Research article

Diapause in the pea aphid (*Acyrthosiphon pisum*) is a slowing but not a cessation of development

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Abstract

Background: Many insects undergo a period of arrested development, called diapause, to avoid seasonally recurring adverse conditions. Whilst the phenology and endocrinology of insect diapause have been well studied, there has been comparatively little research into the developmental details of diapause. We investigated developmental aspects of diapause in sexually-produced embryos of the pea aphid, *Acyrthosiphon pisum*.

Results: We found that early stages of embryogenesis progressed at a temperature-independent rate, characteristic of diapause, whereas later stages of embryogenesis progressed at a temperature-dependent rate. However, embryos maintained at very high temperatures during the temperature-independent stage showed severe developmental abnormalities. Under no temperature regime did embryos display a distinct resting stage. Rather, morphological development progressed slowly but continuously throughout embryogenesis.

Conclusion: Diapause in the pea aphid, and perhaps in many other insects, is a temperature-independent slowing but not a cessation of morphological development. This suggests that the mechanisms limiting developmental rate during diapause may be the same as those controlling developmental rate at other stages of growth.

Background

Many animals have evolved to survive seasonally recurring adverse conditions by entering a diapause stage. Diapause is generally characterized by a slowing of metabolism, a reduction or elimination of cell division and a cessation of morphological development, evident as a 'resting stage' [1]. Diapause occurs in response to stimuli that predict adverse environmental conditions, not in response to the conditions themselves. Once in diapause, development is not resumed even if conditions return to those amenable to growth. It is this lack of responsiveness that is a distinguishing characteristic of diapause [2]. In most animals diapause can occur only at a specific developmental stage, be it as an embryo, juvenile, or adult. Diapause, therefore, represents a discrete developmental process that should be controlled, at least in part, by stereotyped gene expression.

Until recently diapause was generally considered a physiological phenomenon, and much research had concerned the conditions that induce diapause and the endocrinology of its regulation [2,3]. Nevertheless, diapause is also a developmental process, and these previous studies did not address fundamental questions concerning development before, during and after diapause. More recent research has begun to focus on the molecular aspects of diapause,
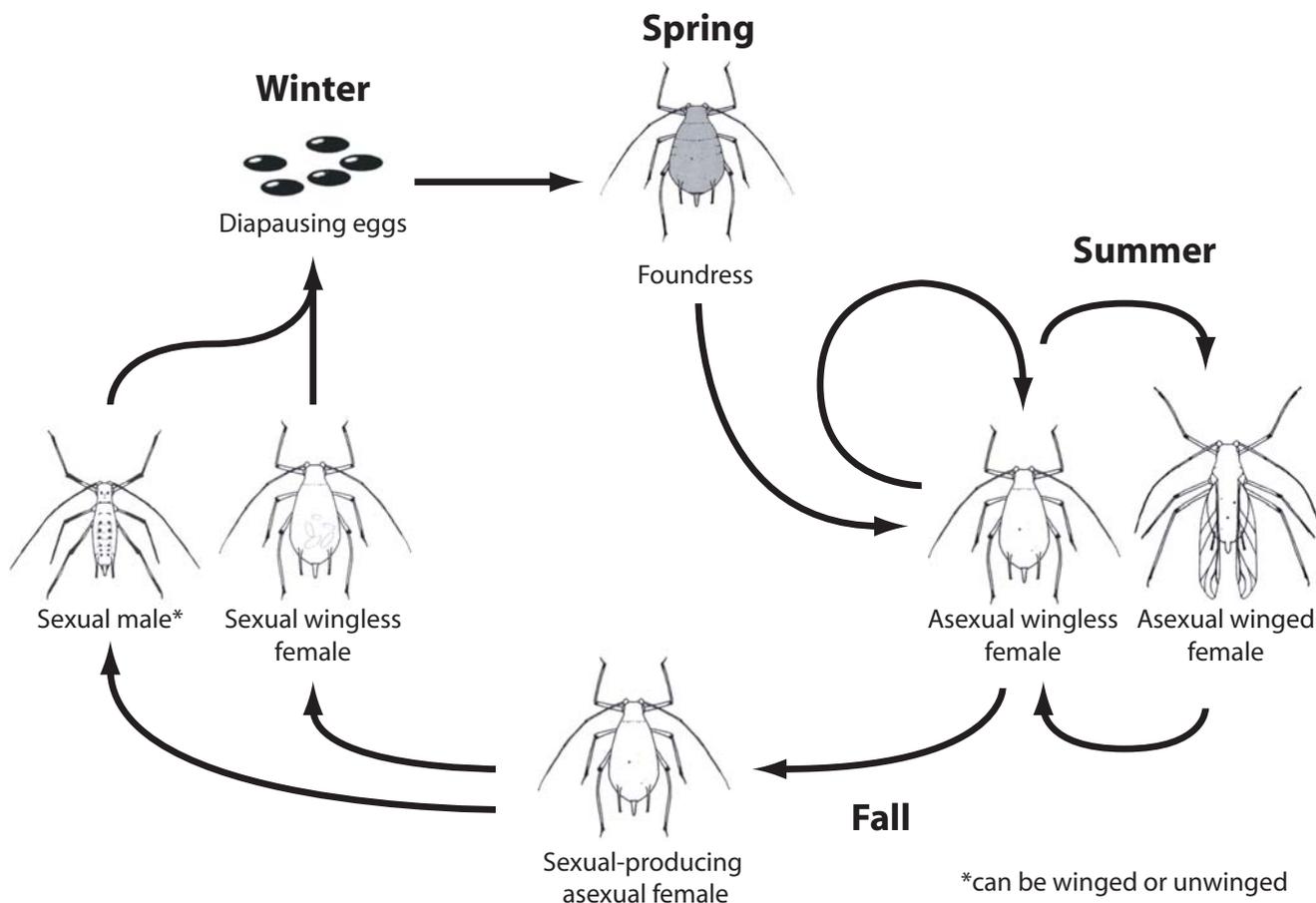
Figure 1
Life cycle of the pea aphid, *A. pisum*. During the spring and summer reproduction is by parthenogenesis. Asexually-produced embryos develop in approximately 10 days. In the fall there is a single generation of sexually-reproducing males and females which produce diapausing eggs. These eggs do not hatch until the following spring, more than 100 days later.

with studies of *Bombyx morii*, *Sarcophaga crassipalis*, and *Lymantria dispar* identifying genes up- and down-regulated during diapause [1,4–7]. In order to complete this picture, however, detailed descriptions of the morphological aspects of development spanning diapause are also necessary.

The pea aphid (*Acyrthosiphon pisum*: Hemiptera, Aphidoidea) ostensibly undergoes embryonic diapause as an overwintering egg (figure 1). During spring and summer, aphids reproduce through viviparous parthenogenesis. In the fall, however, low temperatures and short days result in the production of egg-laying females and males to inseminate them. The females produce eggs that hatch the following spring, when the life cycle is repeated. The total period of egg development is reported as approximately 100 days [8]. In contrast, parthenogenetic embryogenesis

is completed in approximately 10 days. It has been assumed that the order of magnitude difference in developmental rate between the two forms is due to diapause in the sexually-produced embryos [8]. However, detailed embryological studies of sexually-produced embryos has yet to be conducted, so this remains an assumption.

Certain aspects of aphid egg development have been elucidated in other species. The conditions required to induce the production of sexual males and females has been established [8,9]. There has also been work on the influence of genetic background and the environment on the duration of egg development in different species and strains [10,11]. The most comprehensive study of aphid egg development, by Behrandt [12], suggests that it constitutes three stages: (i) a temperature dependent period of initial development, (ii) a temperature independent

'diapause' followed by (iii) temperature dependent development. Additionally, development is only completed successfully if the eggs are chilled for at least a short period of time during early development. The embryonic stage upon which diapause is supposed to impinge is, however, unclear. According to Behrandt, diapause occurs at the end of anatrepsis (germband immersion) in *Aphis fabae*. This has been corroborated by the observation that in *Sitobioum graminum* the majority of embryos also pass winter at the end of anatrepsis [13], and the same may be true of *Aphis pomi* in Virginia USA [14]. Other aphids, however, including German clones of *Aphis pomi*, appear to develop constantly throughout the winter, with no definite resting stage [15]. A resting stage is a widely reported characteristic of diapause [1,2,16–18] and so the extent to which diapause occurs generally in aphids is therefore in some doubt.

Here we present a description of the development of sexually-produced pea aphid embryos. We maintained embryos under conditions that mimic those experienced naturally (0–4°C), and tracked their development through sampling and dissection. In aphids, and most other insects, however, there is a strong relationship between temperature and developmental rate [19]. In order, therefore, to determine whether any resting stage we observed at 0–4°C was truly diapause and not a consequence of low rearing temperatures, we also tracked the development of embryos maintained under warmer conditions (10°C and 16°C). Any putative diapause observed at 0–4°C should also be observed at higher temperatures. Further, to determine whether there was a cessation of cell division at any point during development, we immunostained the embryos with anti-Histone H3, which identifies cells undergoing mitosis. Finally, we tested whether putatively diapausing embryos could resume development and hatch earlier than the reported 100 days [8] by transferring embryos from 0–4°C to 16°C at day 49 and tracking their development. We show that under winter-like conditions, development is continuous but slow, and progresses in a temperature independent manner for part of the time. The data indicate that *A. pisum* undergoes a type of diapause, but one that is different from the 'cessation of development' presented as diapause in many other insects.

Results

Embryonic development at 0–4°C
The first 15 days of development at 16°C of a sexually produced *A. pisum* embryo has been described previously by Miura et al. [20]. By the end of this period the embryo has completed anatrepsis and is lying immersed in the yolk. The embryo is fully segmented and the limb buds are clearly defined (figure 2A) and is orientated such that its anterior lies at the posterior of the egg, and its dorsal

side faces the ventral side of the egg. By day 15 the embryo is assumed to have entered diapause, as subsequent chilling does not prohibit successful hatching [8].

At day 16 all the embryos were transferred from a constant 16°C to one of three different temperature regimes: (1) 4°C in the light and 0°C in the dark; (2) constant 10°C; (3) constant 16°C, all with a 13L:11D photoperiod. Figure 3 shows the changes in length of the body and third leg from day 21 to completion of development for all three temperature regimes. We will discuss each treatment separately.

The sexually-produced *A. pisum* embryo lacked a distinct resting stage when maintained at 0–4°C. Although there was no significant change in the length of the embryo from day 21 to 56 (figure 3), there was continued growth of the legs and further differentiation of the organs, most obviously of the gut (not shown). Figure 2 illustrates the development of an embryo maintained at 4°C days and 0°C nights from day 15 – 85. Staining with anti-Histone H3 revealed cell division in most, but not all, of the embryos collected throughout this period (figures 2B,2C &2D). Cell division was detected in a number of embryos from every sample, indicating there was no uniform resting stage at a particular age. After day 56 the embryo increased in body length and between day 63 and 77 it began to undergo katatrepsis (figures 2G,2H,2I,2J), the process by which the embryo re-orientates in the egg. By the beginning of katatrepsis (day 63) the embryos had moved to the posterior of the egg such that the amnion and serosa lie in contact (figure 2G). Subsequent embryological movements suggest that these membranes fuse, and the head of the embryo emerges from the opening to move up the ventral side of the egg as the serosa shrinks. At day 70 the embryo has curled at the posterior of the egg with its heads against the ventral side of the egg (figure 2H). It was not possible to distinguish between the amnion and the serosa once they had fused so we will collectively refer to them as the amnioserosa. By day 77 the embryo had completed katatrepsis and was positioned with its anterior lying at the anterior of the egg and its posterior lying at the posterior of the egg (figures 2I &2J). At this stage the embryo had a cap-like structure at the front of its head, which appears to be the remains of the amnioserosa. Either during or after this process of embryonic reorientation the embryo underwent dorsal closure. It is unclear how this was achieved, but by day 84 it had been completed, as the embryo had deposited an embryonic cuticle (figure 2K). This cuticle was shed upon hatching and possessed a serrated egg burster at the anterior end. Hatching was almost immediate upon transfer to 16°C at day 98. Sixty nine percent (n= 62) of the transferred eggs hatched, and all within 4 days of transfer (mean = 1.29 days, S.D. = 0.67).
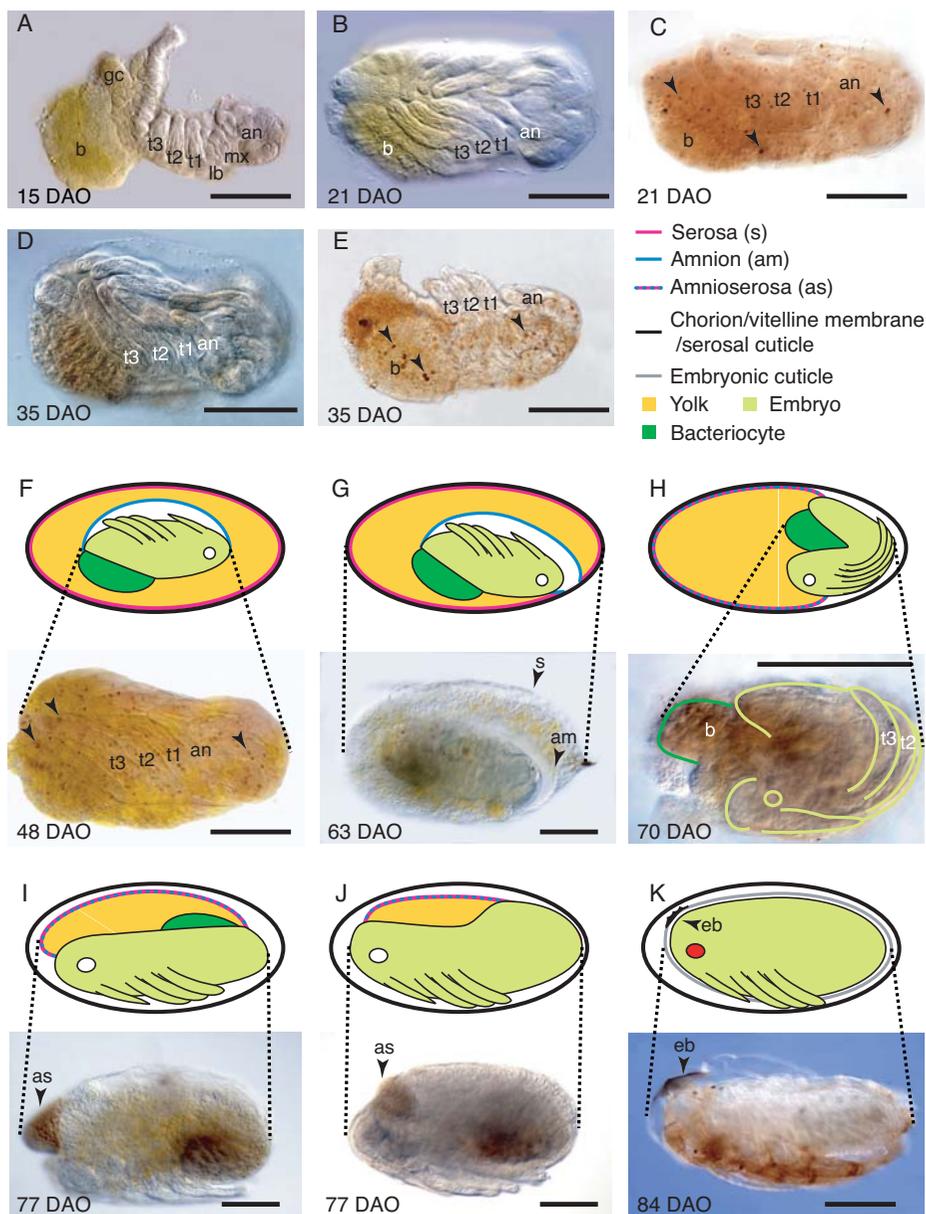
Figure 2
Development the sexually-produced pea aphid embryo, under 'winter-like' conditions. (**A**) At day 15 the antennae (an), mandibles (not visible in this image), maxillae (mx), labium (lb), and thoracic limbs (t1–t3) are visible, as are the germ cells (gc) and bacteriocyte (b). (**B**) By day 21 the embryo has straightened with considerable growth of the appendages. (**C**) Nuclear staining of anti-Histone H3, which can be seen as brown spots (arrowhead) on the embryo, reveals cell division at day 21. (**D**) At day 35 the legs but not the body are longer than at day 21. (**E**) Cell division is also evident at day 35 with anti-Histone H3 staining (arrowhead). (**F**) At day 48 dividing cells stained with anti-Histone H3 are again observed (arrowhead). At this stage the embryo is lying in the centre of the egg. (**G**) At day 63 the embryo begins katatrepsis and has moved to the posterior of the egg, with the amnion (am) in contact with the serosa (s). (**H**) At day 70 the embryo is curled at the posterior of the egg. The amnion and serosa have fused into a single membrane, here called the amnioserosa. (**I**) At day 77 the embryo has completed katatrepsis and has a cap of putative aminioserosa at its anterior end. (**J**) At a slightly later stage another embryo has a reduced "amnioserosal cap". (**K**) At day 84 dorsal closure has been completed and the embryo has deposited an embryonic cuticle, complete with egg burster (eb). All scale bars are 100 μm long. Embryos are orientated as they would be in the egg, with the anterior of the egg to the left.
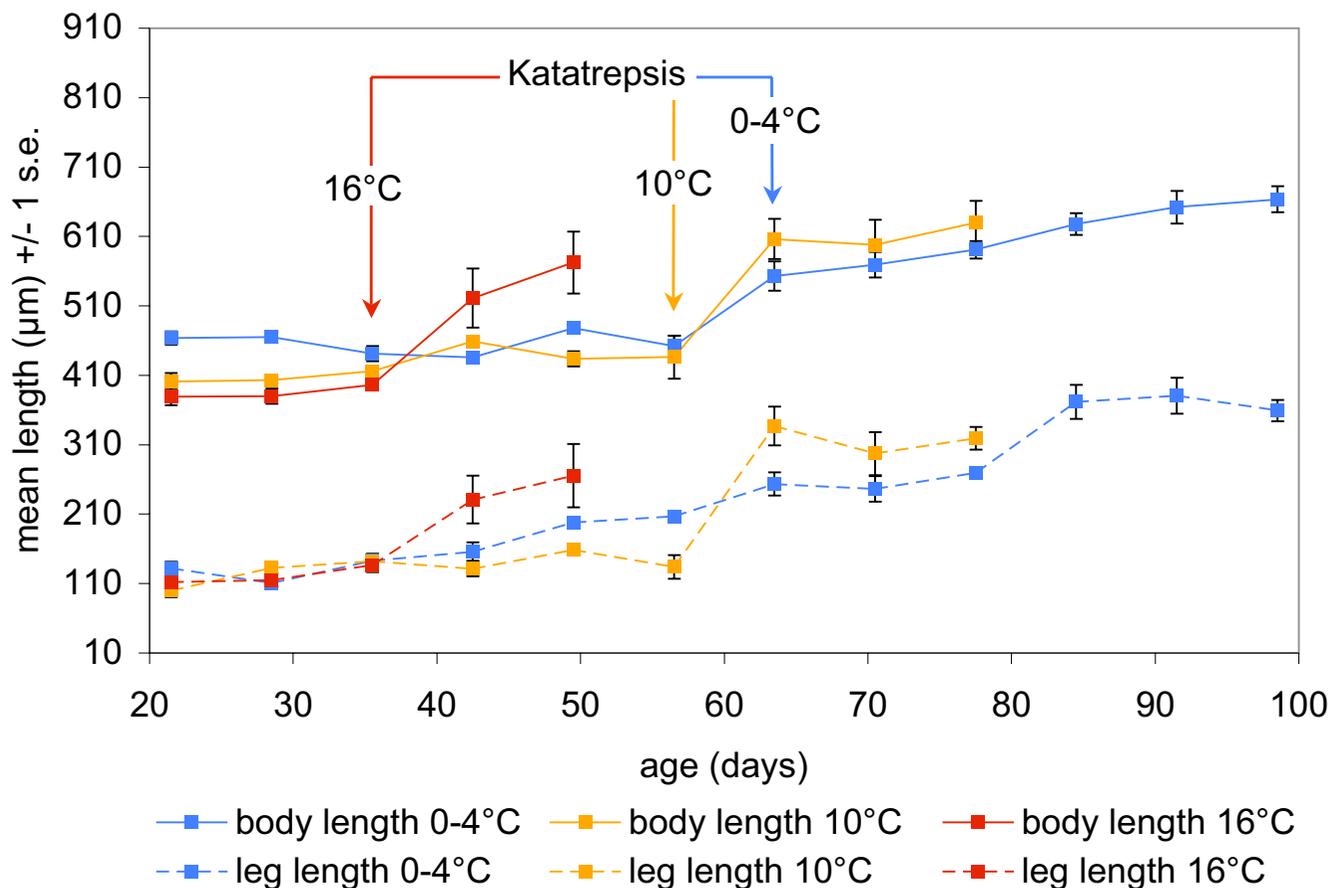
Figure 3
Change in mean length of body and third thoracic leg with age (days) in embryos maintained under different temperature regimes. Arrows indicate the beginning of katatrepsis.

Embryonic development at 10°C
Embryonic development at 10°C suggests that early development of the sexually-produced *A. pisum* embryo, particularly before katatrepsis, is temperature independent. Figure 3 shows the growth of aphid embryos maintained at 10°C. The rate of body and leg growth in embryos prior to katatrepsis was not significantly higher in embryos maintained at 10°C than embryos maintained at 0–4°C (GLM: body *or* leg length = time + temperature + time*temperature: Body; $F_{time*temperature(1, 130)}$ = 0.1357, *p* = 0.7132: Leg; $F_{time*temperature(1, 130)}$ = 3.1184, *p* = 0.0798). Staining with anti-Histone H3 revealed cell division at all ages.

Late development of the sexually-produced *A. pisum* embryo, particularly during and after katatrepsis, appeared to be temperature dependent. Embryos maintained at 10°C underwent katatrepsis earlier than embryos maintain at 0–4°C. Katatrepsis commenced after

day 63 at 0–4°C but after day 56 at 10°C (figure 4). Because the rate of growth prior to katatrepsis was the same at both temperatures, 10°C embryos that undertook early katatrepsis appeared to do so at a less developed stage; that is embryos just prior to katatrepsis had significantly shorter bodies at 10°C than at 0–4°C (figure 5). Nevertheless, embryos that had just completed katatrepsis were the same size at 10°C as at 0–4°C (figure 5). Development was completed by day 77 at 10°C in contrast to day 100 at 0–4°C.

Hatching success in embryos maintained at 10°C was lower than at 0–4°C. Three embryos hatched after 58 days at 10°C, and one more hatched a day later (n = 80). Upon transfer to 16°C on day 78, only 26% of the eggs hatched (n = 31) over a period of 8 days (mean = 3.6 days, S.D. = 3.6).
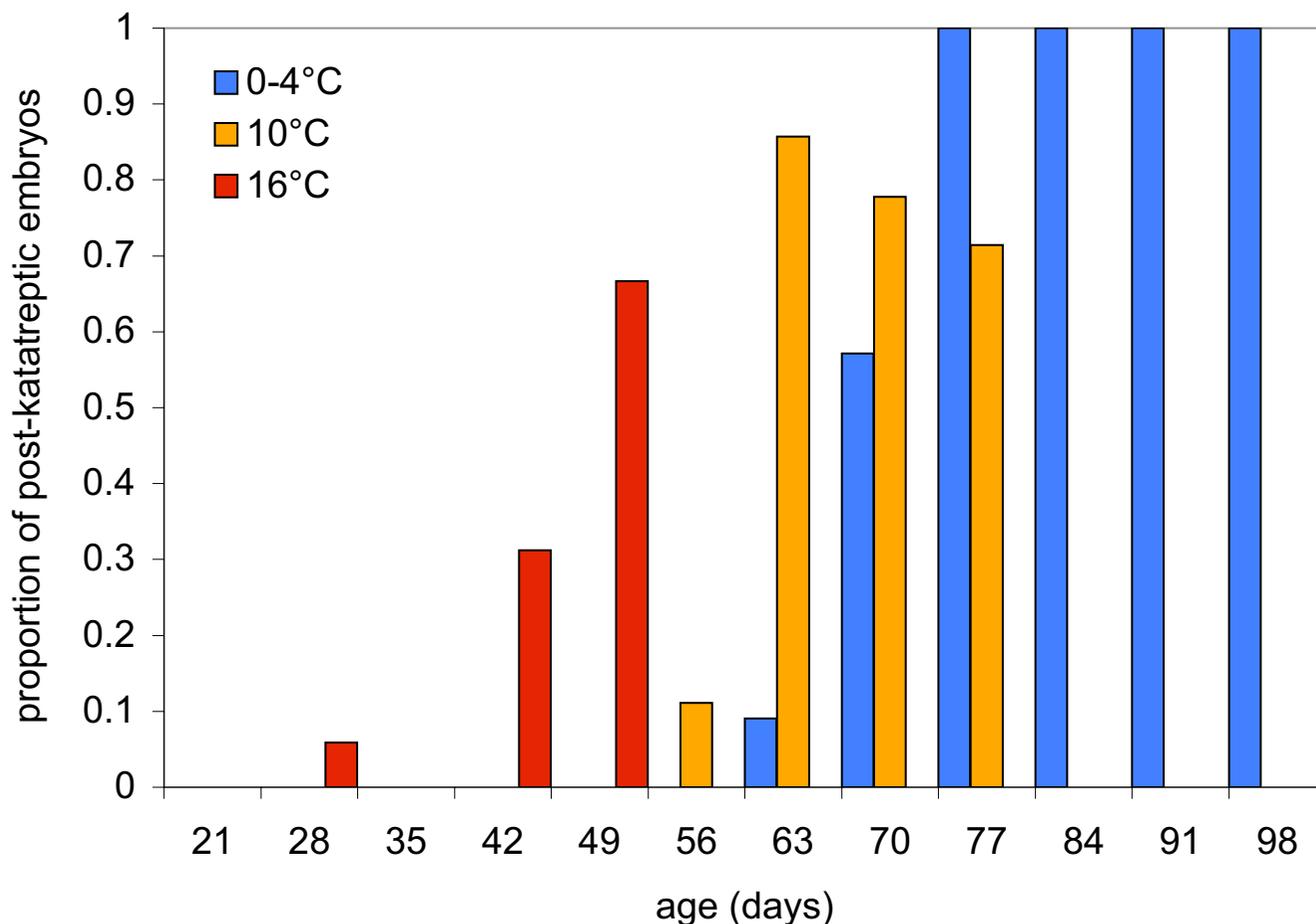
Figure 4
Change in proportion of embryos that have undergone katatrepsis with age (days) in embryos maintained under different temperature regimes.

Embryonic development at 16°C

Embryonic development at 16°C again suggests that early development of the sexually-produced *A. pisum* embryo is temperature independent. Figure 3 shows the developmental progress of aphids maintained at 16°C. Statistical analysis revealed that for those embryos that apparently developed normally, the rate of growth prior to katatrepsis was not significantly higher at 16°C than at 0–4°C for both overall body length and leg length (GLM: body *or* leg length = time + temperature + time*temperature: Body; $F_{time*temperature(1, 122)} = 0.3706$, $p = 0.5438$: Leg; $F_{time*temperature(1, 122)} = 0.1657$, $p < 0.6847$). Embryos maintained at 16°C underwent katatrepsis earlier than those maintained at 0–4°C and 10°C (figure 4). Embryos again appeared to undertake katatrepsis at a less developed stage at 16°C than at 0–4°C; that is embryos just prior to katatrepsis had significantly shorter bodies at 16°C than at 0–4°C (figure 5). Nevertheless, embryos that had just

completed katatrepsis were the same size at 16°C as at 0–4°C (figure 5).

Interpreting these data is, however, problematic, as a high proportion of 16°C embryos showed moderate to severe developmental malformation, making measurement of body and leg length difficult. Malformed embryos were not included in the statistical analysis of growth rates. Figure 6 shows two embryos maintained at 16°C. Typically the embryos showed accelerated development of part but not all of their morphology. In particular many embryos appeared to initiate katatrepsis whilst still under-developed, resulting in embryos with an embryonic cuticle and egg burster, but short or absent limbs. The proportion of malformed embryos increased with time after egg laying. By day 28 10% of sampled embryos showed some abnormality, rising to 90% of embryos sampled on day 49.
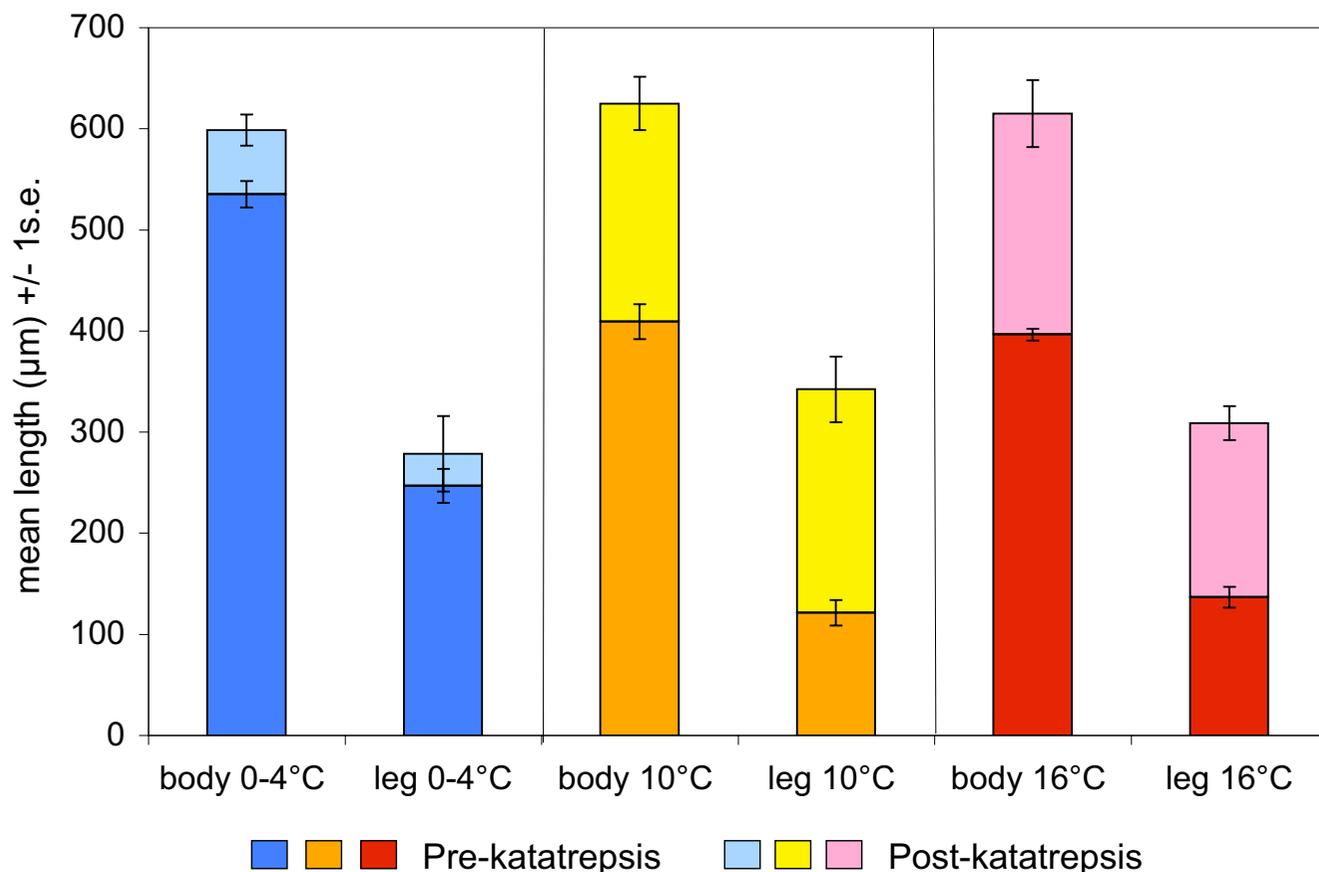
Figure 5
Mean length of body and third thoracic leg of embryos sampled just before and after katatrepsis. Sampling of pre-katatreptic embryos was at day 63 for 0–4°C embryos, day 56 for 10°C embryos and day 35 for 16°C embryos. Sampling of post-katatreptic embryos was at day 70 for 0–4°C embryos, day 63 for 10°C embryos and day 42 for 16°C embryos.

None of the embryos maintained at 16°C either completed development or hatched.

Embryonic development after transfer from 0–4°C to 16°C
Transferring aphids from 0–4°C to 16°C on day 49 resulted in almost immediate katatrepsis followed by a rapid completion of development (figure 7). Aphids began to hatch four days after transfer (mean = 6.8 days, S.D. = 1.4), with 49% of eggs hatching (n = 260).

Discussion
The data suggest that sexually-produced *A. pisum* embryos undergo a period of diapause, although a diapause different to that commonly presented in the literature. Diapause typically involves as a period of developmental arrest during which an insect is resistant to environmental influences on developmental progression [2]. Sexually-produced *A. pisum* embryos do not demonstrate

developmental arrest. Whilst there is little increase in overall body size between anatrepsis and katatrepsis, there is continued growth of the legs and further differentiation of body organs. *A. pisum* embryos do, however, show resistance to environmental influences on developmental progression. Growth rate up to katatrepsis is the same at 0–4°C, 10°C and 16°C. This is in contrast to asexually-produced embryos where growth rate approximately doubles between 5°C and 10°C and between 10°C and 16°C [19]. In sexually-produced embryos the period of temperature independent development, or diapause, ends around katatrepsis. After this point development becomes temperature dependent, with more rapid growth at 10°C than at 0–4°C. Katatrepsis, and the end of diapause, however, occurs earlier in embryos maintained at higher temperatures, (10°C and 16°C) and can be bought forward in embryos maintained at 0–4°C by transferring them to 16°C at day 49.
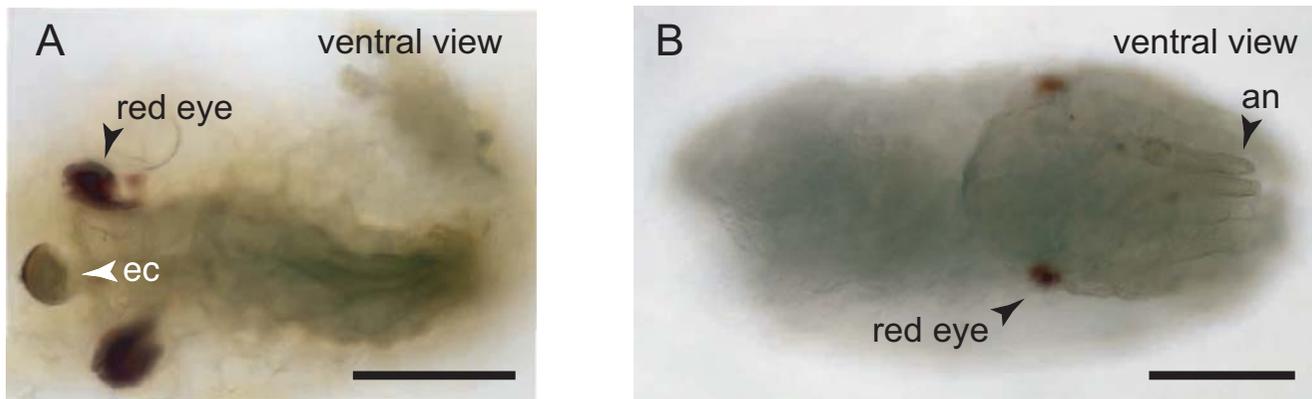
Figure 6
Examples of developmental defects in embryos maintained at 16°C at day 42. (**A**) This embryo has red eyes and an egg burster, both characteristic of embryos about to hatch, but the yolk has not been enclosed through dorsal closure. (**B**) This embryo has red eyes, but is curled at the posterior of the egg, typical of early katatrepsis (see figure 2H). Embryos are orientated as they would be in the egg, with the anterior of the egg to the left. Scale bars are 100 μm long.

At all the temperatures tested there was no cessation of morphological development. Cell division was evident at all ages and, at 0–4°C at least, there was continued growth of the legs. This appears to be somewhat contrary to the traditional understanding of diapause, which typically involves a distinct 'resting stage' [1,21]. Much research has revealed, however, that even during the 'resting stage' there are physiological changes in the diapausing insect, leading to the concept of 'diapause development' [22,23]. Nevertheless, such development appears to be primarily physiological rather than morphological, and was defined as physiogenesis by Andrewartha (1952). It is possible that the morphological development observed in diapausing *A. pisum* is exceptional. Alternatively, diapause in many other insects may also involve a slowing but not a cessation of morphological development. It is difficult to test this notion with the current literature as timed dissections of diapausing embryos, larvae and pupae have rarely been conducted. There are, however, a few examples of continued morphological development during diapause. Diapausing embryos of the orthopteran *Austroicetes cruciata* undergo slow development for the first two months of diapause, before entering a resting stage [24]. Similarly, caterpillars of *Cirphus unipunctata* and *Laphygma exigua* continue to feed and develop during diapause, albeit at a much-reduced rate [25], and caterpillars of the corn-stalk borer *Sesamia nonagriodes* undergo non-stationary moults during diapause [26]. These examples hint that the expression of diapause-controlled dormancy may vary between species [2]. Consequently, diapause could be considered extreme regulation of developmental rate, rather than a shutting down of morphological development. The mech-

anisms that control diapause in *A. pisum* may therefore be the same as those that control the rate of morphological development at other stages of development.

Several insects that diapause over winter are resistant to environmental conditions conducive to growth only at the beginning of winter [27–31]. This prevents premature emergence, be it from egg, larvae or pupae, just at the onset of the cold temperatures the insect is trying to avoid. Once winter has commenced, diapause ends and the insect enters post-diapause quiescence, relying on persistent low temperatures to maintain developmental arrest [27]. This appears to be the case in *A. pisum*. The aphid clone used for the experiment would normally oviposit in mid-October, when the average daily temperature is 9°C with a maximum of 15°C [32]. By day 28 the embryos would be exposed to average daily temperatures of 3°C. Consequently, whilst *A. pisum* maintained at 0–4°C had regained temperature-dependent growth by day 49 (figure 7), it would not normally be exposed to warmer conditions until the following spring, approximately 150 days later. By this time the aphid would be fully developed and ready to hatch. In embryos that remained at 0–4°C until day 98, there was no obvious difference in development rate during and after diapause, and this is consistent with observations in other insects [1,27]. There must, however, be some change in condition to move development from being temperature independent to temperature dependent [1]. This change appears to be at or around katatrepsis. Identifying what this change is, and what, if anything, it has to do with katatrepsis, will be key to understanding

how the pea aphid controls development during diapause.

The finding that *A. pisum* embryos maintained at 16°C showed considerable malformation may be a consequence of surpassing the normal thermal limits of developmental regulation. The genetic pathways involved in the development of *A. pisum* embryos must build an animal under two conditions. The first is slow, temperature independent development at low ambient temperatures in sexually-produced embryos, and the second is rapid temperature dependent development at higher ambient temperatures in asexually-produced embryos. Presumably the same pathways are involved in both processes – it would be surprising if *A. pisum* were to have different sets of genes for the two modes of development. Diapause may therefore act to limit the speed of reactions that would otherwise occur more rapidly at higher temperatures, possibly through the action of an 'inhibitory factor' [22,33]. According to this model, the higher the ambient temperature the greater the required activity of the inhibitory factor [33]. When temperatures are maintained at abnormally high temperatures (16°C), regulation may be compromised with only some aspects of development slowed. This may result in embryos displaying a mosaic of developmental stages.

Alternatively, the malformed phenotypes seen at 16°C may result form differing levels of temperature independence for different developmental processes. There would be no selection on embryos to make later developmental processes temperature independent, as they normally occur during winter. At unnaturally high temperatures, for example 16°C, these later, temperature dependent processes, may occur prematurely, resulting in embryos displaying a mosaic of developmental stages. This hypothesis suggests that the timing of some developmental processes occurs independently, i.e. that the beginning of one developmental process is not contingent on the ending of another. This appears to be the case for katatrepsis, which occurs in smaller embryos at higher temperatures. Premature katatrepsis did not, in itself, cause the malformations. Embryos transferred to 16°C from 0–4°C on day 49 underwent early katatrepsis but displayed a high hatching success.

It is perhaps surprising that many embryos should develop abnormally at 16°C given that they would encounter temperatures as high, or higher, during winter in their natural environment. However, such high temperatures would be experienced only temporarily, and it may be persistent exposure to high temperatures that caused the developmental abnormalities in our experiment. Further research is therefore necessary to determine the influence of fluctuating temperatures on developmental
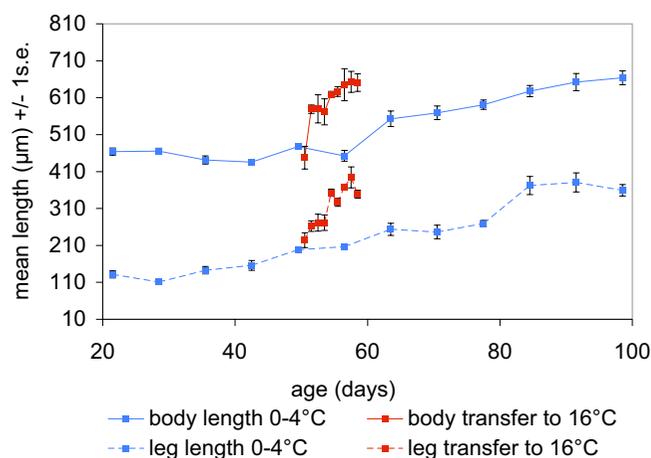

Figure 7
Change in mean length of body and third thoracic leg with age (days) in embryos maintained at 0–4°C and then transferred from 0–4°C to 16°C at day 49.

progression, both with respect to developmental abnormalities and developmental rate.

There has been little progress towards identifying the genetic control of diapause in insects. The one system where diapause has been well elucidated, dauer formation in the nematode *C. elegans*, indicates that the insulin pathway plays an important role [34]. Recent work on mutant *Drosophila* that mimic reproductive diapause in adults also suggests a role for insulin [35,36]. Insulin, which regulates growth in other stages of development in *Drosophila* [37,38], may therefore play a similar role during *A. pisum* diapause. Work on diapause in the flesh fly *Sarcophaga crassipalpis* has identified a second class of genes that may be involved in diapause regulation [39]. These are the heat shock proteins, which are normally expressed during times of stress [40] and which provide cryptoprotection during diapause. Since heat-shock proteins are also involved in cell cycle arrest, however, they may play a more central role in the regulation of diapause [1]. Heat-shock proteins are also typically heat inducible, and so fit with the concept of a temperature dependent 'inhibitory factor' [33]. Additionally, high levels of heat-shock proteins may disrupt development [41], which could help explain the developmental defects observed in embryos maintained at 16°C. Further work on the expression of both insulin and heat shock proteins in *A. pisum* embryos will clarify their respective roles.

Conclusion
Diapause in the pea aphid involves a period of slow growth, the progression of which is temperature inde-

pendent. There is no distinct resting stage, which is characteristic of diapause in many insects. Diapause in the pea aphid represents an extreme regulation of developmental rate and may be controlled by the same mechanisms that control developmental rate at other life stages.

Methods

Egg material

We produced sexually reproducing male and female aphids by exposing asexual females from a single clone (LSR1, collected near Ithaca, NY) to 'autumnal' conditions (short-day illumination, 13L:11D, 16°C) for five weeks. Sexuals were collected and placed at a ratio of two males to eight females in 16 mm diameter petri dishes holding single leaves of *Medicago arborea* inserted into 3 ml of 2% agar with 1gL^{-1} of Miracle-Gro (The Scotts Company, OH). Every day we collected any eggs deposited on the leaves with a soft, moist paintbrush and transferred them to dampened filter paper placed in the lid of a 16 mm diameter petri dish. Each cohort of eggs therefore contained eggs laid over a 24-hour period. The eggs were then kept at 16°C 13L:11D for a further 14 days. On day 16 we sterilised the eggs by immersing them in 1% formaldehyde for 10 minutes and then washing them three times in double-distilled water. Sterilisation and washing took place in a Buchner funnel, with liquids being passed through the filter paper on which the eggs rested. The eggs remained on their original filter paper throughout, which, while still moist, was transferred to a fresh sterile petri dish. We then sealed the dishes with parafilm (United American Can, WI) and placed them in incubators (Model: I-30BLL, Percival Scientific, Inc., IA) for storage.

Egg storage

To determine the influence of temperature on the progression of development we stored the eggs under three temperature regimes, all with the same 13L:11D photoperiod: (1) 4°C in the light and 0°C in the dark; (2) constant 10°C; (3) constant 16°C. The first temperature regime most closely mimics natural conditions and has been previously used to successfully rear eggs to hatching [8]. Under each temperature regime, eggs were sampled for dissection every seven days, starting on day 21. Preliminary experiments indicated that eggs maintained at 0–4°C hatched successfully when transferred to 16°C when approximately 100 days old. Consequently, eggs kept at 0–4°C were transferred to 16°C on day 98 for hatching. Eggs kept at 10°C were transferred to 16°C on day 78 for hatching. Additionally, a subset of eggs maintained at 0–4°C were transferred to 16°C on day 49 and sampled daily from day 50 to 59.

Egg fixing, dissection, and staining

Eggs were dechorionated in 50% bleach for 2 minutes. They were then transferred to a 0.5 mL eppendorf tube

containing 4% paraformaldehyde in PBS:heptane (1:1) and agitated for 45 minutes. The paraformaldehyde was then replaced with methanol and the tube was shaken to pop the vitelline membrane. The embryos were then transferred to absolute methanol and stored at -20°C until dissection. Dissection began with the removal of the black serosal cutical by sticking the eggs to a microscope slide with double-sided sticky tape, covering with PBS, piercing the serosal cuticle and pushing out the embryo and yolk with a hypodermic needle. The embryos were then separated from the yolk using pulled glass-needles, again under PBS. In order to detect cell division during putative diapause, we immunostained the embryos with anti-phospho(S10)-acetyl(K14)-Histone H3 (anti-Histone H3) (rabbit immunoaffinity purified IgG, Upstate Group, Inc., MA). This anti-body recognizes Histone H3 phosphorylated at serine 10 and acetylated at lysine 14, which occurs only during mitotic cell division. Fixed embryos were washed for 30 minutes in 0.3% Triton-X 100 in PBS (PBT), and then blocked for 30 minutes with 2% Normal Goat Serum (NGS)/0.2% Bovine Serum Albumin (BSA) in PBT (BBT/NGS). Embryos were incubated for at least 2 hours at room temperature or overnight at 4°C with the primary antibody in BBT/NGS, and then washed four times in BBT. Embryos were blocked with BBT/NGS for 30 minutes prior to incubation with the biotin-conjugated secondary antibodies in BBT/NGS for at least 2 hours at room temperature or overnight at 4°C. Embryos were washed four times before being developed using the Vectastain ABC kit Elite (Vector, CA) and diaminobenzidine (Sigma Fast DAB). The embryos were subsequently mounted in 70% glycerol in PBS and their image digitized.

Embryo size measurement

The size of embryos was determined from their digitized image using the software IPlab (Scanalytics, Inc. VA). We measured both the total length of the embryo and the length of the third leg. Where the embryo was curled, the length was estimated as the sum of several linear measurements approximating the curvature of the embryo.

Statistical analysis

To determine whether the growth rate was influenced by temperature we plotted body and leg length against age in days under each of the different temperature regimes. The gradient of the line is the rate of growth. We statistically tested whether temperature influenced the rate of growth using the model:

body *or* leg length = $a*$age + $b*$temperature + $c*$(temperature$*$age) + k

We treated time as a continuous variable and rearing temperature as a categorical variable. We fitted this model to

the data using a generalized linear model (GLM) with the program JMP 4.04 (SAS Institute, Inc., NC). This analysis gives estimates of the coefficients *a*, *b*, *c* and *k*, and tests whether they are significantly different from zero. The coefficient *a* gives the slope of the relationship between length and age and is the rate of growth. The coefficient *b* gives the relationship between temperature and length and indicates if embryos reared at a different temperatures are longer or shorter. The coefficient *c* indicates whether there is an interaction between the affects of temperature and age on length. If this interaction term is significantly different from zero it indicates that the slope of the relationship between length and age (rate of development) varies with temperature. All the data were log transformed prior to analysis to uphold assumptions of normality of error and homogeneity of variance.

Authors' Contribution

AWS designed the experiments, participated in sampling, mounting and measuring embryos, conducted the statistical analysis, and prepared the manuscript. GCS assisted in sampling, mounting and measuring embryos. DLS assisted with the design and coordination of the study.

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