence for body size (see McCabe and Partridge (1997), Reeve et al. (2000) for the best evidence so far; but see Huey et al., 2000). Except for one study of butterflies (Fischer et al., 2003), there is also no evidence for the hypothesis that eggs laid at a particular temperature perform best at that temperature (Ernsting and Isaaks, 1997, 2000; Blanckenhorn, 2000). On the other hand, the generality of the physiological constraint hypothesis can best be refuted by finding counter-examples. Blanckenhorn and Hellriegel (2002) recently found that sperm length of the yellow dung fly *Scathophaga stercoraria in*creases (rather than decreases) with temperature. Furthermore, Angilletta and Dunham (2003) recently refuted the generality of the hypothesized underlying physiological mechanism of Bertalanffy (1960) and Perrin (1995).

A number of studies have investigated whether temperature-mediated changes in body size are a mere consequence of corresponding changes in cell size (Partridge et al., 1994; James et al., 1995, 1997; Stevenson et al., 1995; Van Voorhies, 1996; De Moed et al., 1997a, b; McCabe et al., 1997; French et al., 1998; Zwaan et al., 2000; Azevedo et al., 2002). Except for Van Voorhies' (1996) study on the nematode *Caenorhabditis elegans*, these were all on *Drosophila* (primarily *melanogaster*), investigating wing cells as a rule, except for Stevenson et al. (1995), who also studied ommatidia (cf. also Butler and Rogerson (1996), and Timofeev (2001), for data on amoebae and crustaceans). Invariably, these authors found increases in cell size, but also typically in cell number, as temperature decreased. This implies that animals are larger at colder temperatures because they have larger and more body cells. At the same time, developmental biologists have shown in *Drosophila melanogaster* that typically cell size and number are jointly regulated by the same genes (McCabe et al., 1997; Boehni et al., 1999; Stocker and Hafen, 2000; Brogiolo et al., 2001).

Here we investigate the allometry of wing cell and ommatidia size and number with body size as a function of temperarature in the yellow dung fly *Scathophaga stercoraria* (Diptera: Scathophagidae). Strictly speaking, one ommatidium is composed of eight fused rhabdomeric cells, but for the purposes here it was considered as one cell (cf. Stevenson et al., 1995). Previous research

on the yellow dung fly has shown a weak Bergmann body size cline in Europe (Blanckenhorn and Demont, 2004), and that flies have larger bodies (Blanckenhorn, 1997) and eggs (Blanckenhorn, 2000) but shorter sperm (Blanckenhorn and Hellriegel, 2002) at lower temperatures. This study adds to the generality of previous results by investigating two additional cell types in an insect other than *Drosophila* that in the past has shown inconsistent effects of temperature on the size of various body structures.

2. Materials and methods

To assess the effect on ambient temperature on wing cell and ommatidia size and number, flies were raised in the laboratory at five different temperatures (12, 15, 18, 22, and 24 °C) and two larval food availabilities (low and high). The latter treatment served to increase the size range at any particular temperature (cf. Blanckenhorn, 1997). *Scathophaga stercoraria* prefer cooler climates. In Central Europe this species is reproductively active quite early (March) and late (November) in the year (Blanckenhorn, 1997), even at temperatures below 10 °C. The flies regularly disappear from the pastures during the hot summer (Parker, 1970; Blanckenhorn, 1998; Blanckenhorn et al, 2001), because adults, larvae and pupae suffer high mortality at temperatures exceeding 26 °C (Ward and Simmons, 1990; Blanckenhorn, 1998).

Approximately 10 eggs from laboratory-reared females that had copulated with one male were allowed to hatch and develop in 50 ml plastic containers with either an overabundant amount of 20–40 g (high food) or a limited amount or 5–10 g (low food) of defrosted, uniform cow dung. Both dung limitation and high temperatures are known to produce smaller flies (Amano, 1983; Blanckenhorn, 1997, 1998). In one set of families, a given clutch (henceforth called family) was split among the three temperatures 12, 18 and 24 °C as well as the two food levels; in a second set of families, a given clutch was split among the two temperatures 15 and 22 °C as well as the two food levels. In both sets, we measured one female and one male per family and temperature/food level combination. We thus minimized genetic variation, most of the variation in body and cell size among the treatments being environmental. Emerging flies were frozen immediately at −20 °C for later measurement. Sample sizes ranged from 18 to 40 flies of each sex per temperature.

We largely followed the methods of Partridge et al. (1994) and Stevenson et al. (1995). We estimated wing cell size and number in a constant area of one particular region of the right wing by counting the hairs (trichomes) of each cell of one of the two cell layers, using a microscope at 400 × magnification connected to an image analysis system. The surface area, and subsequently the diameter, of wing cells was then calculated by dividing the region's total surface area by the number of hairs counted. The number of cells of the wing was also estimated by measuring the wing's total area (with the same image analysis system) and multiplying it with the number of cells in the region's area. This assumes that cell sizes of a wing are constant, which is not necessarily justified (Partridge et al., 1994), but by always measuring the same region this error would be systematic and probably does not bias differences in cell counts among temperature treatments.

We measured the ommatidia of one (typically the right) eye by first covering the eye with nail varnish. After drying, we peeled off the varnish with tweezers, flattened the slightly spherical imprint by cutting into it, and put it on a cover slip. As above, we now could estimate the number and surface area of the ommatidia as above (at 400 × magnification), always choosing approximately the same central region. To estimate the total number of ommatidia of the eye, we measured the length and the width of the eye (cf. Stevenson et al. 1995). The resulting eye area, calculated using the formula of an ellipse, was an underestimate because the eye of yellow dung flies is somewhat spherical. Therefore, for a subset of samples, we additionally estimated the actual surface area using our nail varnish imprint, yielding an average factor by which the ellipse formula was later corrected upwards.

Lastly, we measured hind tibia length as the standard index of body size in this species, in addition to wing length and eye diameter.

3. Results

ANCOVA of wing length, eye diameter and tibia length with sex as a fixed factor and rearing temperature as the covariate showed that all traits were larger in males than females (as usual in this species; all sex effects: $F_{1,244} > 4.04$, $P < 0.05$), and that trait size decreased as temperature increased (according to the temperature-size rule: temperature effects: $F_{1,244} > 39.2$, $P < 0.001$) equally in the sexes (temperature by sex interaction *ns*; Fig. 1). In general, the decrease in size with temperature was linear (Fig. 1), as quadratic regressions did not yield a better fit. Analogous results were obtained for square-root-transformed wing cell area (sex effect: $F_{1,244} = 6.14$, $P = 0.014$; temperature effect: $F_{1,244} > 34.0$, $P < 0.001$), and square-root-transformed wing cell number (sex effect: $F_{1,244} = 4.06$, $P = 0.045$; temperature effect: $F_{1,244} > 31.5$, $P < 0.001$; both interactions *ns*, Fig. 2). Square-root-transformed ommatidium size did not differ significantly between the

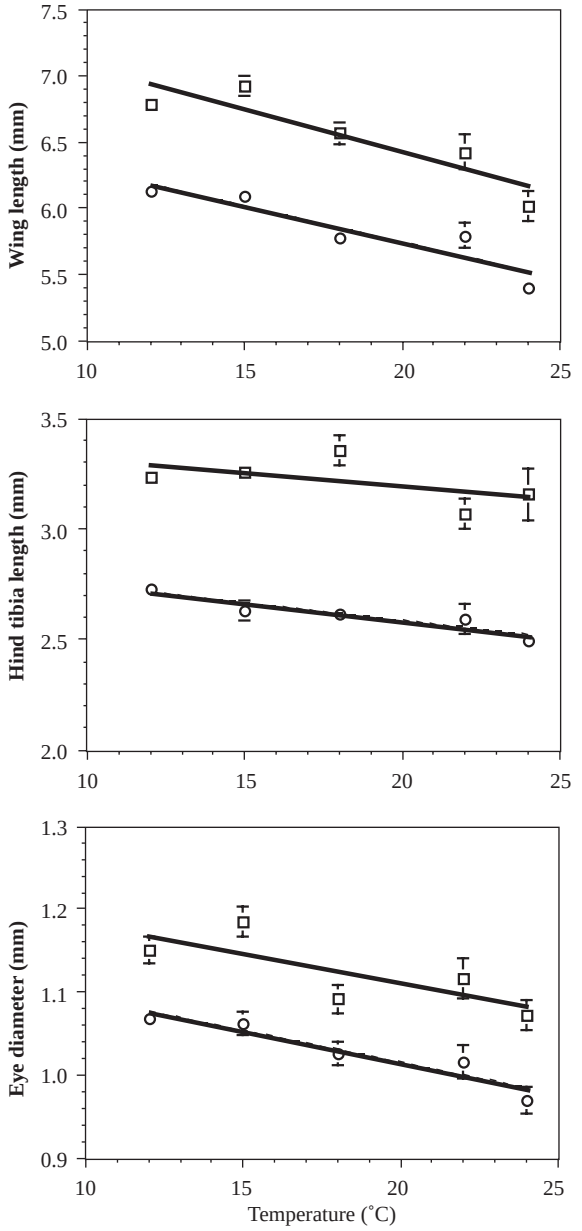

Fig. 1. Mean ± SE wing length (top), hind tibia length (middle), and eye diameter (bottom) of yellow dung flies as a function of rearing temperature (males: squares and unbroken regression lines; females: circles and broken regression lines; $N = 18-40$ per treatment combination).

sexes ($F_{1,244} = 2.38$, $P = 0.124$) but decreased with increasing temperature ($F_{1,244} > 15.5$, $P < 0.001$), whereas square-root-transformed ommatidia number was unaffected by sex and temperature (sex effect: $F_{1,244} = 0.13$, $P = 0.718$; temperature effect: $F_{1,244} = 1.84$, $P = 0.176$; both interactions *ns*; Fig. 2). However, when controlling

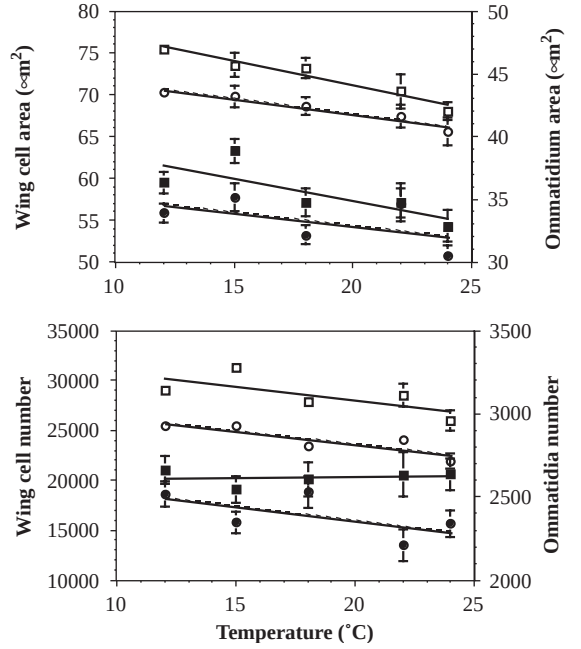

Fig. 2. Mean ± SE wing cell area (left) and ommatidia area (right; top graph), and wing cell number (left) and ommatidia number (right; bottom graph) of yellow dung flies as a function of rearing temperature (open symbols: right scale; closed symbols: left scale; males: squares and unbroken regression lines; females: circles and broken regression lines; $N = 18-40$ per treatment combination).

for body size by including hind tibia length as a covariate in these analyses, the temperature effect disappeared for all the cell traits ($P > 0.1$) except wing cell number ($F_{1,243} = 4.27$, $P = 0.040$). From this we can conclude that the temperature-body size rule is proximately mediated, and thus entirely explained, by effects of temperature on cell size and number.

Analyzing the allometric relationships between cell size or number and organ size as done by Stevenson et al. (1995) showed that for the wing, 73.8% (= allometric slope) of the size increase is due to increases in wing cell number while the remaining 26.2% can be attributed to increases in wing cell size (regression of log wing cell diameter or number on log wing length). The analogous analysis for the eye showed that 66.7% of the size increase is due to increases in ommatidia number and the remaining 33.3% is due to increases in ommatidium size. Both log eye diameter (allometric slope = 0.519 ± 0.031 (SE)) and log wing length (0.682 ± 0.027) scale hypo-allometrically with log body size, here estimated by hind tibia length. All these allometries did not differ for the sexes or among the rearing temperatures (tested by means of ANCOVA), so only the overall results are presented.

4. Discussion and conclusions

Our study shows that in the yellow dung fly the temperature-size rule (Atkinson, 1994; Angilletta and Dunham, 2003) holds not only for body size (Blanckenhorn, 1997) and egg size (Blanckenhorn, 2000), but for wing cell and ommatidia size as well. Moreover, we can conclude that temperature effects on body size are proximately mediated, and can thus in their entirety be explained, by the effects of temperature on cell size and number. As similar studies on *Drosophila* (Partridge et al., 1994; James et al., 1995, 1997; Stevenson et al., 1995; De Moed et al., 1997a, b; McCabe et al., 1997; French et al., 1998; Azevedo et al., 2002) and *C. elegans* (Van Voorhies, 1996) have shown, lower temperatures increase both cell size and number, increases in cell number here making up two-thirds (eye) to three-quarters (wing) of the increase in organ size. Our study thus adds to the generality of previous results by investigating an insect other than *Drosophila* in this regard. Therefore, sperm so far remains the only cell (or organ) type in this or any other species that does not follow the temperature-size rule (Blanckenhorn and Hellriegel, 2002). The physiological constraint hypothesis (van der Have and de Jong, 1996; Van Voorhies, 1996) thus remains viable as a proximate, non-adaptive explanation for the temperature-size rule in ectotherms (but see Atkinson and Sibly, 1997; Angilletta and Dunham, 2003).

In our study the decrease in trait size with increasing temperature was largely linear. This must not necessarily be so, as several studies have shown hump-shaped curvilinear (quadratic) relationships (e.g., David et al., 1997; Imasheva et al., 1997; Karan et al., 1998; Morin et al., 1997, 1999). This may relate to the temperatures tested. Reduced body size can be expected at stressful temperatures below a lower and above an upper threshold beyond which a species is not viable (op. cit.). The lower temperature threshold of yellow dung flies (which is about 5 °C: Blanckenhorn, 1999) was clearly not reached here, however, 24 °C approaches their upper temperature threshold of about 26°C (Ward and Simmons, 1990). It is therefore likely that temperatures outside of the animals' viable temperature range (i.e., higher and lower than those tested here) might have resulted in a drop in trait size as well (cf. Blanckenhorn, 1997, 1999).

Temperature effects on cell size and number can be further tracked back to effects of temperature on gene regulation and/or gene action during development. Joint regulation of cell size and number has been shown in *Drosophila melanogaster* (McCabe et al., 1997; Boehni et al., 1999; Stocker and Hafen, 2000; Brogiolo et al., 2001), and it is easy to envision physiologically how a temperature change may jointly affect the threshold at which cells divide (thus changing cell size) as well as the threshold at which growth of a particular organ, or in fact the whole body, ceases (thus changing cell number). The environmental effects of temperature on cell size and number are apparently completely analogous to the genetic effects of particular body size mutants in *Drosophila melanogaster* (Stocker and Hafen, 2000). So far, there is apparently only one mutation known that affects cell size but not cell number, their regulation being consequently decoupled (Montagne et al., 1999). It would be interesting to investigate the physiological mechanism by which this is possible, because if temperature can differentially affect cell size and cell number regulation and thresholds, deviations from the temperature-size rule can result.

Studies such as ours are important, as they can reveal exceptions to the temperature-size rule, which are necessary to refute the physiological constraint hypothesis as its general explanation (see Introduction). However, whether body, organ and cell size increases at cooler temperatures are adaptive can ultimately not be solved by such experiments, as this requires investigating the fitness consequences of such temperature-mediated changes at the whole-organism level (e.g. Ernsting and Isaaks, 1997; Blanckenhorn, 2000; Reeve et al., 2000).

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