SHORT COMMUNICATION

Urs Schmidt-Ott

# The amnioserosa is an apomorphic character of cyclorrhaphan flies

**Abstract** In developing insect eggs the cells of the blastoderm adopt either an embryonic or an extraembryonic fate. The extraembryonic tissue consists of epithelia, termed amnion and serosa, which wrap the germ band embryo. The serosa develops directly from part of the blastoderm and surrounds the embryo as well as the yolk. The amnion develops from the margins of the germ band and in most insect species generates a transient ventral cavity for the developing embryo. The amniotic cavity and the serosa have been reduced in the course of dipteran evolution. The insect order of Diptera includes the paraphyletic Nematocera, including gnats and mosquitoes, and the more derived monophyletic Brachycera, the true flies. Nematocera develop within an amniotic cavity and the surrounding serosa, whereas cyclorrhaphan Brachycera do not. This observation implies that the amnion and serosa have been reduced before the radiation of the monophyletic cyclorrhaphan flies. Here I show that an amniotic cavity is formed during embryogenesis of the horsefly *Haematopota pluvialis* (Tabanidae) and the dancefly *Empis livida* (Empididae). The results suggest that extraembryonic tissue was reduced in the stem lineage of cyclorrhaphan flies, with consequences for the molecular basis of pattern formation along the anterior-posterior axis of the embryo.

**Key words** Embryogenesis · Amnion · Serosa · Diptera · Orthorrhapha

## Introduction

Insect embryos develop via superficial cleavage leading to the formation of a single-layered epithelium, termed blastoderm, which surrounds the central yolk (Anderson 1972; Johannsen and Butt 1941). The developing egg is divided early into embryonic and extraembryonic anlagen. The embryonic anlagen form the embryo proper, whereas the extraembryonic anlagen form transient epithelia, termed amnion and serosa, which wrap the germ band embryo. The serosa originates directly from the blastoderm. In winged insects it typically forms an epithelium of flattened cells which abuts the extracellular egg shell on the inner side. The amnion develops from an amniotic fold, which is continuous with the margin of the germ band. After midventral fusion the amniotic fold covers the embryo's ventral side. In this way a cavity is formed during the germ band stage, which is delimited dorsally by the germ band and ventrally by the amnion (Anderson 1972; Johannsen and Butt 1941).

The amniotic cavity and the serosa have been reduced during radiation of the holometabolous insect order of Diptera. This conclusion becomes apparent by mapping species with and without an amniotic cavity and a complete serosa on a phylogenetic tree of the Diptera. The taxon of Diptera is usually divided into the paraphyletic Nematocera and the more derived monophyletic Brachycera (McAlpine 1989; Yeates and Wiegmann 1999). Traditionally the Brachycera have been divided into Orthorrhapha, now recognized as a paraphyletic assemblage of several lower brachyceran taxa (see below), and the monophyletic Cyclorrhapha (McAlpine 1989; Yeates and Wiegmann 1999; Fig. 1). In Nematocera an amnion and a serosa are formed during development (Anderson 1972; Johannsen and Butt 1941; Moretti and Larsen 1973; Rohr et al. 1999). This is also the case for most other holometabolous insects, suggesting that the amniotic cavity and the serosa are plesiomorphic characters in Nematocera (Anderson 1972; Handel et al. 1999; Johannsen and Butt 1941). However, in Cyclorrhapha such as *Musca* (Muscidae, Calyptratae), *Drosophila* (Drosophilidae, Acalyptratae) and *Megaselia* (Phoridae, Aschiza), the extraembryonic tissue is composed of a single epithelium only, termed amnioserosa (Anderson 1972; Campos-Ortega and Hartenstein 1997; Johannsen
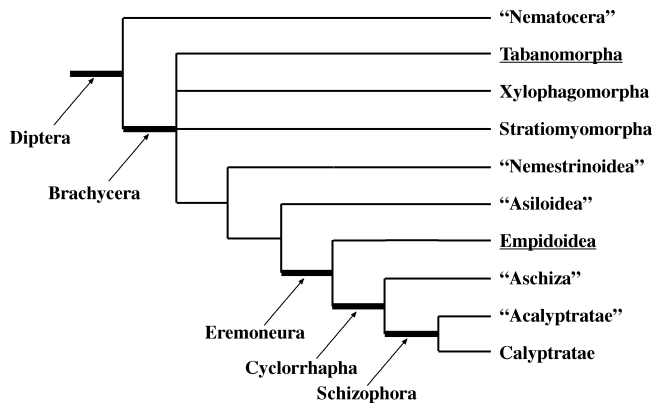
Edited by D. Tautz

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**Fig. 1** Phylogenetic tree of Diptera. *Thick horizontal lines* Well supported monophyletic taxa; *quotation marks* paraphyletic groups. Traditionally the noncyclorrhaphan Brachycera have been called Orthorrhapha. However, the establishment of the Eremoneura as a monophyletic taxon makes this taxonomic unit obsolete. Species from underlined taxa have been analyzed in this study. (Modified after Yeates and Wiegmann 1999)

and Butt 1941; Stauber et al. 1999). The formation of the amnioserosa is restricted to the dorsal side of the embryo, where it transiently covers the yolk. Thus, the amnioserosa of Cyclorrhapha appears as a reduction of the two separate epithelia observed in Nematocera to a single epithelial sheet. Non-cyclorrhaphan Brachycera (Fig. 1) have not been investigated with respect to extraembryonic tissue formation and embryonic development in general. It is therefore not known whether extraembryonic tissue reduction occurred before or during the radiation of the Brachycera. In the present study it is shown that an amniotic cavity is formed during embryogenesis of the horsefly *Haematopota pluvialis* (Tabanidae, Tabanomorpha) and the dancefly *Empis livida* (Empididae, Empidoidea). This finding suggests that the reduction of extraembryonic tissue within the Diptera to a dorsal sheet of cells occurred in the stem lineage of cyclorrhaphan flies. I discuss this result with respect to the evolutionary origin of the anterior determinant gene *bicoid* of cyclorrhaphan flies.

## Materials and methods

### Species

Flies were caught in the surroundings of Göttingen, Germany. Males and females of *E. livida* were kept on humid paper and fed on a honey water diet. After a few days at room temperature some females laid eggs on the bottom of the vial. Females of *H. pluvialis* were fed on human blood and kept for several days on humid paper and a diet consisting of honey water and small pieces of peach. Egg packages were laid after several days on raygrass leaves.

### Fixation and sectioning

*Empis* eggs were fixed in 2% glutaraldehyde and 4% formaldehyde in HEPES buffer (100 mM HEPES; 2 mM MgSO$_4$; 1 mM

EGTA; pH 6.9) for several hours. Fixation of *Empis* eggs is hindered by a very tough chorion which cannot be removed without destroying the embryo. Therefore the eggs were pricked with a glass needle to allow the penetration of the fixative. *Haematopota* eggs were treated with commercial bleach for several minutes, washed with water, fixed in 2% glutaraldehyde in HEPES buffer and *n*-heptane (1:1) for at least 1 h. The vitelline layer was ruptured in a one to one mixture of *n*-heptane and cold methanol (–80°C under vigorous shaking and subsequent heating to +70°C in a water bath. The vitelline layer was then fully removed mechanically, and the embryos were kept over night in 1% osmium tetraoxide in 0.1 M cacodylate buffer. Embedding was carried out in epon following standard protocols. Sections of 2 μm were cut with a diamond knife and stained with a solution of 1% (w/v) toluidine blue and 1% (w/v) Na$_4$Bo·10H$_2$O.

## Results and discussion

The Empidoidea have been proposed as sister taxon of the Cyclorrhapha based on 13 synapomorphic characters (Yeates and Wiegmann 1999; Fig. 1). Together, Empidoidea and Cyclorrhapha have been termed Eremoneura. The amniotic cavity is reduced in representatives of various Cyclorrhapha, suggesting that the reduction predated the radiation of Cyclorrhapha (see "Introduction"), but it is not known whether extraembryonic tissue reduction occurred before the radiation of the Eremoneura. To address this question I have analyzed amniotic cavity formation in a basal eremoneuran species.

Embryos of *E. livida* (Fig. 1) were fixed after germ band formation, sectioned, and analyzed by light microscopy. Midtransversal and midsagittal sections revealed that an amniotic cavity and a serosa are formed at this stage of development (Fig. 2A, B). Both amnion and serosa consist of a single-layered epithelium each. The amnion extends ventrally from the margins of the germ band and forms a large cavity between the ventral egg shell and the embryo ("amniotic cavity" in Fig. 2A). In this way the yolk is located dorsally to the germ band. On the ventral side the amnion abuts the serosa. The serosa continues dorsally and surrounds the yolk (Fig. 2A). It is covered by a very tough chorion/vitelline layer which appears to be insensitive to commercial bleach (Fig. 2A, inset). The large volume of the amniotic cavity seen in Fig. 2A and B may in part be a fixation artifact, as suggested by the bulging of amnion and serosa at the right-hand arrow in Fig. 2C. Such a large amniotic cavity would be an exception among insects in general. Nevertheless, the result leaves no doubt about the existence of an amniotic cavity and a complete serosa in this species.

The organization of extraembryonic tissues in *Empis* is similar to that in Nematocera (Anderson 1972; Johannsen and Butt 1941; Moretti and Larsen 1973; Rohr et al. 1999). This finding suggests that an amniotic cavity was formed in the stem species of Eremoneura, and that the reduction of extraembryonic tissue occurred only after the Empidoidea branched off from the eremoneuran lineage. The result also implies that other orthorrhaphan flies form an amniotic cavity. I tested this prediction by analyzing embryos of *H. pluvialis* (Tabanidae), which

**Fig. 2A–D** Semithin sections
of embryos after germ band
formation (*asterisk*). **A,C** Mid-
transversal and midsagittal
section of *Empis livida*, respec-
tively. *Long arrow* Amnion;
*double arrow* serosa; *short
arrow* egg shell. The large
volume of the amniotic cavity
in **A,C** may in part be a fixa-
tion artifact (see text). *Square*
Magnified inset. **B,D** Midtrans-
versal and midsagittal section
of *Haematopota pluvialis*.
*Long arrows* Amnion. Dorsal
side is *up*; anterior is *to the left*
in longitudinal sections
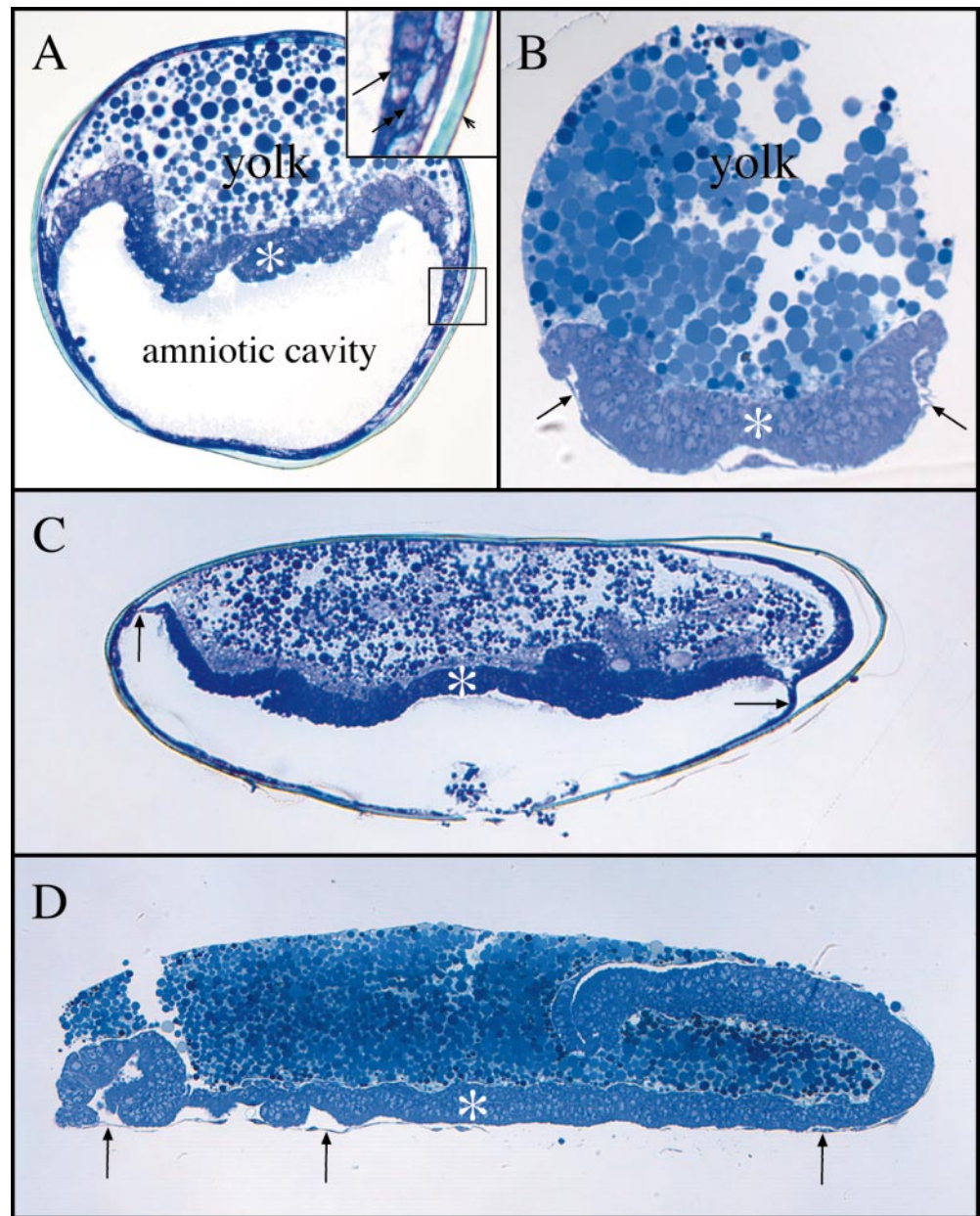


belongs to the basal orthorrhaphan taxon of Tabanomor-
pha (Fig. 1).

Embryos of *Haematopota* were fixed and sectioned
after germ band formation. Such embryos could not be
fixed properly in the presence of the inner egg shell or
vitelline layer. Therefore the vitelline layer was re-
moved mechanically during the fixation procedure (see
"Materials and methods"). By this treatment the abut-
ting serosa was also removed. Figure 2C and D show
that the ventral side of the germ band is covered by a
single layer of stretched cells which extend from the
lateral germ band margins, the head fold, and the
proctodeum to form the amnion along the entire longitu-
dinal axis of the embryo.

The finding that an amniotic cavity is formed during
embryonic development of *Haematopota* and *Empis*

suggests that the reduction of extraembryonic tissue as
observed in Calyptratae, Acalyptratae, and the Aschiza
(see "Introduction") occurred in the stemlineage of
cyclorrhaphan flies. Therefore the change in extraem-
bryonic tissue organization constitutes most likely a de-
velopmental synapornorphic character for cyclorrhap-
han flies. A problem arises from the fact that within the
Cyclorrhapha the basal Aschiza are most likely a para-
phyletic group. Extraembryonic tissue reduction in
Aschiza has been reported for the Phorid *Megaselia* on-
ly (Stauber et al. 1999). This observation suggests that
the loss of the amniotic cavity occurred before the Pho-
rid lineage branched off from the cyclorrhaphan stem
lineage, However, other aschizan families, for example,
Opetiidae, Platypezidae, and/or Lonchopteridae, might
have branched off earlier than the Phorid lineage

(Yeates and Wiegmann 1999). Their embryology is still unknown.

The results presented here have far-reaching implications for early embryonic pattern formation. In species which form an amniotic cavity, such as the flour beetle *Tribolium* and the Psychodid midge *Clogmia* (Nematocera), extraembryonic tissue analgen originate from an anterior and dorsal egg portion (Falciani et al. 1996; Rohr et al. 1999). In fact the *zerknüllt* gene, which has been shown to be required for the specification of extraembryonic tissue in *Drosophila* embryos (Rushlow et al. 1987), is expressed in a dorsally extending anterior cap of the developing *Tribolium* egg (Falciani et al. 1996). In contrast, the reduced extraembryonic anlagen and corresponding *zerknüllt* expression of cyclorrhaphan flies, such as *Drosophila* and *Megaselia*, originate exclusively on the dorsal side of the embryo (Rushlow et al. 1987; Stauber et al. 1999). The different origin of extraembryonic tissue in nematoceran and cyclorrhaphan Diptera implies that a fate map shift or at least a loss of anterior extraembryonic tissue specification occurred in the course of dipteran evolution, most likely concurrently with extraembryonic tissue reduction. Thus the exclusively dorsal origin of extraembryonic tissue might be a new trait of cyclorrhaphan flies.

*Zerknüllt* has a paralogous sister gene in cyclorrhaphan flies, termed *bicoid* (Stauber et al. 1999). In contrast to *zerknüllt*, mRNA the mRNA of *bicoid* is maternally localized to the anterior tip of the egg. The translation of *bicoid* mRNA during early embryogenesis results in an anterior-to-posterior concentration gradient of Bicoid protein, which plays an essential role in patterning the embryo's head and thorax (Dearden and Akam 1999; Driever 1993). No *bicoid* ortholog has been found ouside the Cyclorrhapha (Sander 1996), but *bicoid*-like activity has been postulated in the flour beetle *Tribolium* based on the observation that in transgenic *Drosophila* embryos *Tribolium* orthologs of *caudal* and *hunchback* are regulated by Bicoid in a similar way as the corresponding *Drosophila* genes (Wolff et al. 1998). However, the different functions of bicoid and *zerknüllt*, i.e., embryonic patterning by *bicoid* and extraembryonic patterning by *zerknüllt*, require differential expression of the two genes. Therefore, early anterior specification of extraembryonic tissue might be possible only in species in which *bicoid* does not serve as an anterior determinant. It seems likely then that *bicoid* emerged in the stem lineage of cyclorrhaphan flies or during their early radiation.

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