Developmental Biology 363 (2012) 290–307

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanisms

The embryonic development of the centipede *Strigamia maritima*

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ARTICLE INFO

Article history:
Received for publication 28 July 2011
Revised 18 October 2011
Accepted 10 November 2011
Available online 19 November 2011

Keywords:
Chilopod
Segmentation
Developmental rate
Staging
Arthropod
Geophilomorph

ABSTRACT

The geophilomorph centipede *Strigamia maritima* is an emerging model for studies of development and evolution among the myriapods. A draft genome sequence has recently been completed, making it also an important reference for comparative genomics, and for studies of myriapod physiology more generally. Here we present the first detailed description of myriapod development using modern techniques. We describe a timeline for embryonic development, with a detailed staging system based on photographs of live eggs and fixed embryos. We show that the early, cleavage and nuclear migration, stages of development are remarkably prolonged, accounting for nearly half of the total developmental period (approx 22 of 48 days at 13 °C). Towards the end of this period, cleavage cells migrate to the egg periphery to generate a uniform blastoderm. Asymmetry quickly becomes apparent as cells in the anterior half of the egg condense ventrally to form the presumptive head. Five anterior segments, the mandibular to the first leg-bearing segment (1st LBS) become clearly visible through the chorion almost simultaneously. Then, after a short pause, the next 35 leg-bearing segments appear at a uniform rate of 1 segment every 3.2 h (at 13 °C). Segment addition then slows to a halt with 40–45 LBS, shortly before the dramatic movements of germ band flexure, when the left and right halves of the embryo separate and the embryo folds deeply into the yolk. After flexure, segment morphogenesis and organogenesis proceed for a further 10 days, before the egg hatches. The last few leg-bearing segments are added during this period, much more slowly, at a rate of 1–2 segments/day. The last leg-bearing segment is fully defined only after apolysis of the embryonic cuticle, so that at hatching the embryo displays the final adult number of leg-bearing segments (typically 47–49 in our population).

Introduction

"The myriapods yield up their embryological secrets only as a reward of great patience, ingenuity and histological skills." (Anderson, 1973)

Myriapods are now recognised as an ancient clade among the arthropods, but despite the large number of studies of arthropod development in recent years, we still know very little about their development. Yet if we are concerned to understand the diversity of developmental mechanisms among the arthropods, and how these mechanisms may have evolved, then it is essential to sample all of the major extant lineages within this diverse group.

Myriapods are specifically interesting in that they constitute a basal branch of the mandibulate arthropods that has retained a relatively simple, and possibly ancestral, body plan with only limited trunk segment diversity. They are an appropriate outgroup for comparison with the diversity of all insects and crustaceans, and may retain developmental mechanisms that have been modified or overwritten in these better-studied clades.

There are two major groups of myriapods—the grazing millipedes, which have two pairs of legs on most trunk segments, and the carnivorous centipedes, which are uniquely characterised by the transformation of the first trunk segment into a venomous poison claw or forcipule. In this paper, we provide the first detailed modern description of embryonic development in a centipede, using as our model *Strigamia maritima*, the only myriapod for which a complete genome sequence is now available.

The order of centipedes to which *Strigamia* belongs, the Geophilomorpha, is exceptional in that adult segment number is established at hatching, but is highly variable, both between species and within populations. This makes it particularly amenable for studies both of the mechanisms of segmentation (Chipman and Akam, 2008; Chipman et al., 2004a), and of the factors underlying variation in final segment number. In *Strigamia*, these factors include both environmental temperature during embryogenesis (Vedel et al., 2008, 2010), and genetic factors (Vedel et al., 2009).

The list of publications on the embryonic development of centipedes is not long. From the nineteenth century on, these are: (organised by order, from more basal to more derived) Scutigeromorphs (Dohle, 1970; Knoll, 1974), Lithobiomorphs (Hertzel, 1983, 1984; Kadner and Stollewerk, 2004), Scolopendromophs (Dawydoff, 1956; Heymons, 1898, 1901; Ivanov, 1940; Sakuma and Machida, 2002,

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doi:10.1016/j.ydbio.2011.11.006

2003, 2004, 2005; Whitington et al., 1991) and Geophilomorphs (Chipman et al., 2004b; Metschnikoff, 1875; Sograff, 1882, 1883).

Only three of these studies provide detailed descriptions of centipede development from the very early stages (Heymons, 1901; Knoll, 1974; Sograff, 1883), and none provides a timeline for development. All major reviews on centipede development, and indeed of myriapods as a whole, have been based exclusively on Heymons' decription of *Scolopendra* (Anderson, 1973; Gilbert, 1997; Johannsen and Butt, 1941).

Both Heymons' description of *Scolopendra*, and Sograff's description of a geophilomorph similar to *Strigamia* are excellent detailed studies, but both are limited by the tools of the nineteenth century, and indeed by the preconceptions of their authors. One significant error in Heymon's description has already been pointed out by Chipman et al. (2004b). (Heymon inverted the orientation of the AP axis at early germ band stages, which led him to the incorrect inference that the most anterior segments form in a posterior to anterior direction). A modern description is clearly needed to form the basis for more specific studies of all aspects of myriapod development.

We have previously published a brief description of *Strigamia* development (Chipman et al., 2004b). At that time, it was thought that the eggs of these centipedes would not survive for long when removed from the care of the mothers, but we have found this not to be true. We can now rear them reliably from the earliest stages to hatching, and beyond. This has allowed us to determine a timeline for development under standard conditions, and to provide a far more detailed description of development from sequential and time-lapse observations. This reveals that some assumptions in that earlier work were incorrect (e.g. that segment addition is complete at the time of sinking).

The only other staging series published for centipedes is that published for the scolopendromorph *Ethmostigmus rubripes* (Whitington et al., 1991). However, there are considerable differences in the relative timing of developmental events in scolopendromorphs as compared with geophilomorphs, so the two staging series are not directly comparable.

In defining stages of development, we have considered two main criteria:

1) That the stages represent a distinct phase of development, clearly distinguishable from the preceding and following stages.
2) That, in so far as possible, the stage should be recognised from the external morphology through the chorion, so as to provide a useful and practical tool to stage and score both live and fixed eggs.

Although much of what we report confirms and extends earlier studies, we also correct some earlier misapprehensions, concerning for example the timing of segment addition, and highlight some unusual aspects of geophilomorph centipede development not previously appreciated.

Materials and methods

Field collection and culturing of eggs

Strigamia maritima eggs were collected from a previously studied population (Chipman et al., 2004b) near Brora (Scotland) in late May or early June across the years 2006 to 2010. Individual clutches of eggs were collected from the brooding females into 6 cm diameter plastic Petri dishes, either on filter papers moistened with Locust Embryo Saline (LES, Ho et al., 1997) or into a shallow layer of mineral oil (Sigma M8410, embryo tested, Vedel et al., 2010). Petri dishes with clutches were placed into closed water saturated boxes within insulated cooled containers and kept between approx 10 and 20 °C until returned to the laboratory (typically 24–36 h after field collection). In the lab, the boxes were kept in incubators set at temperatures between 8 and 25 °C. We used 13 °C as the standard incubation temperature for the work described here, unless stated otherwise. Some clutches were initially kept at 4 °C, a temperature at which development almost stops. This extended the period of availability of embryos for experimental work.

Potentially gravid females were also collected in the field into small egg lay chambers (2 ml tubes or small boxes with some matrix collected from the egg lay site) and returned to the laboratory, where they were inspected after 2–16 days. A few of these (7/38) laid clutches of eggs in the lab, which were cultured on moist paper as above.

Egg fixation, staining, sectioning and visualisation

Eggs maintained on LES soaked filter papers were fixed in 4% formaldehyde in 0.5X PBS (1–3 days at room temperature), after incubating them for 30 min in 0.5X PBS (to increase turgidity and reduce shrinkage during fixation). Eggs cultured under mineral oil were fixed in 50:50 heptane: 4% formaldehyde in 0.5X PBS, on a nutating mixer for 1–3 days at room temperature. For storage after fixation, eggs were rinsed in PBS, de-hydrated through a PBT:methanol series over 20′–40′, and kept in 100% methanol at −20 °C. Eggs where then manually dechorionated using fine tweezers (Dumont 55) and rinsed in PBT (PBS + 0.1% Tween 20). Even after long fixation, it is difficult to dechorionate the earliest stages without damaging the egg surface. The chorion is freely permeable to water and formaldehyde, but not to any of the stains that we have tried (see Results).

Selected dechorionated eggs were stained with nuclear stains—Hoechst 34580 (2 μg/ml), Sybr Green (1X) or Sytox Green (1 μM), and stained for actin with FITC phalloidin (SIGMA, 1 μg/ml), in all cases for 1 h on a shaker at room temperature in PBT.

Pictures of whole eggs (both live and fixed) were taken with a Leica MZFLIII stereomicroscope with a Leica DFC 500 Camera (Leica Firecam software), with lateral light and black background. Pictures of some fixed eggs were taken with a Leica TCS SP5 confocal microscope, immersed in an anti-bleaching medium (85.5% glycerol, 5% DABCO, 9.5% PBS) on a microscope cavity slide with a cover slip.

Selected blastoderm and germ band stage embryos were manually dissected from the yolk with fine tweezers and very fine brushes and flat mounted in 15 μl of 70% glycerol in PBT on a microscope slide with a cover slip.

For sectioning, fixed eggs were dehydrated through a PBT:ethanol series. From 100% ethanol, they were washed in 1:1 100% ethanol:acetone for 15′, then in acetone 2X for 15′, then overnight in 1:1 acetone:catalysed resin (Araldite 502, 19%, EMbed 812, 24%, DDSA, 57%, Electron Microscopy Sciences), all at room temperature and in sealed containers. Embryos were then placed in 100% resin for around 5 h in open containers, mounted in 100% resin in embedding moulds and left to polymerize for 2 days at 60 °C. Semithin sections were made with an ultramicrotome and stained with toluidine blue.

Pictures of flat mounted preparations and sections were taken with a Zeiss Axiophot compound microscope with a Leica FC3 FX camera. Contrast and colour of the photos were adjusted using Adobe Photoshop.

Chorion thickness was measured on transverse sections of fixed eggs.

Time-lapse microscopy

To follow development directly, eggs were viewed by time lapse microscopy using a Zeiss Axioskop 2 mot plus compound microscope, with an Axiocam MRm camera and Axiovision rel 4.6 software. To mount the egg, a ring of silicone grease (approx 2 cm in diameter and 1.5–2 mm high) was prepared on a glass slide, with an additional small droplet of grease at the centre of the ring to hold the egg in position. The egg was placed on the central droplet, covered with