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Embryogenesis of *Aphidoletes aphidimyza* (Diptera: Cecidomyiidae): Morphological markers for staging of living embryos

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Abstract. Determination of embryonic stages is an important prerequisite for the long-term cryopreservation of eggs and embryos of the predatory gall midge *Aphidoletes aphidimyza*. This paper describes the embryonic development of this insect based on light microscopy. Gall midge embryogenesis lasts, on average, 102 h at 17°C and 144 h at 15°C. Living embryos can be quickly separated into ten stages that are clearly defined by specific morphological markers. The necessity for selecting definite embryonic stages for cryobiological storage is discussed.

INTRODUCTION

The predatory gall midge *Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae) is an effective biological control agent that has been used for the biological control of aphids in greenhouse crops (Asyakin, 1974; Markkula et al., 1977; Havelka, 1982; Kulp et al., 1989) since 1973.

Long periods of storage of insects at low temperatures is an important component of mass laboratory rearing and crucial for establishing population banks of selected species.

Diapausing larvae in cocoons are used in biological control practice.

Storage at 3°C under continuous darkness extends shelf life of stored insects for up to 7 month, with survival rates above 50%. Diapause of most specimens is terminated within 120 days of chilling (Košťál & Havelka, 2001). The exposure to low positive temperatures ensures cold reactivation (Havelka, 1996).

The discovery of great variability in many bionomical characteristics among the geographic populations of *A. aphidimyza* (Havelka & Zemek, 1988, 1999) revealed that the efficiency of this biological control agent can be improved by selection of suitable parameters (e.g. high fecundity, short oviposition period, non-diapausing type of development, preference for low relative air humidity). Such improvements based on selection and hybridization need the simultaneous maintenance of multiple breeding populations. It is costly and can lead to changes in population characteristics due to long term laboratory rearing. The cryopreservation of gall midge embryos can solve this problem.

Cryopreservation of insects in liquid nitrogen has been achieved over the last ten years using embryos at well-defined developmental stages. The main groups of dipterous insects studied in detail are as follows: Drosophilidae – *Drosophila melanogaster* (Myers et al., 1988; Leopold, 1991; Mazur & Cole, 1992; Mazur et al., 1992, 1993), Muscidae – *Musca domestica* (Heacox et al., 1985; Wang et al., 2000), Tephritidae – *Ceratitis capitata*, *Anastrepha suspensa*, *A. ludens* (Rajamohan et al., 2003), Calliphoridae – *Lucilia cuprina* and *Cochliomyia hominivorax* (Leopold & Atkinson, 1999; Leopold et al., 2001), Culicidae – *Anopheles gambiae*, *A. quadrimaculatus* (Valencia et al., 1996a, b; Liu et al., 2001, 2003; Liu & Mazur, 2003) and Ceratopogonidae – *Culicoides sonorensis* (Nunamaker & Lockwood, 2001).

Development of a cryopreservation method includes five steps:

1. Staging of embryos using characteristic markers; 2. Selection of the most appropriate developmental stage for cryopreservation; 3. Selection of cooling rate to –205°C (liquid nitrogen) after permeabilizing and loading embryos with hypertonic ethylene glycol; 4. Selection of a warming rate for revival; 5. Finding the conditions optimal for the completion of embryonic development (hatching of larvae). The purpose of this study is to determine the embryonic stages of *A. aphidimyza* reared under constant laboratory conditions and identify stage-specific markers that can be used in the cryopreservation of this species (i.e. the 1st and 2nd steps).

MATERIAL AND METHODS

Egg collection and staging

The laboratory population was established from larvae collected in a colony of the aphid *Impatientinum asiaticum* Nevsky on *Impatiens parviflora* (L.) at České Budějovice, Southern Bohemia (49°N, 14°E) in August 1997. The aphidophagous gall midges were reared at 17 ± 1.0°C and a photoperiod of 18L : 6D. This constant temperature is at the lower limit of this species optimum zone (mortality near zero) and results in slow

TABLE 1. Embryonic development of *Aphidoletes aphidimyza*.

Stages of embryonic development of *Drosophila melanogaster* (Wieshaus & Nusslein-Volhard, 1986)		Stages according to simplified scheme of embryonic development of *Aphidoletes aphidimyza*	
No.	Some characteristic processes and features	No.	Characteristic markers
1	Cleavage process; egg cytoplasm is homogenous	1	Egg cytoplasm is homogenous
2	Cleavage process; clear cap of posterior polar cytoplasm (retraction of cytoplasm from vitelline membrane at poles)	2	Cleavage process; clear cap of posterior polar cytoplasm (retraction of cytoplasm from vitelline membrane at poles)
3	Cleavage process; pole cell nuclei form buds at posterior end	2	Cleavage process; buds at posterior end of egg
4	Cleavage process; pole cell nuclei cellularize to form pole cells over blastoderm nuclei (syncytial blastoderm), peripheral migration of nuclei	2	Cleavage process; buds at posterior end of egg
5	Cellularization of blastoderm; pole cell migration begins	3	Cellularization of blastoderm
6	Early gastrulation; invagination of mesoderm and endoderm primordia, dorsal plate forms; cephalic and ventral furrows form	4	Early gastrulation
7	Gastrulation complete; invagination of anterior and posterior midgut; formation of dorsal folds	4	Invagination of anterior and posterior midgut; formation of dorsal folds
8	Amnioproctodeal invagination elongation of germ band mesoderm segmented	4	Amnioproctodeal invagination elongation of germ band, mesoderm segmented (parasegmental furrows)
9	Stomodeal plate formation; dorsal folds and cephalic furrow disappear	4	Stomodeal plate formation
10	Maximum elongation of germ band stomodeal invagination	4	Maximum elongation of germ band; stomodeal invagination; clear segmentation
11	Parasegmental furrows appear, germ band shortening begins at end of stage	5	Germ band shortens
12	Germ band shortens; clear segmentation	5	Germ band shortens; clear segmentation
13	End of germ band shortening; head involution begins	6	End of germ band shortening; the clypeo-labrum is distinct
14	Head involution; midgut and dorsal closure	7	Head range involution; midgut and dorsal closure begins
15	Completion of dorsal closure; anal plates and posterior spiracles distinct	8	Completion of dorsal closure; anal plates and posterior spiracles distinct
16	Intersegmental grooves clear in mid-dorsal region; dorsal ridge overgrows tip of clypeolabrum	8	Intersegmental grooves clear in mid-dorsal region; dorsal ridge overgrows tip of clypeolabrum
17	Muscular movements; air in tracheae; condensation of central nervous system	9	Muscular movements
18	Hatching of larva	10	Hatching of larva

development (Havelka, 1980). Newly emerged adults of *A. aphidimyza* were placed in 1250 ml plastic container. Adults were fed by placing tissue paper soaked in 5% saccharose solution under the lid. Individual cultures contained about 400 gall-midges of both sexes. Ventilation was ensured by two nylon-covered holes on the sides of the bottomless container, which rested on a perforated lid of a plastic cup filled with water. A bean seedling *(Vicia faba* L.), about 3 cm long, infested with *Aphis fabae* Scop. (approx. 40 aphids per plant), was placed into a hole in the lid to stimulate the gall midges to oviposit. Eggs were collected at night after 8 h exposure to the aphid stimulus (from 22 p.m. till 6 a.m.). Gall midges (minimum 200 females) laid eggs in the bean-aphid colony. The plants with eggs were transferred into plastic cups covered with a layer of tissue paper and nylon netting. Larvae were fed on the pea aphid *Acyrthosiphon pisum* (Harris). High relative air humidity was maintained in the rearing cups by the pieces of plant, or by fixing a wall of wet cotton wool to the upper part of the cup. Prior to pupation, mature larvae produced cocoons in a 2–3 cm layer of fine, cleaned quartz sand, sterilized with boiling water. The cocoons of *A. aphidimyza* were passed through a set of sieves, cleaned of organic debris and placed in incubators with a constant humidity close to 100% (Havelka & Zemek, 1988).

The eggs for experiments were collected from the plant surface using a very fine needle and were immediately transferred for 2 min into a drop of 2.5% sodium hypochlorite, or 50% solution of commercial bleach solution SAVO (with 5% of sodium hypochlorite) to remove the chorion. The treatment time was selected after preliminary experiments to determine the treatment that gave the best egg survival.

Microscopy of living embryos

The whole embryonic development was observed and photographed using living eggs submerged in various media under a dissecting microscope, using transmitted light (column a in the figures – dorsoventral view, column b – lateral view, and column c – phase contrast microphotograph with descriptions of markers). A collection of washed eggs was placed on a plate or small Petri dish. Terasaki plates proved very suitable. The solution used for the cultivation of mammalian embryos, PBS (phosphate buffer solution) was used for the embryonic development studies. Transfer and manipulation of embryos were done with

TABLE 2. Rate of embryonic development of *Aphidoletes aphidimyza* at constant temperatures.

Stage	Approximate age of egg (h) at 15 ± 1.0°C	Approximate age of egg (h) at 17 ± 1.0°C	Pivotal markers observable using transmitted light microscopy
1	1–3	1–2	Egg cytoplasm is homogenous
2	3–9	2–6	Pole buds at posterior end of egg
3	9–21	6–12	Cellularization of blastoderm
4	21–57	12–33	Elongation of germ band; stomodeal invagination; clear parasegmentation
5	57–66	33–48	Germ band shortens
6	66–75	48–60	End of germ band shortening
7	75–90	60–72	Head involution
8	90–117	72–84	Dorsal ridge overgrows tip of clypeolabrum; midgut and dorsal closure
9	117–144	84–102	Muscular movements; white "vacuole" in the hind gut region
10	>144	>102	Hatching of larva

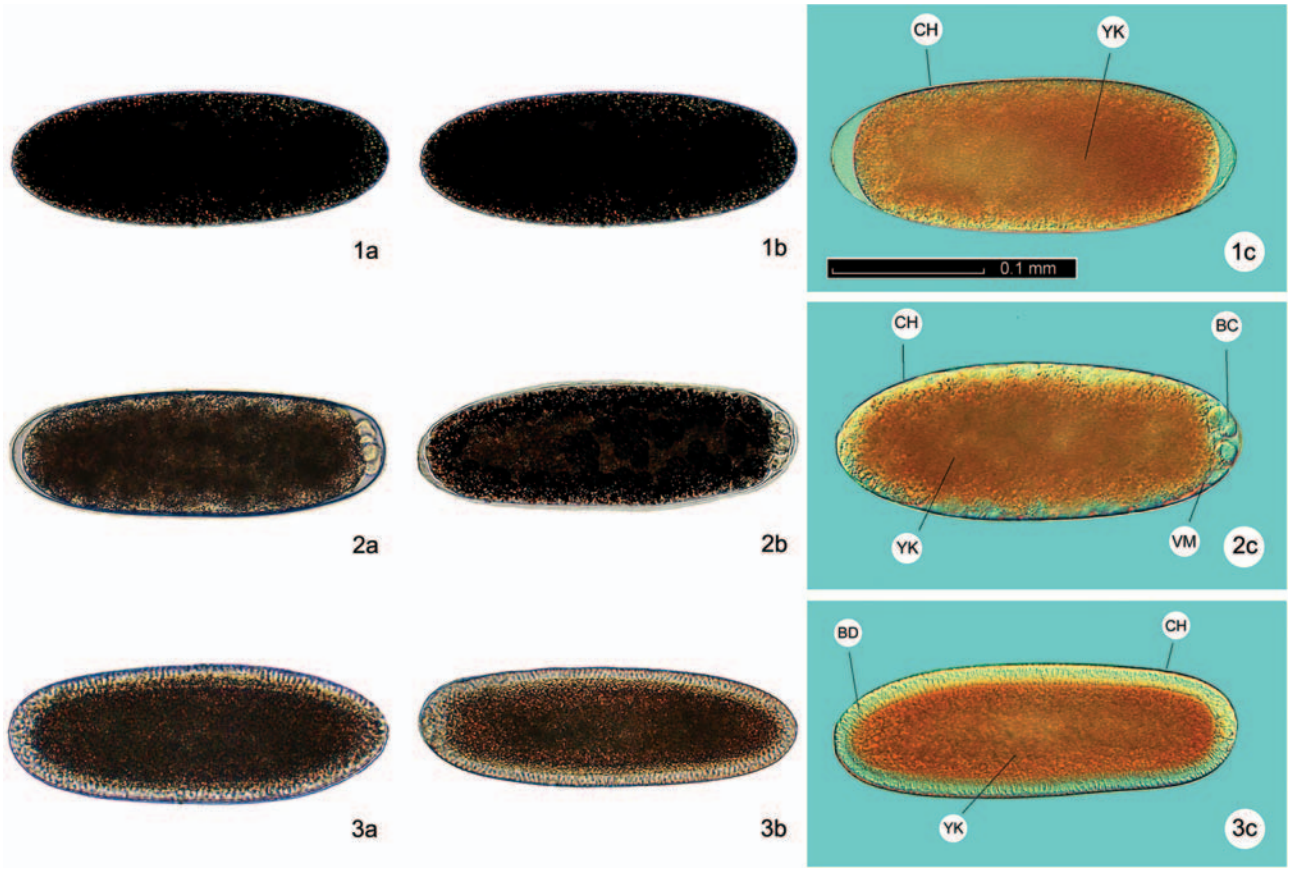
the aid of a capillary glass tube. This method, which is suitable for handling large numbers of embryos, is described in more detail in another paper (Landa et al., in prep.). Zeiss Amplival optics (Zeiss Semiplan 10× objective) and an Olympus BX41 microscope (Olympus Plan N 10× objective) with phase contrast and an Olympus DP 10 camera were used for digital image documentation. Structures in living eggs were compared with those in other Dipteran species – particularly *Drosophila* (Poulson, 1950; Sonnenblick, 1950; Van der Starre-Van der Molen, 1972; Wieshaus & Nüsslein-Volhard, 1986; Ashburner, 1989).

RESULTS

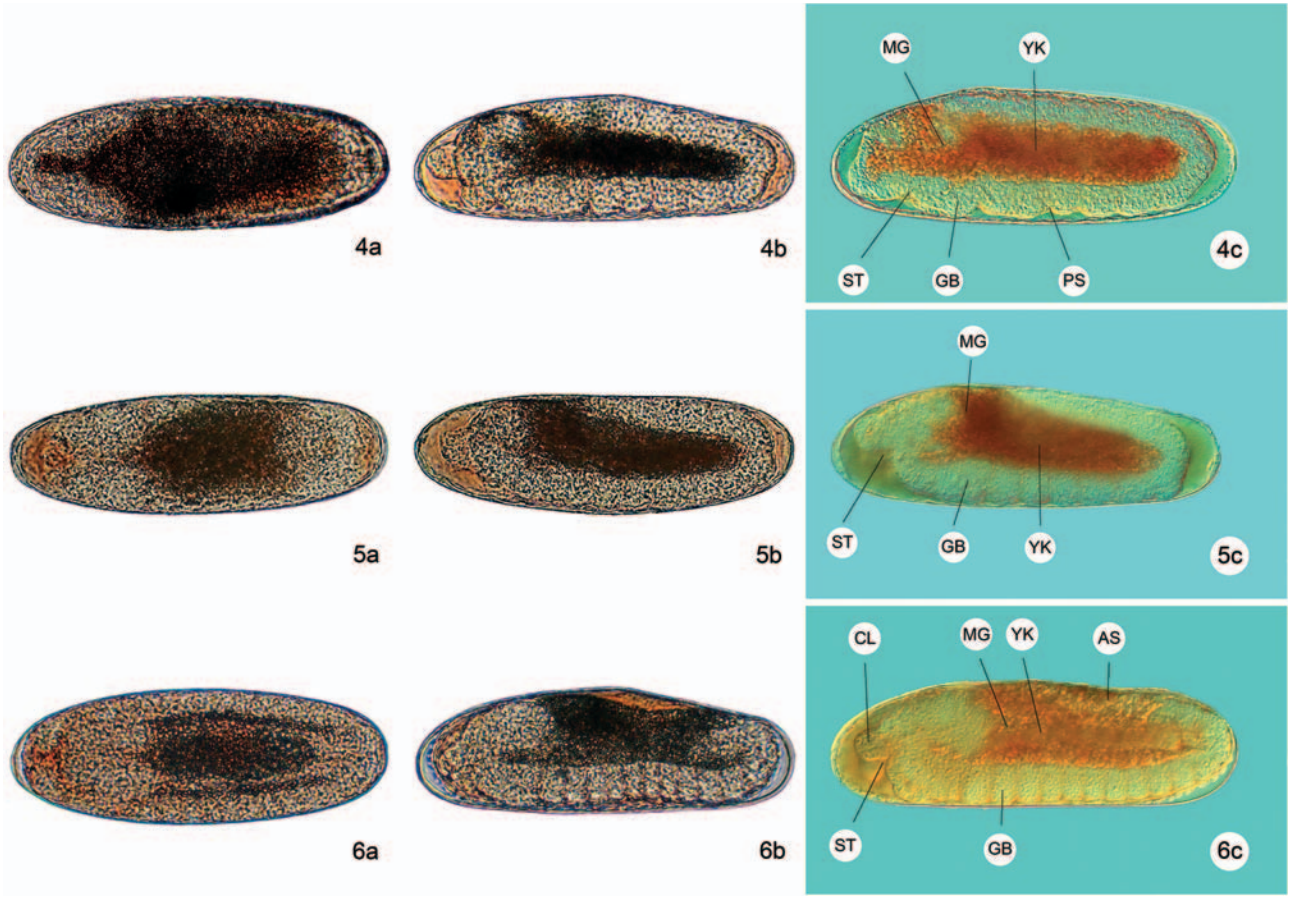
Progress of embryonic development

The shape of a newly laid fertilized egg is elongate-ellipsoidal and is bright orange, ca. 0.23 mm long and 0.05 wide. The chorion (CH) is smooth, colourless, translucent and very thin.

The duration of embryonic development from oviposition to hatching depends on temperature (Havelka, 1980). For the geographical population investigated it lasts about



Figs 1–3. Stage 1: Cleavage process; egg cytoplasm is homogenous. CH – chorion; YK – yolk. a – dorsoventral view; b – lateral view; c – phase-contrast. Stage 2: Cleavage process; syncytial embryo with three pole buds; clear cap of posterior polar cytoplasm. CH – chorion; YK – yolk; VM – vitelline membrane; BC – posterior pole buds. Stage 3: Cellularization of blastoderm. BD – blastoderm; CH – chorion; YK – yolk.



Figs 4–6. Stage 4: Gastrulation: stomodeal invagination, dorsal folds form; elongation of germ band; clear parasegmentation. GB – germ band; SP – stomodeal plate; MG – midgut; YK – yolk; PS – parasegments. Stage 5: Germ band shortens. GB – germ band; ST – stomodeum; MG – midgut; YK – yolk. Stage 6: End of germ band shortening; head involution begins. GB – germ band; ST – stomodeum; CL – clypeo-labrum; MG – midgut; YK – yolk; AS – amnio-serosa.

102 h (about 4 days) at 17 ± 1.0°C and about 144 h at 15 ± 1.0°C. This period can be divided into ten stages, on the basis of changes in microscopic embryonic features. The most convenient markers are summarized in Table 1 and the progress through these stages is shown in Table 2. Dorsoventral and lateral planes of the embryos (Figs 1–10) are shown in longitudinal section. Stage 1–3 are pregastrula stages. Embryonic features – markers are very similar to those in other dipterous species.

Stage 1 (1–3 h at 15°C): The yolk (YK) is a homogenous granular mass but in some eggs clear caps of polar cytoplasm occur (Fig. 1a, b, c). The very detailed studies on *D. melanogaster* (Wieshaus & Nüsslein-Volhard, 1986) revealed that nuclei multiply exponentially in the central region of an egg during early cleavage. The majority of dividing nuclei surrounded by protoplasmic islands start their migration outwards to the periplasm, leaving the future yolk nuclei behind.

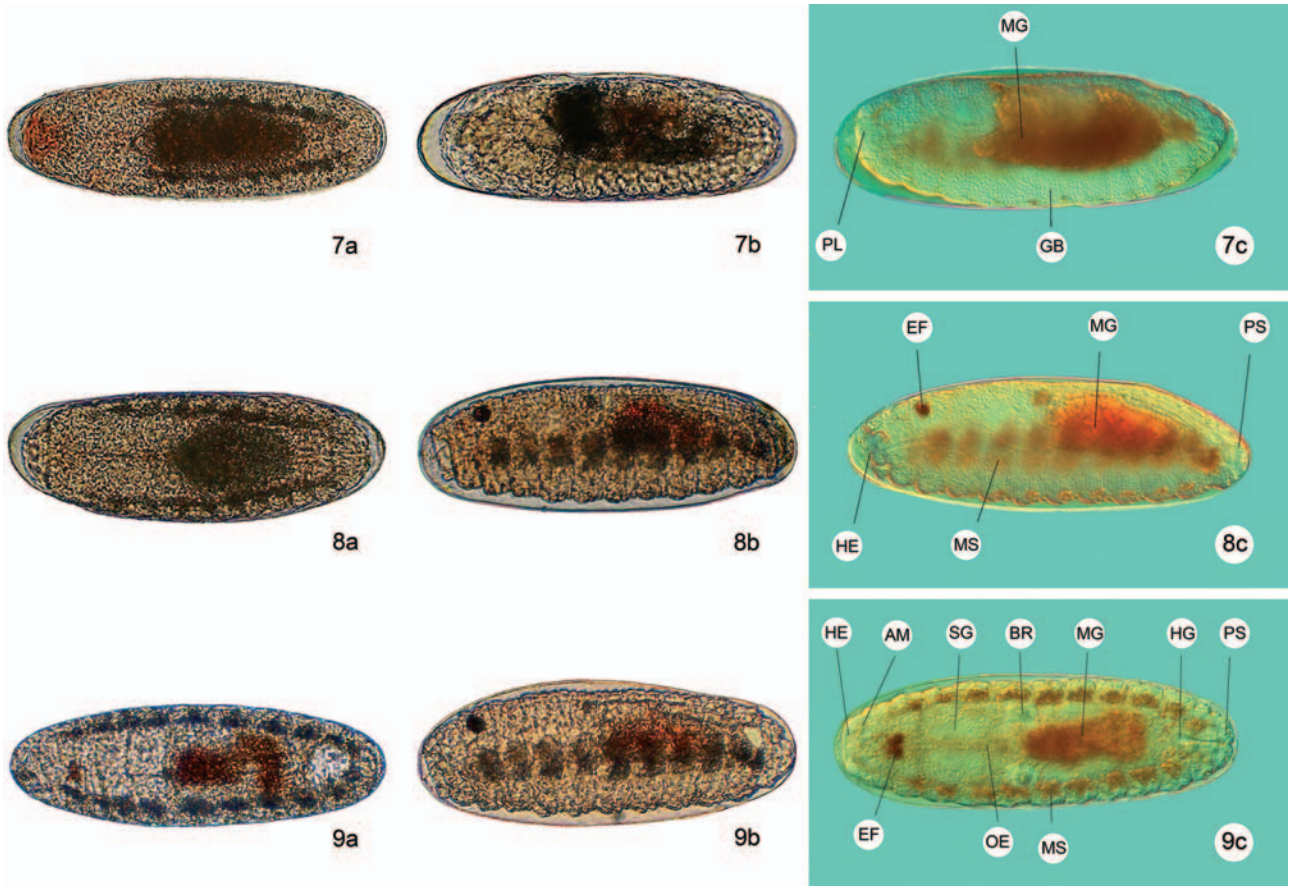
Stage 2 (3–9 h): The depth of yolk-free periplasm increases at the expense of the central yolk region (YK). There are clear caps of posterior polar cytoplasm resulting from the retraction of cytoplasm from the vitelline membrane (VM) at the poles. The pole buds (BC) divide to produce the pole cells (Fig. 2a, b, c).

Stage 3 (9–21 h): Blastoderm cell layer becomes thick and clearly demarcated from the yolk. The cellular blastoderm (BD) is a characteristic marker of this stage. The gastrulation movements begin (Fig. 3a, b, c).

Stage 4 (21–57 h): Gastrulation stage: about one-fourth of the distance down from the anterior pole is a section of the cephalic furrow (anterior oblique cleft) and the invagination at the posterior pole is part of the posterior midgut furrow (also called the amnio-proctodeal invagination). The cephalic folds gradually disappear and the stomodeal plate (ST) is formed. The gut opening has reached the head region three quarters along the egg length and the cephalic furrow is no longer visible. The germ band (GB) extends to maximum size. Parasegmental furrows (PS) appear (Fig. 4a, b, c).

Stage 5 (57–66 h): Germ band shortens. When the stomodeum (ST) forms the anterior tip of the embryo bends ventrally and an invagination appears and deepens. The anterior midgut anlage moves posteriorly reaching and passing the level of the posterior gut opening at the same time flattening out along the ventral germ band. The head bends dorsally and a gap at the anterior ventral region of the head enlarges (Fig. 5a, b, c).

Stage 6 (66–75 h): The germ band (GB) is completely contracted. Ectodermal parasegmentation becomes clear.



Figs 7–9. Stage 7: Head involution. GB – germ band; PL – procephalic lobe; MG – midgut. Stage 8: Dorsal ridge overgrows tip of clypeolabrum; midgut and dorsal closure. HE – head; EF – eye-fleck; MG – midgut; MS – muscles; PS – posterior spiracles. Stage 9: Muscular movements; white vacuole in the hindgut region. HE – head; AM – Antennal-mandibullary complex; EF – eye-fleck; MS – muscles; SG – salivary glands; OE – oesophagus; BR – brain; MG – mid gut; HG – hind gut; PS – posterior spiracles.

The yolk sac (YK) is initially concave dorsally and changes to a convex shape at the end of this stage. The posterior midgut (MG) opening moves posteriorly and the yolk sac, covered by the amnio-serosa (AS), extends to the dorsal surface of the embryo. The head region is distinct. A ridge forms dorsally at the posterior margin of the head. The clypeo-labrum (CL) and stomodeum (ST) are distinct (Fig. 6a, b, c).

Stage 7 (75–90 h): The germ band (GB) stretches anteriorly and a head portion (PL) involutes into the interior of the embryo and is continuous with the stomodeal opening. Ventral layers become apparent and gradually flatten out along the yolk sac (MG). The hindgut grows anterodorsally. Dorsal closure continues (Fig. 7a, b, c).

Stage 8 (90–117 h): Intersegmental grooves are clear in the mid-dorsal region; dorsal ridge overgrows the tip of clypeo-labrum. Head (HE) involution and dorsal closure of the ectoderm occur. Frontal sac formation begins as the dorsal ridge and moves anteriorly covering the head region. There is a well-marked antennal-mandibullary complex on the head. The eye-fleck (EF) is clearly distinct. In the frontal part of embryo there are salivary glands (SG), oesophagus (OE) and brain (BR). The posterior midgut (MG) broadens dorsally. During this stage dorsal closure of the musculature and gut occurs. The midgut initially assumes an "elliptical shape". Subsequently constrictions divide the midgut (8c) into three regularly spaced subdivisions (Fig. 8a, b, c).

Stage 9 (117–144 h): muscular (MS) movements begin in the gut and are soon apparent in the somatic musculature as well. White coloured spherical hindgut (HG) appears. Posterior spiracles (PS) are distinct (Fig. 9a, b, c).

Stage 10 (>144 h): Newly hatched larva appears after disruption of the chorion as a result of convulsive movement in late embryogenesis (Fig. 10a, b, c).

Comparison with *Drosophila*

In contrast to the seventeen stages distinguished in *Drosophila* embryonic development (Campos-Ortega & Hartenstein 1985; Wieshaus & Nüsslein-Volhard, 1986 and Ashburner, 1989), we (to simplify the description) distinguished only ten stages (Table 1). Only five stages are defined by means of distinct markers before the termination of gastrulation (in comparison to the seven stages in *Drosophila* embryonic development) and only six before completion of germ band shortening (compared to thirteen in *Drosophila* embryos).

Embryonic development in *A. aphidimyza* is slower than in *D. melanogaster* and there are morphological differences between these two species in the late stages of

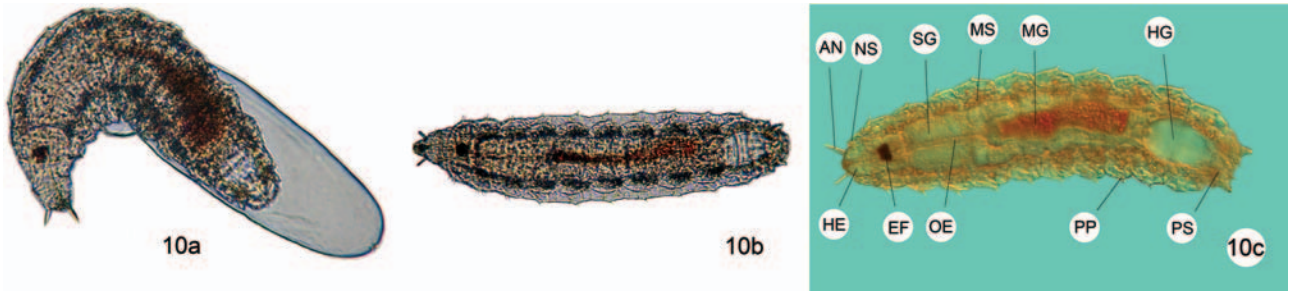


Fig. 10. Hatched larva. AN – antenna; HE – head; NS – neck segment; EF – eye-fleck; MS – muscles; SG – salivary glands; OE – oesophagus; MG – mid gut; HG – hind gut; PP – pleural papilla; PS – posterior spiracles.

embryogenesis (well-differentiated embryo). Among others, the eye fleck, the hindgut form and distinct muscles, characteristic of 8th stage *A. aphidimyza* embryos, are missing in *Drosophila*.

Length of the gall-midge embryonic development is more than twice that of *Drosophila melanogaster* – about 102 h at 17°C and 144 h at 15°C (compared to 46 h for *Drosophila* reared at 17°C).

DISCUSSION

There is only one paper on the embryonic development of Cecidomyiid flies (Kahle, 1908). It is a well-illustrated work, but it focuses on parthenogenetic reproduction of larvae of *Miastor metraloas* Meinert.

The numbering of the embryonic stages and the morphological criteria for their identification depend on the aim of investigators. A total of 17 stages are used in embryological studies of *Drosophila* (Campos-Ortega & Hartenstein, 1985; Wieshaus & Nüsslein-Volhard, 1986; Ashburner, 1989), and only eleven morphological stages in the blowfly, *Calliphora erythrocephala* (van der Starre-van der Molen, 1972).

Detailed study of embryogenesis makes the selection of the most appropriate developmental stage possible. It is very difficult to evaluate results of experiments on cryoprotection without reference to embryonic development. For example, Miles & Bale (1995a) tested cryopreservation using glycerol and methanol on a limited number of eggs of *A. aphidimyza*. They had only three groups of 10 eggs, 2 to 17 h old (probably kept at 22°C). It is obvious, that they used a mixture of early embryonic stages (probably stages 1–3), with high yolk contents. According to Mazur et al. (1992), early embryos are chill-sensitive and this was probably the cause of cryopreservation failure in the experiments of Miles & Bale (1995a). Mathematical modelling (Miles & Bale 1995b) of the effect of the cooling rate and exposure to low temperatures (0–20°C) for short periods on egg survival are meaningless if the cryo-treatment is applied to an unsuitable embryonic stage of *A. aphidimyza* eggs.

The papers on cryopreservation of dipteran embryos (mentioned above) include information that indicate the importance of using embryos at a particular stage in their development or exhibit a particular "markers", or give reasons why the latter stages of development are better for treatment than earlier stages. For example – in *Lucilia cuprina* (Calliphoridae) the best are embryos at the point of dorsal closure (Leopold & Atkinson, 1999). For the Mediterranean fruit fly (*Ceratitis capitata*) the optimal stage is after incubation for 27 h at 29°C (Rajamohan et al., 2003). For *Drosophila melanogaster* it is freshly laid eggs kept for 20 h at 17.5°C (at which time 90% of embryos are generally at stage 14) (Mazur et al., 1992). A critical evaluation of the published results indicates that, except for the *Anopheles* mosquito, cryopreservation of the other dipteran embryos is only possible if they can survive permeabilization, there is little yolk left, and the embryo has not formed an impermeable body wall in the form of a cuticle.

The staging system of living embryos of the predatory gall midge *A. aphidimyza* presented here includes ten stages characterized by distinct morphological markers. Stage 6, appears to be the most appropriate for cryopreservation.

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