Zygote **13** (May), pp. 139–144. © 2005 Cambridge University Press
doi:10.1017/S0967199405003163 Printed in the United Kingdom

Visualization of early embryos of the butterfly *Bicyclus anynana*

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Date submitted: 08.10.04. Date accepted: 25.01.05

Summary

We report on the first attempts, using both light and fluorescence microscopy, to visualize the developing embryo of the butterfly *Bicyclus anynana*. We developed a new protocol that enabled the clear visualization of the internal egg structures in early embryogenesis (1–24 h after egg laying). Dechorionation was followed by fixation and physical dissection of the external egg structures. Observations of embryonic and extra-embryonic cells were made using a Hoechst nuclear stain that fluoresces in the blue spectrum when bound to DNA and excited with ultraviolet (UV) light under a fluorescence microscope. Preliminary data on the developmental rate of the early embryo are also presented.

Keywords: *Bicyclus anynana*, Development, Embryo, Embryogenesis, Lepidoptera

Introduction

In order to study the embryonic development of insects, it is important to be able to see the developing embryo within the egg. Data that can be obtained from these studies are relevant for comparative insect embryogenesis and to our understanding of insect body plan evolution. In many insect species the outer egg shell, or chorion, is opaque or pigmented and must be removed in order for the inner contents to be seen. These, in turn, must be fixed in order to preserve their structure and relative positions. In the moth *Bombyx mori*, fixation with Carnoy's fixative, an alcohol-based solution, followed by physical dechorionation with surgical instruments, lead to successful embryo visualizations (Nakao, 1999). In the butterfly *Precis coenia*, similar success was achieved with chemical removal of the chorion using a detergent (Nijhout & Emlen, 1998).

The purpose of the current research was to determine the relative position of the developing *Bicyclus anynana* butterfly embryo within the egg using nuclear stainings. The location of the embryo within the egg, and its relative development rate, are important for research involving *B. anynana* transgenic manipulations (Marcus *et al*., 2004), as it will allow better targeting of injections of plasmids containing transgenes into the germ cells of developing embryos.

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Materials and methods

Animals

B. anynana were reared inside a temperature- and humidity-controlled insectary at 28 °C, 85% humidity, and under a 12:12 h light:dark cycle. Adults were fed mashed banana and kept in 25 cm diameter and 40 cm tall cylindrical hanging cages at a density of 50–100 individuals per cage. Young maize plants (~15 cm tall) were placed inside these population cages for 1 h periods for egg collections. Thus, the absolute age after egg laying (AEL) for the experiments described here will vary from the time mentioned up to 1 h afterwards.

Hoechst staining protocol

After collection, the eggs were transferred into a small Petri dish and returned to the insectary for a variable period of 1–24 h. After the desired development time the eggs were immersed in 10 ml Carnoy's fixative (Table 1) and fixed for 2 h. Subsequently, the eggs were carefully removed from the Petri dish with curved dissection forceps, and placed in a 75% ethanol/25% ddH_2O solution for 1 h. Most of the ethanol solution was then decanted out of the Petri dish, leaving enough to keep the eggs moistened but not submerged. The chorion and vitelline membranes were removed with

Table 1 Components of Carnoy's fixative and volumes used

Component	% of total volume	Millilitres used
Acetic acid (glacial)	10	1
Chloroform	30	3
Ethanol (200 proof)	60	6

fine tungsten needles (Word Precision Instruments, cat. #500134) under a Nikon dissection microscope, and the dechorionated eggs were then transferred to a 75% ethanol/25% phosphate-buffered saline (PBS) (10 ml) solution contained in another Petri dish. The eggs were easily moved, as they adhered to the side of the needle. After 30 min the eggs were transferred to a 50% ethanol/50% PBS solution with a 200 µl pipette using wide-tip 'cell saver' tips. The previous step was repeated with 25% ethanol/75% PBS and 100% PBS solutions within 30 min intervals. Longer rehydration steps were somewhat more effective, and overnight rehydration in 100% PBS in the refrigerator was also beneficial. The eggs were then transferred, again with the cell saver tip, into small, glass-bottomed microscope Petri dishes with minimal PBS solution. Several drops of Hoechst 33258 staining dye (Molecular Probes), in a concentration of 1 mg powdered dye to 1 ml of ddH_2O, were added with a Pasteur pipette. The Petri dishes and Hoechst dye were immediately wrapped in foil to decrease photodegradation and stored at 4 °C until ready for viewing. The Hoechst dye when complexed with DNA emits blue light with a peak at 492 nm when excited with ultraviolet (UV) light with a peak at 356 nm (Sambrook *et al*., 2001). Eggs were observed under a Leica DM IRE HC inverted compound fluorescence microscope using a UV DAPI filter.

Microinjection techniques

Eggs were collected as above, placed inside a small Petri dish, and incubated from 1 to 48 h inside the insectary. Strips of double-sided adhesive tape (approximately 40 mm × 4 mm) were attached to glass slides. Eggs were anchored to the strips 3 mm apart and injected with the Hoechst staining solution until they plumped up slightly. The injections were done using a hand-held pulled glass needle attached to a Picospritzer III microinjection apparatus, under a dissection microscope. Eggs were then viewed under the fluorescence microscope.

Non-invasive techniques

Eggs were collected and incubated as above (1–48 h incubation times), then treated with acetic acid in aqueous concentrations ranging from 10% to 50% and treatment durations ranging from 5 min to 12 h. The eggs were then stained in the Hoechst solution and viewed under the fluorescence microscope. This procedure was repeated with identical concentrations and durations using sodium hydroxide solutions instead of acetic acid. Heptane-containing solutions with identical concentrations to those of the acetic acid and sodium hydroxide treatments were utilized with the same protocol as above. Glycerol was added to 50% of the eggs prior to staining in an attempt to make the chorion more translucent. In addition, some series of eggs were treated with 6.5% carbamide peroxide (an organic wax solvent) alone or following the various acid and sodium hydroxide solutions and prior to staining with Hoechst dye.

Chemical chorion removal

B. anynana eggs were collected and incubated as above (1–48 h incubation times), then treated with diluted aqueous bleach solutions (with concentrations ranging from 5% to 50% and times ranging from 30 s to 45 min) utilizing two separate immersion techniques. The first was uninterrupted immersion in the bleach. The second involved placing the eggs on a screen and immersing them in bleach for 5 s, then immersing them in ddH_2O for 5 s and repeating. Eggs were then stained with Hoechst dye and observed.

Results

Bicyclus anynana embryonic development is around 4–5 days at 28 °C and 80% relative humidity. Depending on the batch of eggs collected, 24–76% of the eggs emerge on day 4, whereas the rest emerge on day 5 (day 0 is the day of oviposition). For the purpose of relative staging and comparison with other species, we can say that 1 h of embryonic development corresponds approximately to 1% of total embryogenesis.

The chorion of the egg contains two reference points that aid in the orientation of the otherwise spherical egg. These are the micropyle (a small hole where sperm cells enter the egg just prior to oviposition; Fig. 1A) and the silk pad, a slightly flattened area where silk is deposited to help glue the egg onto the plant; Fig. 1B). Being able to visualize the inside of the egg while keeping the chorion intact would therefore be ideal. This, however, was difficult to accomplish. The semi-translucent chorion allows the clear visualization of embryos in their last stages of development (Fig. 1C), but earlier non-pigmented stages are more difficult to distinguish. Embryos can sometimes be seen when the eggs are lit from below while submerged in glycerol. In these conditions a lighter,

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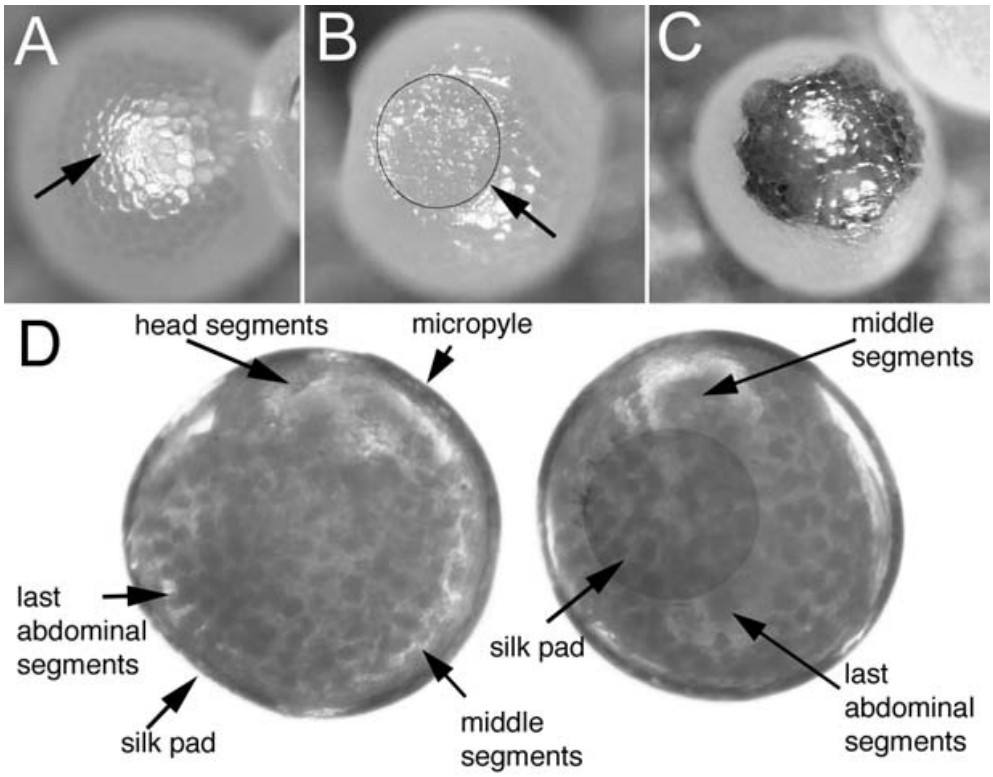

Figure 1 Light microscopy of *B. anynana* eggs. Egg diameter is approximately 1 mm. (*A*) Surface of the egg chorion showing the position of the micropyle. (*B*) The silk pad, a patch of silk on the attachment point of the egg to the plant. (*C*) The end of embryogenesis, with a first instar larva showing a melanized head capsule and about to eclose. (*D*) Eggs emerged in glycerol and lit from below showing the developing embryo in a lateral view (left) and dorsal view (right) and its position relative to the micropyle and silk pad.

translucent mass of cells becomes visible adjacent to the chorion and surrounded by the darker yolk (Fig. 1D).

Under UV light, however, and following microinjection of Hoechst 33258 into the egg, the stained nuclei were not visible through the chorion. The microinjection itself caused damage to the contents of the egg. Cellular cytoplasm leaked through the puncture and the force of the injection was likely to have altered the positioning of the cells inside, especially in very early embryos.

Attempts to make the chorion more transparent and/or permeable to the aqueous Hoechst dye were also unsuccessful. Treatments of various durations and concentrations of acetic acid and sodium hydroxide to alter the pH and structure of the chorion yielded either no staining results or destroyed the entire egg. Despite some success with permeabilization of the *Drosophila melanogaster* egg using alkanes (Mazur *et al*., 1992), heptane solution treatments had no effect on the *B. anynana* chorion. Treatments using carbamide peroxide (an organic wax solvent) were used to remove the wax coating that renders the chorion impermeable, but these were unsuccessful as well. Combinations of the aforementioned treatments, as well as variations of staining time (by soaking in Hoechst dye) and addition of glycerol were all unsuccessful. We concluded that the chorion must be removed in order to allow us to view the nuclei inside the egg.

We attempted chemical removal of the chorion using bleach either by soaking or repeatedly immersing the eggs in and out of solution. In some cases the chorion was successfully dissolved, andthe stained nuclei were visible. The success rate for observable nuclei was approximately 3%, but there was considerable physical distortion of the eggs in all cases, making it impossible to determine the relative position of the embryo. The remaining, non-stained eggs were either destroyed by the bleach or not stained at all (implying that the chorion had not been removed).

Physical removal of the chorion in an iso-osmotic PBS solution, effective in *Bombyx mori* chorion removal (Hajime Nakao, personal communication), led to the successful observation of the stained nuclei inside the egg. In the initial trials, the vitelline membrane appeared to adhere to the chorion and ruptured when the chorion was split with the tungsten needles. Subsequently, Carnoy's fixative (Table 1) was used prior to

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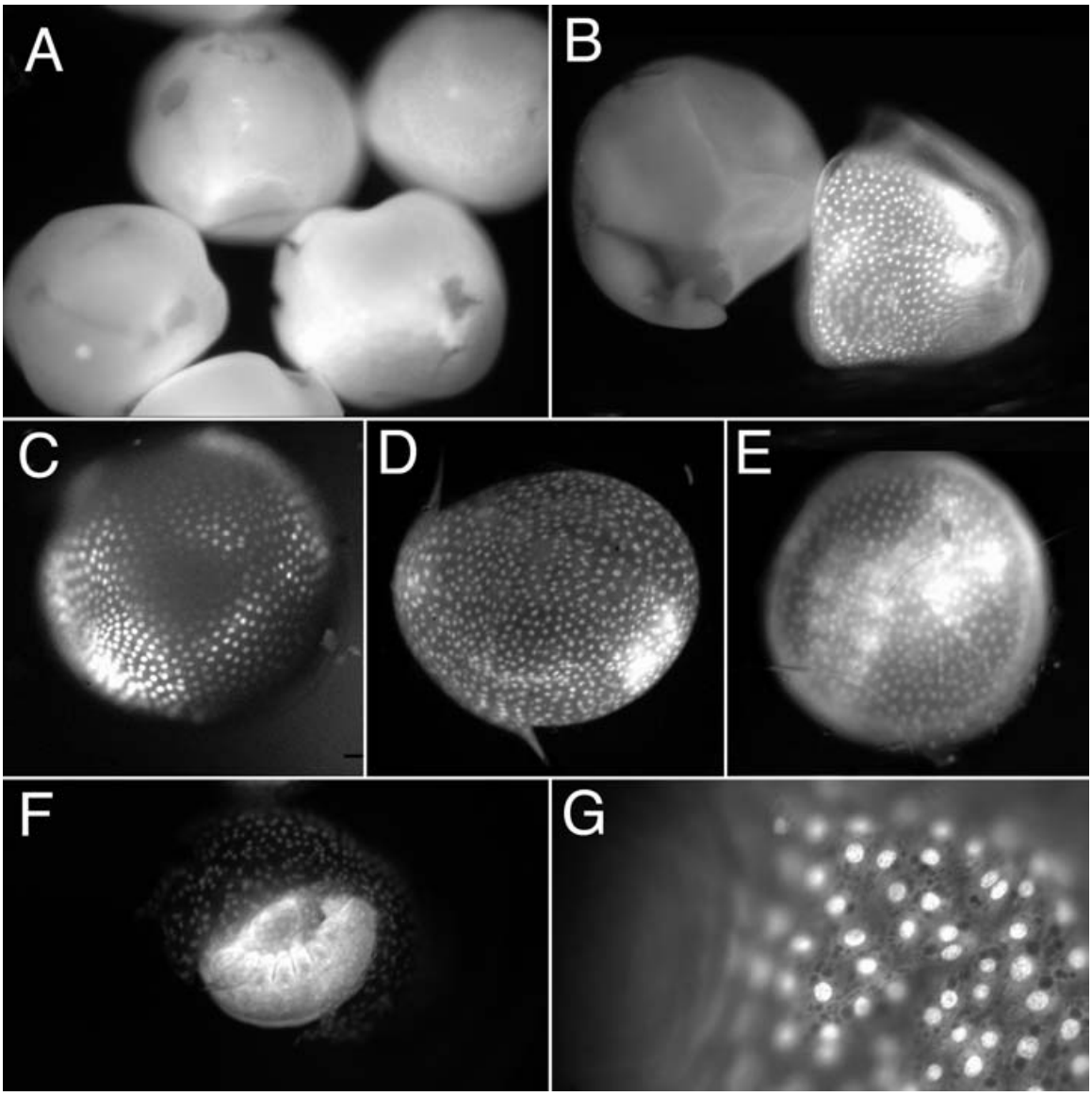

Figure 2 Fluorescence microscopy of *B. anynana* dechorionated embryos stained with Hoechst 33258 and viewed under UV light, ×100 magnification. (*A*) Dechorionated eggs showing underlying opaque vitelline membrane. (*B*) Partially removed vitelline membrane of egg on the right-hand side, showing the underlying blastoderm nuclei. (*C*) Egg at 1 h after egg laying (AEL) and *(D)* at 4 h AEL showing many blastoderm nuclei and a putative condensation of nuclei at the bottom to form the germ anlage. (*E*) At 24 h AEL a fairly well developed embryo, with clear segments, is seen. The embryo is surrounded by mononucleated serosal cells. (*F*) Also at 24 h AEL, a different embryo already shows some well-developed appendages in most of its segments. (*G*) Detail of the matrix-like structure surrounding the blastoderm nuclei, 1 hour AEL at × 200 magnification.

the dissections to toughen the vitelline membrane and detach it from the overlying chorion.

Once the chorion is removed via physical means, the vitelline membrane becomes visible. This is a very fragile membrane that is susceptible to osmotic changes which can greatly alter its size, shape and texture. The vitelline membrane is initially translucent and permeable which allows for both the absorption of the aqueous staining dye and the viewing of the stained nuclei under UV light. We have observed, however, that after fixation the membrane proteins appear to become cross-linked making the membrane opaque (Fig. 2A).

Chemical removal of the fixed and opaque vitelline membrane was attempted with acetic acid and heptane. The repeated immersion technique was used (as in the bleach treatments described previously) with various total duration times of heptane and acetic acid. The concentration of acetic acid was also varied. The heptane had no effect in removing the membrane, implying that the vitelline membrane may not be made up of non-polar lipids. The acetic acid immersions were

https://doi.org/10.1017/S0967199405003163 Published online by Cambridge University Press

very minimally successful, with most of the eggs being partially degraded or destroyed. With practice, however, it was possible to remove the vitelline membrane through strictly physical means, although the overall success rate was low. Embryos in various stages of development were then visible (Fig. 2B–F).

Condensations of the blastoderm nuclei to form the embryo proper appear within 4 h AEL (Fig. 2C, D). At 24 h AEL (24% of embryogenesis) a more developed embryo is already apparent. Although the early germ band was not visible in most of the photographs, with one apparent exception (Fig. 2D), some important observations could be made. First, segmentation and limb development can be seen clearly (Fig. 2D, E). In addition, eggs up to 4 h AEL have a meshwork or matrix of some sort within the cytoplasm that is not visible in more mature eggs (Fig. 2G). We could not observe any other nuclei beyond those directly adjacent to this matrix, either because all blastoderm nuclei are at the periphery of the egg at this developmental stage, or because this meshwork prevents visualization beyond the periphery of the egg. It is well known from other insects, however, that nuclei from the first few zygotic divisions will migrate to the periphery of the egg and form a syncytial preblastoderm (Heming, 2003).

Discussion

Due to the opaque and impermeable nature of the *B. anynana* chorion, it is necessary to remove it to allow any type of observation of the cells within the egg. Microinjection of Hoechst stain into the eggs does not enable us to visualize the nuclei inside. Attempts to make the chorion more permeable or translucent through chemical means were unsuccessful. Chemical manipulations to remove the chorion with bleach and acetic acid resulted in distortion of the egg. In many cases, despite successful chorion removal, the chemicals severely damaged or destroyed the much more sensitive underlying structures. It seems that these methods are too unreliable and destructive to warrant further investigation.

Fixation of the internal egg structures (vitelline membrane, cytoplasm, proteins, etc.) is necessary to allow physical removal of the chorion. This can be accomplished consistently with Carnoy's fixative, although this fixation treatment results in some egg shrinkage due to dehydration of the egg, presumably because of the ethanol contained in the fixative. The eggs can be effectively returned to their original shape by ethanol/PBS rehydration steps, with only minimal shape distortion. Avoiding distortions is important for determining the position of the embryonic cells relative to landmarks on the egg. The dechorionated, membraneless egg does not retain any landmarks such as those present on the chorion, i.e. the silk pad or micropyle. We have found, however, that if a small incision in the chorion is made prior to submerging the eggs in Carnoy's fixative it leaves a mark on the internal structures that can be seen after fixation. It may be possible, then, to make an incision into the egg through the micropyle or silk pad before fixation and later use this mark as a reference point.

The early eggs, at roughly 1% of their total gestational time, seem to contain a single cell layer around the periphery of the egg. This could be the blastoderm layer, as found in *D. melanogaster* and other insect species (Heming, 2003). The matrix observed at 1–4 h AEL may correspond to the partial staining of the cell membranes of the blastoderm. In future studies, it may be useful to include a cell membrane stain to determine whether the peripheral nuclei observed at 1–4 h AEL are in the syncytial stage or whether a cytoplasmic membrane already surrounds them. In the hawkmoth species *Manduca sexta*, however, the formation of the species' cellular blastoderm only occurs at 5% of its total gestational age (Broadie *et al.*, 1991).

Taking into account the current insect embryology literature, there are two main types of embryo: those with long- and short-germ bands. Characteristics of long-germ embryos include meroistic (nurse cell) organogenesis, relatively short duration of embryogenesis, the inability to regulate embryogenesis in response to environmental perturbation, and a large germ band within the egg. It has been observed that derived lepidopteran species, including other butterfly species (Nagy, 1995), follow this development paradigm. More primitive lepidopteran species have panoistic (no nurse cells) organogenesis, short germ bands, and a longer duration of embryogenesis with the accompanying ability to respond favorably to environmental changes during embryogenesis. It is believed that the fate of each cell in the germ band is determined later in the development of short-germ embryos, allowing for adjustments in cell fates (Nagy, 1995). In addition, the serosal cells that remain from the early blastoderm, after the germ disc has differentiated, are many times multinucleated. In contrast, the long-germ species tend to have mononucleated serosal cells that surround the early embryo (Wall, 1973; Kobayashi & Ando, 1982, 1884). In general, the germ rudiment of a short-germ, basal Lepidopteran species is formed by the deep invagination of the small germ disc into the yolk, in a U shape (Ando & Tanaka, 1980). In the more derived Lepidopteran lineages, such as Monotrysia and Ditrysia, the germ disc is cut off along the margin of the serosal area, then sinks into the yolk. It later coils as the embryo develops (Kobayashi & Gibbs, 1995). Overall developmental duration varies greatly from species to

species, and does not appear to follow any evolutionary trend, but rather is dependent on the environmental challenges facing each particular species.

From our limited data it appears that *B. anynana* conforms to a long-germ band insect. Embryogenesis occurs quite rapidly, as the earliest pictures, 1 h AEL, show a fully formed blastoderm (Fig. 2B). There is also some visual evidence to imply that the (segmented) germ band itself is relatively long (Fig. 2E). The twisted form observed in these later (24 h AEL) embryos is consistent with the shape of embryos of the other higher-order lepidopteran species (Wall, 1973; Tanaka *et al*., 1983; Tanaka, 1987). In addition, the serosal cells appear to be mononucleated.

In conclusion, the current protocol developed here allows us to visualize embryos from 1 h to 24 h after egg laying, and probably at later developmental stages as well. Due to the variable development time observed in *B. anynana* embryos (between 4 and 5 days) the precision with which we are able to estimate the absolute gestational time of each embryo is limited. Embryos of similar age (Fig. 2E, F) can show marked differences in their development. As more data are collected, at smaller gestational time intervals, we may be able to describe more accurately the levels of variability for each important developmental stage. The protocol developed here will prove useful in this endeavour and will enable us to investigate further the embryogenesis of *B. anynana*.

Acknowledgements

This work was supported by NSF award #29339 to A.M. We thank William Piel for reading and revising the manuscript.

References

Ando, H. & Tanaka, M. (1980). Early embryonic development of the primitive moths, *Endoclyta signifer* Walker and *E. excrescens* Butler (Lepidoptera, Hepialidae). *Int. J. Insect Morphol.* **9**, 67–77.

Broadie, K.S., Bate, M. & Tublitz, N.J. (1991). Quantitative staging of embryonic development of the Tobacco Hawkmoth, *Manduca sexta*. *Roux Arch. Dev. Biol.* **199**, 327–34.

Heming, B.S. (2003). *Insect Development and Evolution*. Ithaca: Cornell University Press.

Kobayashi, Y. & Ando, H. (1982). The early embryonic development of the primitive moth, *Neomicropteryx nipponensis* Issiki (Lepidoptera, Micropterygidae). *J. Morphol.* **172**, 259–69.

Kobayashi, Y. & Ando, H. (1984). Mesodermal organogenesis in the embryo of the primitive moth, *Neomicropteryx nipponensis* Issiki (Lepidoptera, Micropterygidae). *J. Morphol.* **181**, 29–47.

Kobayashi, Y. & Gibbs, G.W. (1995). The early embryonic development of the Mnesarchaeid Moth, *Mnesarchaea fusilella* Walker (Lepidoptera, Mnesarchaeidae), and its phylogenetic significance. *Aust. J. Zool.* **43**, 479–88.

Marcus, J.M., Ramos, D.M. & Monteiro, A. (2004). Germ line transformation of the butterfly *Bicyclus anynana*. *Proc. R. Soc. Lond. B (Suppl.)* **271**, S263–5.

Mazur, P., Cole, K.W. & Mahowald, A.P. (1992). Critical factors affecting the permeabilization of *Drosophila* embryos by alkanes. *Cryobiology* **29**, 210–39.

Nagy, L.M. (1995). A summary of lepidopteran embryogenesis and experimental embryology. In *Molecular Model Systems in the Lepidoptera*. Cambridge: Cambridge University Press.

Nakao, H. (1999). Isolation and characterization of a *Bombyx vasa*-like gene. *Dev. Genes Evol.* **209**, 312–16.

Nijhout, H.F. & Emlen, D.J. (1998). Competition among body parts in the development and evolution of insect morphology. *Proc. Natl. Acad. Sci. USA* **95**, 3685–9.

Sambrook, J. & Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Tanaka, M. (1987). Primary dorsal organ in eggs of *Parnassius glacialis* Butler and *Luehdorfia japonica* Leech (Lepidoptera: Papilionidae). *Int. J. Insect Morphol. Embryol.* **16**, 91–4.

Tanaka, M., Kobayashi, Y. & Ando, H. (1983). Embryonic development of the osmeteria of Papilionid caterpillars, *Parnassius glacialis* Butler and *Papilio machaon* Hippocrates C. et R. Felder (Lepidoptera: Papilionidae). *Int. J. Insect Morphol. Embryol.* **12**, 79–85.

Wall, C. (1973). Embryonic development in two species of *Chesias* (Lepidoptera: Geometridae). *J. Zool.* **169**, 65–84.