Fate and nature of the onychophoran mouth–anus furrow and its contribution to the blastopore

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The ancestral states of bilaterian development, and which living groups have conserved them the most, has been a controversial topic in biology for well over a hundred years. In recent years, the idea that gastrulation primitively proceeded via the formation of a slit-like blastopore that then evolved into either protostomy or deuterostomy has gained renewed attention and some molecular developmental support. One of the key pieces of evidence for this ‘amphistomy’ theory comes from the onychophorans, which form a clear ventral groove during gastrulation. The interpretation of this structure has, however, proved problematic. Based on expression patterns of forkhead (fkh), caudal (cad), brachyury (bra) and wingless (wg/Wnt1), we show that this groove does not correspond to the blastopore, even though both the mouth and anus later develop from it. Rather, the posterior pit appears to be the blastopore; the posterior of the groove later fuses with it to form the definitive anus. Onychophoran development therefore represents a case of ‘concealed’ deuterostomy. The new data from the onychophorans thus remove one of the key pieces of evidence for the amphistomy theory. Rather, in line with other recent results, it suggests that ancestral bilaterian development was deuterostomic.

1. Introduction

The phylum Onychophora is closely related to the Arthropoda [1]. Because of the phylogenetic position of the onychophorans at the base of the highly divergent arthropods, understanding of onychophoran development is critical for the unravelling of ancestral developmental traits of the common ancestor of onychophorans and arthropods. However, it is still the case that relatively little is known about onychophoran biology, including their development. Despite their close relationship to arthropods, the development of onychophorans is in some aspects crucially different from arthropod development. One such difference concerns the fate of the blastopore and the development of mouth and anus. Because arthropods belong to the protostomes the blastopore should in theory [2] only develop into the mouth. Careful studies of early arthropod development, however, revealed that a number of arthropods undergo deuterostomic development [3–5]. As a probable adaptation to yolk-rich eggs, the arthropod mouth and anus often form secondarily after complete closure of the blastopore [3,6,7]. This developmental mode can also be observed in some onychophorans [8,9]. In other onychophorans, as for example in Euperipatoides kanangrensis, the model organism of this study, the blastopore is believed to first develop into one single opening, a slit-like furrow along the anterior–posterior axis. After median closure, the remnants of this furrow give rise to mouth and anus [9–12].

The nature of the onychophoran blastopore is thus, despite several studies, still not fully resolved. While some authors believe indeed that the so-called mouth–anus furrow represents the blastopore [10–13], others suggest that the pit posterior to the mouth–anus furrow represents the blastopore [8,9]. These diverging views are owing to different opinions on whether the furrow is involved in endoderm formation [9].
There is consensus about the position and origin of the onychophoran mouth; it is believed to originate from the anterior part of the furrow, regardless of the furrow’s nature [9–12]. Most authors agree with the idea that the anus is formed from the posterior opening of the furrow [9–12]. A recent study by Eriksson & Tait [13], however, showed that in E. kanangrensis the mouth–anus furrow is initially continuous with the posterior pit, the blastopore sensu [8,9]. It suggests further that the onychophoran anus forms posterior to the mouth–anus furrow [13]. The authors argue that the posterior opening of the furrow does not form the anus, but that this opening disappears at some point during embryogenesis. This conclusion is mainly based on the expression of the segment polarity gene orthologue wingless (wg/Wnt1) that is used to homologize the position of the anus of arthropod embryos with that of onychophoran embryos.

These varied forms of gastrulation and gut opening development can be set in a broader picture of how bilaterian development as a whole has developed. The puzzling presence of classical ‘protostomes’ and ‘deuterostomes’, in which the blastopore develops either into the mouth (or mouth and anus) or anus, respectively (figure 1a), led some authors to conclude that the through gut evolved at least twice in bilaterians [2]. More recently, the repeated observation of slit-like blastopores in a variety of organisms, such as annelids [14], onychophorans [9], arthropods [15] and nematodes [16], has led to the ‘amphistomy’ theory [17] in which the protostomous and deuterostomous states are both derived from a primitive slit-like blastopore that closed centrally to leave the mouth at one end and the anus at the other (figure 1b) (e.g. fig. 22.2 of [18]). Nevertheless, others have argued that the reported examples of ‘amphistomy-like’ development in fact represent special cases of protostomy or deuterostomy rather than true amphistomy as seen in annelids [5,19]. The (disputed) case in onychophorans has been suggested to have arisen as a result of terrestrialization [5].

In order to clarify these issues, we here document the fate of the onychophoran mouth–anus furrow in a series of successive embryonic stages. Our data show that the posterior of the furrow moves into a position very close to the posterior pit region (i.e. the position of the anus), and that the indention of the posterior pit disappears. The proctodaeal marker genes forkhead (fkh) and caudal (cad) are not expressed in the posterior pit but in the posterior of the mouth–anus furrow.

Exclusive expression of the blastoporal marker genes brachyury (bra), and indeed also wg/Wnt1, in the posterior pit suggests that this region represents the blastopore. This result contradicts the assumption that the ventral furrow is derived from the blastopore.

2. Material and methods

(a) Embryo collection, fixation and embryonic staging
Specimens of the onychophoran E. kanangrensis [20] were collected in New South Wales, Australia. The embryos were collected, staged and prepared for in situ hybridization experiments according to previously published methods [21,22].

(b) Gene cloning
Total RNA was isolated from E. kanangrensis embryos of different stages using TRIZOL (Invitrogen). In a subsequent step, poly-A RNA was extracted from total RNA (PolyATtract mRNA isolation system III, Promega) and reverse transcribed into cDNA.

In situ hybridization was performed as described in [23]. Digoxigenin-labelled RNA probes for all genes were transcribed from

(c) Whole mount in situ hybridization and nuclear staining

Figure 1. The fate of the blastopore during development. The blastopore either gives rise to the anus (a; deuterostomy), the mouth (m; protostomy) or both, the anus and the mouth (amphistomy). (b) The amphistomy theory is based on the idea that a slit-like furrow was present in the last common ancestor of all bilaterians. This furrow then gave rise either to the anus of deuterostomes (via anterior closure), the mouth of protostomes (via posterior closure) or both, mouth and anus (via median closure). Grey ovals represent embryos. The blastopore and its remnants are in white. Closure of the blastopore is indicated in black. a, anus; m, mouth.
the entire cloned fragments. Cell nuclei were stained by incubation of the embryos in 1 μg ml⁻¹ of the fluorescent dye 4-6-diamidino-2-phenylindol (DAPI) in phosphate-buffered saline with 0.1% Tween-20 (PBST) for 30 min followed by extensive washes in PBST.

(d) Data documentation
Embryos were analysed under a Leica dissection microscope equipped with a Leica DC100 digital camera. The image processing software Adobe PHOTOSHOP CS2 (v. 9.0.1 for Apple Macintosh) was used for linear corrections of brightness, contrast and colour values in all images.

3. Results
(a) Expression of marker genes
Gene expression analysis in *E. kanangrensis* represents a well-established technique. The level of background is usually...
Figure 3. Expression of *E. kanangrensis* brachyury (*bra*). For all panels, anterior is to the left and ventral view. Panels (a’–c’) represent DAPI-stained embryos corresponding to embryos shown in (a–c). Green arrows point to the position of the anus. Abbreviations as in figure 2; proct, proctodaeum.

*Euperipatoides kanangrensis* forkhead (*Ek-fkh*) is expressed strongly in the germ disc around the mouth–anus furrow, but not the posterior pit (figure 2a). When the two halves of the embryo grow towards the anterior on either side of the mouth–anus furrow, transcripts of *fkh* are detected in all tissue in between (figure 2b,c). Stronger signal, however, is along the ventral margins of the germ band(s) and close to the furrow (figure 2b,c). When the mouth–anus furrow closes, expression disappears from the tissue that has grown together, but remains in the openings, i.e. the mouth and the posterior part of the mouth–anus furrow (figure 2d,e,e’). Anterior expression is at later stages, after the mouth opening has disappeared behind the outgrowing head bulbs, at either side of the mouth in the posterior rim of these bulbs (figure 2d–f). At stage 16, the posterior signal is in the anus, but does not extend into posterior adjacent tissue of the posterior pit (figure 2f,f’). At later developmental stages, *fkh* is also transcribed inside the head bulbs. Expression in and around the mouth, in the anus and along the ventral margin of the split germ band is present at all developmental stages, but never extends into the posterior pit (figure 2, all panels).

At all investigated stages, *E. kanangrensis* brachyury (*Ek-bra*) is expressed in the posterior pit, but is absent from the mouth–anus furrow (including the anus; figure 3). At later stages, *Ek-bra* expressing cells contribute to the developing hindgut (figure 3d,e). No other signal of *Ek-bra* has been observed at any of the investigated developmental stages.

*Euperipatoides kanangrensis* caudal (*Ek-cad*) is expressed in the posterior part of the developing embryo, but not in the centre of the posterior pit (figure 4a–d) (cf. [22] supplement). Its expression surrounds the pit and extends anteriorly into the posterior of the mouth–anus furrow, and a few last-formed posterior segments (figure 4a–d). Expression of *cad* in the posterior segments is strongest in the dorsal edge of the germ band and as transverse stripes between the coelomic pouches (figure 4b,c). The segmental expression may form a short-range gradient, but this is unclear from the available data. At later stages, *Ek-cad* is expressed in the ventral portion of the anal valves and also in tissue that possibly turns into the hindgut (figure 4c,f).

(b) Successive developmental stages showing origin and migration of the anus

At approximately stage 7/8, the mouth–anus furrow splits into mouth and anus; this is the result of median closure of the furrow. The part of the mouth–anus furrow that develops into the mouth soon after appears as a small round structure. The part of the furrow that develops into the later anus remains for some time as a slit-like structure (figure 5a). The suture of the closing central part of the mouth–anus furrow is still visible in figure 5a. At this stage (stage 9), the pit that is located posterior to the furrow appears as a round anus-like indentation (figure 5a). At the subsequent stage 10, the pit is compressed. Now, the posterior part of the mouth–anus furrow is round and looks like a typical anus (figure 5b). At stage 11, the anus ‘moves’ further towards the posterior pit. The latter does not appear as a typical anus anymore. This becomes clear at the subsequent stages 12–14 (figure 5d,e). The former indentation has fully disappeared, whereas the opening of the anus (the former posterior part of the mouth–anus furrow) persists. At stage 15, the anal valves form, and the anus is now in a position posterior and between these structures (figure 5f). This is the position of the anus as seen at later developmental stages and adult onychophorans.

4. Discussion

(a) The onychophoran anus forms from the posterior of the mouth–anus furrow

We carefully observed formation and fate of the mouth and the anus in *E. kanangrensis*. Our observations reveal that the
slit-like groove which forms early during development [13,22] closes from its centre to the outer edges leaving an anterior opening and a posterior opening (figure 5). On purely morphological grounds, this could be considered an instance of amphistomy. However, our gene expression results do not support this interpretation (discussed below).

Posterior to the posterior remnant of this furrow is a region of high cell density. In the centre of this area an indentation forms. This is the blastopore sensu [9] (discussed below). The anterior opening of the furrow forms the future mouth as concordantly suggested by authors [8–13]. The posterior opening remains throughout development and does not, as previously suggested by Eriksson & Tait [13], disappear at later developmental stages. By contrast, the indentation in the centre of the posterior pit region disappears. Eriksson & Tait [13] suggested that this latter structure turns into the anus, but our observations show that this is not the case. The main argument that led [13] to suggest that the anus forms in the region posterior to the mouth–anus furrow is that the ostensible anus-marker *wg/Wnt1* is expressed in this region. Although it is true that *wg/Wnt1* serves as an anus marker in arthropods (and other bilaterians), this is merely, because, in these animals, the anus forms together with the invaginating proctodeal tissue. It has to be taken into account that *wg/Wnt1* (as a member of the Wnt pathway) is rather a blastopore marker, or a marker of invaginating cells, than a marker of the anus *per se* [24]. We investigated the expression of two molecular markers, *fkh* and *cad*. In arthropods, these genes are regularly associated with the formation of the anal valves and the anus [25–33]. As expected, *cad* is strongly expressed in the onychophoran anal valves. In addition to that, and in contrast to *wg/Wnt1*, it is also expressed in the tissue surrounding the posterior opening of the mouth–anus furrow. The *fkh* gene is exclusively expressed in the posterior of the slit anterior to the posterior pit (figure 2). At later developmental stages, *fkh* is clearly expressed in the anus, and because this expression can easily be followed throughout development, it can unambiguously be associated with the posterior of the furrow. A look at early stages reveals that neither *cad* nor *fkh* are expressed in the centre of the posterior pit, the structure that, according to Eriksson & Tait [13], would form the anus. This shows that the onychophoran anus originates from the posterior opening of the mouth–anus furrow and not the region posterior to it.

(b) Where is the blastopore?

The blastopore is generally referred to as the site of endomesoderm formation (reviewed in [5]). It has been argued that the mouth–anus furrow in onychophorans may represent the blastopore, or at least part of it [9–13]. The early claims for this view were eventually grounded on the findings of [34,35], who claimed that cells which originated from the mouth–anus

![Figure 4. Expression of *E. kanangrensis* caudal (*cad*). For all panels anterior is to the left and ventral view, except panels (c,f; lateral view). Panels (d’–d”) represent DAPI-stained embryos corresponding to embryos shown in (a–d). Green arrows point to the position of the anus. White arrows point to the position of the posterior pit. Asterisks in panels (c) and (c’) mark transverse segmental stripes of expression. Abbreviations as in figure 2; av, anal valve.](image-url)
mouth–anus furrow implies that this structure is not part of the blastopore. Therefore, we suggest that the blastopore sensu [9], i.e. the posterior pit, indeed, represents the onychophoran blastopore. It is, however, possible that endodermal tissue that comes from the edges of the furrow is not under control of the canonical endomesodermal gene regulatory network, and does for this reason not express bra and/or wg/Wnt1 (or any other Euperipatoides Wnt gene) [39].

(c) Onychophoran development: another case of ‘concealed’ deuterostomy

The evidence we present here shows that the ventral slit which appears in onychophoran development is not the blastopore, even though the mouth and anus both develop from it. The blastopore (as defined in this article) exclusively develops into proctodaeal (hindgut) derivatives. In addition, none of the typical mouth or anus marker genes are expressed in the complete mouth–anus furrow in onychophorans. This differs from an annelid, where for example bra is at least weakly expressed along an elongate blastopore that develops into both mouth and anus ([40], but see [41] for different expression patterns in another annelid).

The onychophorans thus have neither protostomous nor amphitomous development. If only the region posterior to the mouth–anus furrow represents the blastopore in onychophorans, then the developmental mode is deuterostome-like. Although the blastopore does not develop into the anus, the fact that it gives rise to the hindgut suggests that onychophoran ancestors developed their anus from the blastopore (deuterostomy), and that the blastopore-independent formation of the anus (and the mouth) evolved secondarily, possibly as an adaptation to yolk-rich embryos. Protostomy and deuterostomy may thus represent a similar case of adaptive variation in early development that has also been suggested for other features such as the varying origin of the coelom [42].

The developmental mode observed in E. kanangrensis may be called ‘concealed deuterostomy’ in order to distinguish it from true deuterostomy and amphistomy. ‘Amphistomy’ in onychophorans and annelids, in the sense that both mouth and anus develop from a ventral slit-like structure, is thus not
Provision of a permit SL100159 to collect onychophorans at Kanangra Boyd National Park, and to the Australian Government Department of the Environment, Water, Heritage and the Arts for export permits WT2009-4598 and WT2012-4704. We wish to thank Jean Joss, Robyn Stutchbury, Rolf Ericsson and especially Noel Tait for their help during onychophoran collection. The onychophoran transcriptome was analysed with the kind support of Nico Posnien and Alistair McGregor.

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