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1 **Do chitons have a brain? New evidence for diversity and complexity in the polyplacophoran**
2 **central nervous system**

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10 **Running title:** Do chitons have a brain?

11 Abstract

12 Molluscs demonstrate an astonishing degree of morphological diversity, and the relationships
13 among molluscan clades have been debated for more than a century. Molluscan nervous
14 systems range from simple 'ladder-like' arrangements of nerve cords to the complex brains
15 of cephalopods. Chitons (Polyplacophora) are assumed to retain many molluscan
16 plesiomorphies, lacking neural condensation and ganglionic structure, and therefore a brain.
17 We reconstructed three-dimensional anatomical models of the nervous system in eight
18 species of chitons in an attempt to clarify chiton neuroarchitecture and its variability. The
19 specimen material incorporated both new data and digitised historic slide material originally
20 used in the work of malacologist Johannes Thiele (1860-1935). Reconstructions of whole
21 nervous systems in *Acanthochitona fascicularis*, *Callochiton septemvalvis*, *Chiton olivaceus*,
22 *Hemiarthrum setulosum*, *Lepidochitona cinerea*, *Lepidopleurus cajetanus*, and *Leptochiton*
23 *asellus*, and the anterior nervous system of *Schizoplax brandtii*, demonstrated a consistent
24 and substantial anterior concentration of nervous tissue in the circumoesophageal nerve ring.
25 This neural mass is further organised into three concentric tracts, corresponding to the paired
26 lateral, ventral, and (putatively) cerebral nerve cords. These represent homologues to the
27 three main pairs of ganglia found in other molluscs. The relative size, shape and organisation
28 of these components is highly variable among the examined taxa, but consistent with
29 previous studies of select species, and we formulated a set of neuroanatomical characters for
30 chitons. These characters are parsimony-informative for reconstructing chiton phylogeny at
31 the ordinal and subordinal levels; the identification of robust detailed homologues in neural
32 architecture will be central to future comparisons among all molluscs, and more broadly in
33 Lophotrochozoa. Modern evolutionary thinking, and modern tomographic technology, bring

34 new light to an old problem. Contrary to almost all previous descriptions, the size and
35 structure of the chiton anterior nerve ring unambiguously qualify it as a true brain with cordal
36 substructure.

37 **Key words:** Molluscs, neuroanatomy, evolution, complexity.

38 **Research highlights**

39 3D reconstructions from historic histological slides reveal unappreciated complexity in chiton
40 nervous systems. The concentration and organisation of nervous tissue in the
41 circumoesophageal nerve ring in eight species unambiguously qualify it as a true brain.

42 Introduction

43 Chitons are benthic marine molluscs found from the intertidal to abyssal depths across the
44 globe. The class is characterised by eight articulated dorsal shell valves, which protect the
45 foot, viscera, and pallial cavity. Most species graze the substrata using a biomineralised radula
46 (Sigwart & Schwabe, 2017). They lack cephalic eyes and tentacles, but possess an extensive
47 network of sensory pores in the valves, of which some have evolved to form ‘shell eyes’
48 capable of true image formation (Omelich, 1967; Speiser, Eernisse, & Johnsen, 2011). Their
49 simple body plan (dorsal shell, ventral foot; anterior mouth, posterior anus) has been
50 purported to reflect a plesiomorphic or ‘primitive’ state within molluscs (Hyman, 1967), with
51 many classical projections of a common ancestor to this incredibly diverse phylum resembling
52 living chitons (Haszprunar, 1992; Salvini-Plawen, 1981, 1985). This places chitons in a unique
53 position of interest to the evolution of animal body plans.

54 As a phylum, molluscs demonstrate some of the wildest morphological diversity and disparity
55 found in the animal kingdom, and conclusively resolving molluscan relationships remains a
56 fundamental challenge (Sigwart, 2017; Telford & Budd, 2011). Molecular studies provide
57 reasonably robust support for certain groupings, including a monophyletic Aplacophora and
58 its sister relationship with chitons (Aculifera; Kocot et al., 2011; Smith et al., 2011, 2013), and
59 recent fossil findings also support a chiton-like ancestor for the clade Aculifera (Caron,
60 Scheltema, Schander, & Rudkin, 2006; Sigwart & Sutton, 2007). But other nodes in molluscan
61 phylogeny remain uncertain, and recovered topologies vary with taxonomic coverage and
62 choice of outgroups (Kocot et al., 2011; Sigwart & Lindberg, 2015; Smith et al., 2011; Telford
63 & Budd, 2011). Another substantial problem with the interpretation of unstable phylogenies
64 is the interpreted polarity of key characters: among so much diversification, including radical

65 changes, convergence and reversals, which features are plesiomorphic and which are
66 derived?

67 One key aspect of the interpreted simplicity of polyplacophorans is their nervous system.
68 Chitons have no ganglia; the distinct cerebral, pedal and pleural ganglia that compose the
69 brain or circumoesophageal nerve ring in most other molluscan classes are entirely lacking
70 (Faller, Rothe, Todt, Schmidt-Rhaesa, & Loesel, 2012; Moroz, Nezlin, Elofsson, & Sakharov,
71 1994; Sigwart & Sumner-Rooney, 2015). Chiton nervous system architecture comprises two
72 pairs of medullary cords, lateral and ventral, running longitudinally through the body and
73 forming a visceral loop, and an anterior circumoesophageal nerve ring (Eernisse & Reynolds,
74 1994; Hyman, 1967; Moroz, 2009). The anterior part of this ring is thought to be homologous
75 to the cerebral ganglia in other molluscan classes (Sigwart & Sumner-Rooney, 2015;
76 Voronezhskaya, Tyurin, & Nezlin, 2002). Chitons are widely considered to lack a true brain
77 (Moroz, 2009; Sigwart & Sumner-Rooney, 2015), and their nervous system is invariably
78 described as “primitive” and “ladderlike” (Arbas, Levine, & Strausfeld, 2011a; Morton &
79 Yonge, 1964). Though they occupy a position of particular neuroevolutionary significance, the
80 assumption of a homogenously primitive neural architecture may explain why they have been
81 understudied neurobiologically, with the exception of their sensory aesthete and shell eye
82 structures.

83 Historical anatomical illustrations of different chiton species demonstrate reasonable
84 variation in key features, such as the shape of the circumoesophageal nerve ring and the size
85 and position of the buccal ganglia (Heath, 1904; Plate, 1899). In almost all schematic
86 depictions, the circumoesophageal nerve ring is slender, often no wider than the individual
87 lateral or ventral cords, with slight postero-lateral swellings at the origin of the cords (Bullock

88 & Horridge, 1965). The majority of previous works illustrate dissected material, which
89 naturally results in disruption to the specimen; it is not clear how much of the variation seen
90 in depicted nervous systems is *bona fide*. Despite this, objective comparisons of nervous
91 system architecture and neuroanatomy across the class are almost entirely absent.

92 The advent of three-dimensional anatomical imaging techniques has helped morphological
93 studies to blossom in the digital age (Sumner-Rooney & Sigwart, 2017). The ability to
94 accurately reconstruct internal structural characters of whole organisms has revolutionised
95 the field, and provides crucial insight to organ systems that have previously been observed
96 through potentially disruptive dissections or individual tissue sections, which can be
97 challenging to quantify. It is a testament to the astonishing skill and diligence of the classic
98 morphologists that many of their decades- or even centuries-old observations using these
99 methods still remain at the forefront of our knowledge today, but the application of new
100 technology to morphological studies is constantly improving accessibility, resolution and
101 efficiency in anatomy.

102 Morphology continues to play a crucial role in phylogenetics and systematics in the face of an
103 increasingly molecular future. Nervous system characters have already shed significant light
104 on arthropod phylogeny (Strausfeld & Andrew, 2011), and are also emerging as an important
105 tool in resolving molluscan relationships (Friedrich, Wanninger, Brückner, & Haszprunar,
106 2002; Shigeno, Parnaik, Albertin, & Ragsdale, 2015; Shigeno, Sasaki, & Haszprunar, 2007;
107 Sumner-Rooney et al., 2015; Wollesen, Rodríguez Monje, McDougall, Degnan, & Wanninger,
108 2015). Relationships within Polyplacophora are reasonably well understood, which provides
109 a phylogenetic backbone to test questions and models of character evolution; there is a well-
110 established divide between the two living orders, Lepidopleurida and Chitonida, and the

divisions of major subclades are increasingly robust (Eernisse, 2008; Okusu, Schwabe, Eernisse, & Giribet, 2003; Sigwart, Stöger, & Schwabe, 2013). Remaining uncertainty is due in part to the relatively low morphological variation found within the class, which can hamper morphological phylogenetic analyses (Sigwart et al., 2013). New morphological character sets that exhibit sufficient variation to be phylogenetically informative will contribute to the resolution of long-standing questions at different taxonomic levels in molluscs, and neurocladistics could potentially form a core part of future analyses (Faller et al., 2012; Sumner-Rooney et al., 2015). Crucially, morphological data also provide insight to ancestral conditions and characters, beyond pure relationships between taxa, which molecular data often cannot. Such insights are critical to resolving high-level relationships as well as evolutionary changes in body plan and organisation on a macroevolutionary scale.

Indeed, interest in molluscan nervous systems has already helped shed light on inter- and intra-class molluscan evolutionary relationships (Faller et al., 2012; Friedrich et al., 2002; Haszprunar, 1988; Shigeno et al., 2007, 2015; Sumner-Rooney et al., 2015; Wanninger & Haszprunar, 2003). Among the resulting publications are several studies of chiton nervous systems, of which some found greater than expected size or complexity of the circumoesophageal nerve ring (Faller et al., 2012; Gantner, 1989; Sigwart et al., 2014); however, capacity for diversity remains underappreciated as a fundamental characteristic of the chiton nervous system. A departure from previous descriptions could undermine the narrative consensus of polyplacophoran nervous systems as undifferentiated and primitive.

Here, we pursued two specific aims to move beyond the assumptions of “primitive” chitons and provide a more objective identification and assessment of relevant characters. First, we evaluated the available characters within the chiton nervous system and how these vary

among species in the class. Second, we examined neuroanatomical structures within the chiton nervous system that represent homologies to standard features in other molluscan classes, such as the main pairs of ganglia. A robust description of the chiton nervous system and its range of morphological variation will be critical to understanding the relationships between chitons and other molluscan classes and potentially between molluscs and other phyla (Kocot, 2016).

Materials and Methods

Specimen material

Slides of serial histological sections, produced by Prof. Johannes Thiele between 1890-1910, were drawn from the Malacology collection at the Museum für Naturkunde, Berlin (ZMB/Moll 230880-230999). Series of eight species were sufficiently complete and of sufficient quality to reconstruct digital models of the nervous system: *Acanthochitona fascicularis* (Linnaeus 1767), *Callochiton septemvalvis* (Montagu 1803), *Chiton olivaceus* Spengler 1797, *Hemiarthrum setulosum* Carpenter 1876, *Lepidochitona cinerea* (Linnaeus 1767), *Lepidopleurus cajetanus* (Poli 1791), *Leptochiton asellus* (Gmelin 1791), and *Schizoplax brandtii* (Middendorff 1847). These species represent each of the five major clades of living chitons (Sigwart et al., 2013); the order Lepidopleurida, and all four superfamilies in the order Chitonida: Chitonoidea, Callochitonoidea, Mopalioida and Cryptoplacoidea (Table 1).

Data on the anterior nervous system in *Leptochiton asellus* were taken from modern histological sections, produced by Sigwart and colleagues (Sigwart et al., 2014), as the anterior sections were missing from Thiele's collection.

156 *Tomographic modelling and digital analysis of the nervous system*

157 Slides were visualised on a Zeiss Axioskop stereomicroscope, using a Leica DFC490 mounted
158 camera and Leica LAS Core software for image capture. In total, 3699 histological sections
159 were digitised. Where sections or slides (sets of sections) were missing or damaged, preceding
160 or succeeding sections were duplicated to maintain voxel size during modelling. Sections of
161 *Leptochiton asellus* used for anterior nervous system modelling by Sigwart et al. (2014; slides
162 deposited to the Bavarian State Collection of Zoology, Munich) were also used in new
163 analyses: the original image stack was re-examined and re-modelled alongside the other
164 historic material.

165 Sampled images were processed and contrast-enhanced in Adobe Photoshop CS4 before
166 loading into AMIRA (v.5.3.3, FEI Visualisation Group). Image stack alignment, segmentation,
167 surface rendering, smoothing, and volume calculations were all performed in AMIRA.

168 All visible nervous tissue in the digitised images was segmented and included in
169 reconstructions. However, in some species, the identification of smaller transverse nerves or
170 commissures was hampered by section thickness, quality or stack sampling (where fine
171 nerves appeared in single sections only but the image stack sampled alternate sections). As
172 these are fine structures running parallel to the cutting plane, they often appear in only one
173 or two sections and thus are particularly vulnerable to these confounding factors. This
174 affected *Callochiton septemvalvis*, *Hemiarthrum setulosum* and *Lepidochitona cinerea*. These
175 minor nerves and their patterning are therefore not evaluated as potential characters herein,
176 for any taxa.

The degree of anterior concentration of nervous tissue was assessed by extracting nerve volumes from the oesophageal nerve ring in AMIRA and comparing these with nerve volumes taken from an equivalent section further posterior in the specimen; i.e. if the circumoesophageal nerve ring was present in 25 images in the stack, a comparative nerve volume was taken from a 25-image section at the anteroposterior midpoint of the animal. We also calculated anterior nerve volumes as a proportion of total nervous system volumes, and measured the relative lengths of the reconstructed oesophageal nerve ring and the whole nervous system. Buccal ganglia were excluded from volume calculations as they do not form part of the nerve ring and we were not able to reconstruct these in all taxa.

In order to document the variation in neural structures among the sampled taxa we formulated 17 discrete morphological cladistic characters describing the chiton nervous system from rendered models and literature (Table 2), and constructed a coded matrix for the eight taxa examined here (Table 3).

Results

Nervous systems in all species examined were consistent with the expected general chiton neural architecture: two pairs of medullary cords, the ventral and the lateral nerve cords, run longitudinally through the foot and the roof of the pallial cavity respectively, and an anterior circumoesophageal ring surrounds the oesophagus (Figures 1 and 2). Paired buccal ganglia, linked posteriorly by the buccal nerves, and subradular ganglia, located posterior to the nerve ring, were also identified in line with existing descriptions.

All species demonstrated concentration of nervous tissue in the anterior part of the animal in the circumoesophageal nerve ring. The volume of the nerve ring ranged in various species

between 50–144% greater than the volume of an equivalent part at the midpoint of the nervous system (Table 1). The nerve ring occupied between 22–28% of total nervous system volume in different species, and extended along 13–18% of total nervous system length (Table 1). These figures exclude the volume of the buccal ganglia and minor nerves as these were not reconstructed in all species (see Methods).

All examined species showed distinct mediolateral organisation of the circumoesophageal nerve ring, giving the impression of three concentric tracts. The ring itself comprises central neuropil and surrounding cell bodies, as described by many previous authors (reviewed in Bullock & Horridge, 1965; Sigwart & Sumner-Rooney, 2015); however, the central neuropil is incompletely subdivided by interjecting veins of cell bodies (Figure 3). This broadly divides the anterior and lateral regions of the nerve ring into three concentric tracts, corresponding to the lateral (outermost) and ventral (intermediate) nerve cords as well as a third central tract, which we interpret to be homologous to the cerebral ganglia (Gantner, 1987).

From their origins in the circumoesophageal ring, the ventral nerve cords project posteriorly, turning slightly medially prior to their entry to the foot, and then remaining roughly parallel until slightly anterior of the anus, where they converge. A series of transverse nerves (commissures) join the ventral cords at fairly regular intervals; these were not captured in all subsampled image stacks and so do not appear in all reconstructions, but inspection of complete slide series confirms they are a common feature across all eight species, as well as many previous illustrations (Bullock & Horridge, 1965; Faller et al., 2012; Gantner, 1989; Heath, 1904; Hyman, 1967). The lateral nerve cords extend posterolaterally from the nerve ring and enter the roof of the pallial cavity, running very close to the surface within the gill row, before joining posterior to the anus (sometimes referred to *in litt.* as the suprarectal

commissure, but in fact this appears to be a conjoining of the nerve cords, in agreement with Faller et al. 2012). Smaller nerves connect the lateral nerve cords to the adjacent ventral cords, but unlike the ventral commissures they do so at an angle, projecting posteriorly as well as medially to the ventral cords. Again, these nerves were not captured in all reconstructions but appeared in complete material for all taxa. The lateral and ventral nerve cords are roughly evenly distributed mediolaterally. Distinct buccal ganglia and medioposteriorly-projecting buccal nerves were visible dorsal to the circumoesophageal nerve ring in all specimens except *Lepidochitona cinerea* and *Schizoplax brandtii*.

The descriptions below summarise the neuroanatomical features observed in each of the eight species examined, with particular note of variations on the above generalised plan. A summary of neuroanatomical characters and their occurrence is included in Tables 2 and 3.

Descriptive neuroanatomy

The circumoesophageal nerve ring in *Acanthochitona fascicularis* (Figure 1A-C) is large, comprising 18% of the length of the nervous system and shows exaggerated posterolateral expansion, with the posterior margin being three times the thickness of the anteriormost part (sometimes referred to as the anterior commissure, Figure 1A,B). All three tracts are at their widest in this region. The ventral (pedal) and cerebral tracts are much finer at the anterior side of the ring, whereas the cerebral tract appears to be incomplete at the anterior side of the nerve ring. The tracts of the circumoesophageal nerve ring are organised on a mediolateral plane in the posterior two thirds, but the anterior part is slightly flexed so that the cerebral and ventral tracts are ventral to the lateral tract, rather than medial to it. The

buccal ganglia are situated around three-quarters of the way up the circumoesophageal nerve ring (Figure 1B). They are visible in the models as slight expansions of the buccal nerves, which project posteriorly and appear to converge well posterior of the nerve ring; however, this was not recovered in the available sections. Subradular ganglia are small and bean-shaped, curving slightly laterally, and are located directly dorsal to the posterior edge of the cerebral tract. Ventral-lateral commissures were not visible in the examined sections, but have been illustrated in congeneric species (Pelseneer, 1898; Plate, 1899). The lateral and ventral nerve cords originate in parallel (at the same point on the antero-posterior axis), at the posterior edge of the nerve ring. The ventral cords are slightly thicker than the lateral cords, particularly in the anterior half of the foot, and several ventral commissures were reconstructed, though more may be present that were missed as a result of histological section thickness and stack subsampling (Pelseneer, 1898; Plate, 1899).

Callochiton septemvalvis (Figure 1D–F) has a slightly shorter, ovoid circumoesophageal nerve ring, occupying 14% of total nervous system length. The three tracts of the ring are organised dorsoventrally as well as mediolaterally, and are of consistent thickness throughout, except the anteriormost portion of the cerebral tract, which appears to be absent. The lateral and ventral tracts are of similar thickness, and larger than the cerebral tract. The buccal ganglia and nerves are situated quite anteriorly; the ganglia are dorsal to the anterior margin of the nerve ring, and *C. septemvalvis* was the only species whose short, thick buccal nerves converged within the circumoesophageal nerve ring, anterior to its posterior margin (Figure 1E). Subradular ganglia were small and triangular, located directly dorsal to the posterior edge of the cerebral tract and joined posteriorly by a slim commissure that was free of cell bodies. The ventral and lateral nerve cords are of similar thickness, and several ventral and ventral-

lateral commissures were reconstructed intermittently throughout the length of the body; more were observed in complete section series but were not recovered in reconstruction.

Chiton olivaceus (Figure 1G–I) has a regularly organised circumoesophageal nerve ring occupying 17% of the length of the nervous system. All three tracts are anteriorly joined and of consistent thickness, and while the cerebral and ventral tracts are slightly ventral to the lateral tract, the ring is mostly organised mediolaterally. The ventral tract is thicker than the others, followed by the lateral tract. The buccal nerves and ganglia extend beyond the nerve ring both anteriorly and posteriorly, with the centres of the buccal ganglia located dorsal to the anteriormost edge of the nerve ring, and the slender buccal nerves conjoin posterior to the base of the cerebral ring, in line with the subradular nerves. Paired triangular subradular ganglia are situated ventral to the posterior margin of the nerve ring. The ventral and lateral nerve cords are of similar thickness throughout the length of the body, and the ventral nerve cords are slightly closer to each other than to the lateral nerve cords. Some ventral commissures were reconstructed, though more were visible in the full series of histological sections. We did not reconstruct any ventral-lateral commissures; however both types are illustrated as numerous but very fine structures present along the whole length of the animal in *Chiton olivaceus* (as *C. siculus*) by Haller (1882), so it is likely these were not captured by thick sections and subsampling.

Hemiarthrum setulosum (Figure 1J–L) is the only species to show a potentially incomplete lateral tract in the circumoesophageal nerve ring, with the ventral and cerebral tracts both projecting further anterior than in other species (see Figure 1K). The nerve ring accounts for 14% of total nervous system length. The ventral tract of the circumoesophageal nerve ring is again thicker than the lateral and cerebral tracts. The ring is flattened dorsoventrally, with

only a slight ventral projection of the medial regions. The lateral and ventral nerve cords originate almost in parallel, with the ventral nerve cords being slightly further posterior. The buccal nerves are quite short, with the buccal ganglia being positioned dorsal to the anterior side of the circumoesophageal nerve ring, and the buccal nerves converging in line with the posterior margin of the ring. The buccal ganglia are also innervated by two parallel nerves projecting anterodorsally from the posterior side of the cerebral ring. Subradular ganglia are clearly defined, and are located significantly posterior to the circumoesophageal nerve ring, in line with the first ventral commissure. The ventral nerve cords are laterally distributed, closer to the lateral nerve cords than to each other. The ventral and lateral nerve cords are similar in diameter. A few ventral-lateral and ventral commissures were reconstructed, with several more visible in the complete section series along the length of the specimen.

The condition of sections of *Lepidochitona cinerea* (Figure 2A–C) hampered the identification of structures much beyond the basic architecture of the nervous system, but the differentiation between the lateral, ventral and cerebral tracts of the circumoesophageal nerve ring was still visible. The nerve ring is relatively short, occupying just 13% of the length of the nervous system. The lateral tract is of roughly uniform thickness, but both the ventral and cerebral tracts are narrower at the anterior side of the ring. The origins of the lateral nerve cords are slightly anterior to those of the ventral nerve cords. No buccal or subradular ganglia, or buccal nerves, were visible, but these have been described by Faller et al. (2012). Lateral nerve cords appear to be slightly thicker than the ventral nerve cords. The ventral and lateral nerve cords were both distributed laterally, with both pairs being wide-set within the body cavity in comparison with the circumoesophageal nerve ring (Figure 2A). Only the anterior and the two posteriormost ventral commissures were visible, but further

commissures have been observed along the length of the foot by other authors (Faller et al., 2012; Gegenbaur, 1878). Ventral-lateral commissures are faintly visible (Faller et al., 2012) or implied (Gegenbaur, 1878) in previous images of *L. cinerea*, but are presumably much finer than the ventral commissures as they are not fully reconstructed in these, or the current, studies.

Lepidopleurus cajetanus (Figure 2D–F) shows complete lateral, ventral and putative cerebral nerve tracts in the circumoesophageal nerve ring, with the latter being greatly narrowed at the anterior side. The nerve ring is quite short, accounting for 13% of the nervous system length. The anterior and lateral sides of the ring are flexed slightly dorsally. The buccal nerves converge posterior to the circumoesophageal nerve ring, but the buccal ganglia were not visible. Triangular swellings are found at the lateral posterior margins of the putative cerebral tract. The subradular ganglia are drop-shaped and curve slightly medially; they are located dorsal and posterior to the circumoesophageal nerve ring (Figure 2E). The origins of the lateral nerve cords are anterior to those of the ventral nerve cords, around three quarters of the way to the posterior edge of the nerve ring. The ventral nerve cords were very closely associated and connected by many visible commissures. The ventral nerve cords were thicker than the lateral nerve cords, particularly at the anterior end of the foot. No ventrolateral commissures are visible in the sections examined, but Plate described them as present in small numbers and difficult to find (Plate, 1897).

Leptochiton asellus (Figure 2G–I) was reconstructed from two separate slide series: one for the anterior nervous system (material from Sigwart et al. 2014) and one for the rest of the body (from the Thiele collection, MfN), so nerve volumes were not comparable. Recently-produced sections show the same concentric partitioning of the circumoesophageal nerve

ring, with all three tracts being joined anteriorly and of uniform thickness throughout. The cerebral tract is much narrower than the ventral and lateral tracts, and the lateral tract is slightly smaller than the ventral. The ring is flexed dorsally at the anterior edge. The lateral nerve cords originate significantly anterior to the ventral nerve cords, with the latter separating from the cerebral tract at the posterior side of the nerve ring. A pair of buccal ganglia is located dorsal to the anterior edge of the nerve ring, turning anteromedially towards each other. The buccal nerves briefly extend laterally before turning, projecting posteromedially and converging in line with the posterior margin of the cerebral ring. The large, distinct subradular ganglia are dorsal and posterior to the posterior margin of the circumoesophageal ring. The lateral and ventral nerve cords are evenly distributed, though the lateral nerve cords begin to turn medially anterior of the midpoint in the body. Both ventral commissures and the ventrolateral connectives were prominent and could be reconstructed throughout the body.

Only the anterior part of *Schizoplax brandtii* (Figure 2J, K) was available, but reconstruction showed that the same concentric arrangement was discernible in the circumoesophageal nerve ring. The ring is widest at the lateral sides, and flexes dorsally out the outer margins. The cerebral tract is prominent and slightly wider at the lateral sides, with a slender anterior completion. It was not possible to trace the posterior completion of the cerebral tract. By contrast, the ventral tract appears to be incomplete anteriorly, and is broadest in the lateral part of the ring even in comparison to the anterior part of the ventral cords. The lateral tract is also widest at the postero-lateral edge of the circumoesophageal nerve ring. The origins of the lateral nerve cords are situated definitively anterior to those of the ventral nerve cords. We could not identify the buccal or subradular ganglia in the subsampled image stack.

359

360 **Discussion**

361 The chiton nervous system shows significant anterior concentration in terms of volume, and
362 the circumoesophageal nerve ring is in fact composed of three concentric regions that
363 correspond to the cerebral, ventral and lateral nerve cords, i.e. these tracts are likely
364 homologues of the ganglia found in other molluscan classes. This represents a far higher level
365 of neural structure than is reflected in the established literature, particularly in “textbook”
366 summaries of chiton biology. Further, we found that the architecture of the nervous system,
367 including the composition of the circumoesophageal nerve ring, is not constant between the
368 eight species studied herein, which includes representatives of all the major extant
369 polyplacophoran clades. Chiton neuroanatomy is demonstrably not homogeneous across
370 taxa. Our very simple cladistic analysis was intended only to test whether the variation
371 observed among the identified characters has any correlation with established phylogenetic
372 relationships. The details that characterise these neural structures correspond to known
373 divergences in chiton phylogeny. These structures are large, and can be characterised by
374 multiple morphological features. Based on the evidence of substructure within it, we propose
375 that the circumoesophageal nerve ring of chitons represents a true brain.

376

377 *The chiton anterior nerve ring as a brain*

378 Two main metrics can be used to assess and define central nervous systems and brains. The
379 first is simply size, relative to the rest of the nervous system. Richter et al. (2010), for example,
380 define a brain as ‘the most prominent anterior condensation of neurons’; and Moroz (2009)

endorsed the classification of the brain as a ‘concentration of neurons within a defined organ-type structure’. The concentration of nerve tissue at the anterior end of the body in chitons is clear and, in terms of the proportion of nervous system volume and length, relatively consistent across multiple taxa (Table 1). The circumoesophageal nerve ring in chitons therefore certainly meets this criterion to qualify as a brain.

The other potentially defining character of a brain is complexity; although this term can be used ambiguously, size alone is not a robust indicator of complexity or ‘advanced’ brain development (Chittka & Niven, 2009). This can be related to compartmentalisation and processing of tasks or information (e.g. Riebli & Reichert, 2015). At its simplest, this implies a subdivision of the nerve mass into distinct parts. In some cases this may be the division of the cerebral ganglia into distinct neuropil compartments (e.g. Faller et al., 2012), or the amalgamation of the cerebral, pleural ganglia and, in the case of cephalopods, the pedal ganglia into clusters or even fused structures contributes to a multipartite brain structure (Young, 1965). The oesophageal nerve ring also demonstrates a level of spatial organisation unappreciated in much of the existing, and particularly the modern, literature. Gegenbaur (1878: p.344) described the chiton anterior commissure as ‘a nervous band formed of two chords’. (The two cords are those identified herein as the lateral and the ventral tracts; Gegenbaur did not identify the innermost tract as separate.) Plate (1897) later depicted the medio-lateral partitioning of the circumoesophageal nerve ring into three distinct sections in *Acanthopleura echinata* (Figs 104 and 105, plate 10). Gantner (1989) also observed subdivisions of the neuropil in the nerve ring of *Lepidochitona monterosatoi* and identified ventral, lateral and subcerebral parts in his thesis, and Faller et al. (2012) described partitioning of the neuropil in at least the anterior part of the nerve ring in *Acanthochitona*

crinita and *Lepidochitona cinerea*. This is evidently the same organisational patterning we have identified in representatives of all the major living clades of chitons. However, this hugely significant feature is not reported in the majority of chiton literature, and the consensus that chitons lack a brain has persisted (Arbas et al., 2011; Eernisse, 2007; Moroz, 2009; Sigwart & Sumner-Rooney, 2015). The circumoesophageal nerve ring in chitons is large, and contains well described complex sub-structure (herein, and in previous studies), two major criteria to qualify anterior neural mass as a *bona fide* brain.

In light of the classification of the nerve ring as a brain in the true anatomical sense, questions immediately arise concerning its function and capacity. Almost nothing is known about nervous system physiology in chitons. The extent of centralisation and processing in the anterior nervous system has never been examined to our knowledge. The widespread belief that such centralisation would be minimal based on the apparent absence of a brain would limit motivation for such studies. Electrophysiological techniques have been deployed only for studies of muscle physiology and pericardial innervation (Burnstock, Greenberg, Kirby, & Willis, 1967; Matsumura & Kuwasawa, 1996), and no published recordings have ever been taken directly from the nervous system itself. Exploratory recordings taken from the anterior nervous system of *Leptochiton asellus* indicated potential concentration of nerve activity in the circumoesophageal nerve ring compared to the lateral and ventral nerve cords (Sumner-Rooney, 2015); spike frequency and amplitude were dramatically increased in the nerve ring. This requires further investigation, but could represent the first physiological evidence of potential cephalisation in chitons, beyond using volume as a proxy. Additionally, chiton behaviour has traditionally been viewed as largely driven by localised reflexes (Arey & Crozier, 1919), but it is possible that the brain does play a more dominant role in centralised

processing, comparable to other molluscs. We also previously identified a putative vibration stimulus response localised to a specific region in the anterolateral part of the ring, which was consistent across subsequently tested animals (n=5) (Sumner-Rooney, 2015). Chitons lack statocysts, and no specific vibration-sensitive organs are found in the immediate vicinity; it is possible that this finding represents evidence of centralised processing of information from mechanosensors elsewhere in the body (e.g. ciliary tufts in the pallial cavity), but we stress the preliminary nature of these findings. It has been suggested that the shell eyes of some species may also integrate information across multiple eyes (Speiser et al., 2011); however, centralisation of this kind has never been physiologically observed.

The chiton nervous system and phylogeny

Neuroanatomical characters show promise in resolving longstanding questions of deep molluscan phylogeny (Friedrich et al., 2002; Sumner-Rooney et al., 2015; Wanninger & Haszprunar, 2003; Wollesen et al., 2015), but the perceived ambiguous arrangement of the chiton nervous system has confounded comparisons with other classes (Sigwart & Sumner-Rooney, 2015). The recognition of the cordal nature of the chiton brain is crucial to comparisons across the phylum. As the chiton brain is clearly not an undifferentiated neural mass, but shows a structure of concentric layers, the next question of interest is in identifying homologies with the typical aspects of ganglionic nervous systems in other molluscan classes. Species in most of the other molluscan classes show discrete or fused ganglionic organisation (Sigwart & Sumner-Rooney, 2015), and we propose that the three nerve tracts identified here are homologous, and therefore directly comparable, to the cerebral, lateral, and pedal ganglia of other molluscs.

The development of the chiton nervous system has been studied in several species, and previous findings also support our proposed model that concentrically-organised neural tracts within the circumoesophageal nerve ring are homologues to the cerebral, lateral, and pedal ganglia in other molluscs. Voronezhskaya et al. (2002) and Friedrich et al. (2002) showed that the cerebral aspect of the anterior commissure originates in the larval apical cells and expands laterally and posteriorly. The ventral nerve cords (or pedal system) appear several hours later, followed by the lateral nerve cords, with both expanding bidirectionally from further posterior in the developing animal (Friedrich et al., 2002; Voronezhskaya et al., 2002). Images from Voronezhskaya et al. (2002) show the oesophageal nerve ring comprising discrete parts as early as 42 hours post-fertilisation, when the ventral and lateral cords have made contact with the cerebral region, with the “cerebral ganglia” labelled at the inner edge of the nerve ring. Faller et al. (2012: p.166 figs. 9B, 10B) and Voronezhskaya et al. (2002: figs. 2F,G, 4F,G) depict juvenile *Acanthochitona crinita*, *Lepidochitona cinerea* and *Ischnochiton hakodadensis* with clear division in the circumoesophageal nerve ring that correspond to the three regions we find here. Among all molluscs, cerebral ganglia originate from ectodermal invagination of the apical region and this is considered a molluscan symplesiomorphy; additional ectodermal invaginations give rise to lateral and pedal cords (Raven, 1959). The sequential and positional development of the cerebral, ventral and lateral cords could support the formation of three concentric tracts within the circumoesophageal nerve ring as indicated in a schematic diagram (Figure 4).

The concentric ring arrangement of the chiton brain is not unique, and this architectural configuration raises important questions regarding nervous system evolution in molluscs. Shigeno et al. (2015) demonstrated that the brain of *Octopus bimaculoides* also develops as

a series of concentric cords, and not as discrete ganglionic structures. This is reminiscent of the proposed model for the chiton nervous system, but significant differences between the two discourage speculation about shared ancestral conditions. Developmental patterning in the chiton and cephalopod nervous systems differ substantially (Fritsch, Wollesen, & Wanninger, 2016; Fritsch, Wollesen, de Oliveira, & Wanninger, 2015), and the dominant outermost region of the brain in cephalopods is homologous to the smaller inner cerebral tract in chitons (Shigeno et al., 2015). Other complex brains follow this pattern of fusing ganglionic structures into layers, including the vertebrate brain (Raven, 1959). The appearance of two such systems among molluscs may reflect the relative ease of patterning a concentric cordal brain in place of a ganglionic one as separately derived conditions. Interestingly, there is evidence from within Gastropoda that molluscs exhibit a high degree of neural plasticity, with varying levels of neural fusion in the brains of even relatively closely related species (Haszprunar, 1988). However, without conclusive evidence regarding the polarity of characters that describe molluscan nervous system architecture, we cannot eliminate entirely the possibility that this cordal state is plesiomorphic.

The nature of the plesiomorphic state in molluscs requires careful, objective consideration. The chiton nervous system has been compared to that of monoplacophorans (Lemche & Wingstrand, 1959), and both monoplacophorans and chitons are colloquially considered 'primitive', but it is not clear to what extent that is informative. In contrast to chitons, the monoplacophoran anterior nervous system is apparently composed of ganglia (Sigwart & Sumner-Rooney, 2015). Indeed, in the original description of *Neopilina galathea*, the cerebral ganglia are explicitly described as "complex" and "tripartite, with swellings at the base of the pedal cord, the lateral cord, and the cerebral commissure" (Lemche & Wingstrand,

1959), but whether this tripartite structure can be compared to the cordal structure seen in the chiton circumoesophageal nerve ring is not clear from published histological sections (Ruthensteiner, Schropel, & Haszprunar, 2010; Schaefer & Haszprunar, 1997).

The best-supported relationship among molluscan classes is the clade Aculifera, which includes clades with ganglionic (aplacophoran) and non-ganglionic (chiton) nervous systems as sister-taxa, and no fossils preserve the central nervous system, so there is a limited basis to infer character polarity. The topology of the remaining molluscan classes is largely unclear, but among these clades, most groups have a ganglionic arrangement (Lindberg & Sigwart, 2015), which speculatively suggests the cordal structure found in cephalopods may be derived. Chitons are popularly thought to be primitive, and cephalopods are perceived as ‘advanced’, but it is not entirely parsimonious to infer that a cordal brain structure is plesiomorphic for aculiferans, yet derived in conchiferans. Nonetheless a convergent nervous system evolution between chitons, cephalopods, and vertebrates would be highly unexpected, given that the convergence between the two latter groups is attributed to their similarly active lifestyles (e.g. Budelmann, 1996).

Characters of the chiton nervous system

In order to be useful in finer phylogenetic analyses, it is important to evaluate the consistency of the nervous system characters we describe herein. Our findings can be closely compared to descriptive results from other closely-related chiton species. In particular, our observations of *Acanthochitona fascicularis* closely resemble those of *Acanthochitona crinita* made by Faller et al. (2012): aligned origins of the ventral and lateral nerve cords at the posterior margin of the nerve ring, lateral thickening of the circumoesophageal nerve ring and an apparently anteriorly incomplete cerebral tract. *Hemiarthrum setulosum*, another member of

the Cryptoplacoidea, also exhibits origins of the two pairs of nerve cords almost in parallel, but shows a complete cerebral ring. Despite some quality issues with the historic material of *Lepidochitona cinerea*, we identified several features in line with the findings of Faller et al. (2012) and Gegenbaur (1878) in the same species, and of Gantner (1989: Fig. 72) in *Lepidochitona monterosatoi*, including a short circumoesophageal nerve ring, origins of the lateral nerve cords anterior to those of the ventral nerve cords, and a prominent first ventral commissure (both species), thicker lateral than ventral nerve cords, and anteriorly narrowed ventral and cerebral tracts (*L. cinerea* only).

Chitons also possess notable diversity in sense organs, which have previously been used as standard characters for differentiating major clades. The aesthetes are a system of innervated shell pores that infuse the exposed dorsal shell layer in all chiton species (Eernisse & Reynolds, 1994; Sigwart & Sumner-Rooney, 2015). The proximal and distal termini of these nerve channels are apparent as pores on the ventral and dorsal shell surface, which are used as taxonomic characters. Fine differences in the arrangement of dorsal aesthete pores are used in identifying lepidopleuran species and broader differences in the ventral patterning and the points of penetration at slits in the shell insertion plates correspond to major clades (Sirenko, 1997, 2006). Another sense organ, the Schwabe organ, is an anatomical synapomorphy of Lepidopleurida (Sigwart et al., 2014). The separate molluscan “osphradium” is a nonspecific term for epithelial sense organs described from some chitons; in chitons this structure represents a “posterior sense organ” that is not homologous to the osphradium *sensu stricto* (Lindberg & Sigwart, 2015).

We proposed a set of identified neuroanatomical characters (Table 2; Table 3) for future use in chiton phylogenetics analyses at the ordinal and subordinal level. Previous cladistic

analyses of chitons emphasised the need for input from diverse character sets (Sigwart, 2009). These characters do not supplant other morphological or molecular data, but additional independent evidence from neuroanatomical characters could provide useful additions to larger analyses. In particular, there are features that separate Lepidopleurida from other species, not only in the known features of the sense organs (Schwabe organ present and posterior sense organ absent as likely apomorphies in Lepidopleurida; *vice versa* in Chitonida), but also in features of the overall neural architecture. However, there are no clear synapomorphies from the present data that would apparently support a monophyletic Chitonida. The genus *Callochiton* is well known to be significantly different to other members of the order Chitonida based on morphological and molecular data (Okusu, Schwabe, Eernisse, & Giribet, 2003; Sigwart, Stöger, & Schwabe, 2013), and this is also reflected in its neuroanatomy.

The limited taxon sampling and missing data available for both *Lepidochitona cinerea* and *Schizoplax brandtii* may hinder the resolution of finer relationships. There are several specific features that could represent synapomorphies of established groupings (Table 2), that provide hypotheses to test with additional relevant taxa. The relative positions of the origins of the lateral and ventral nerve cords (6), for example, are consistent within the major clades, with the Cryptoplacoidea having origins in parallel (*Acanthochitona*) or near-parallel (*Hemiarthrum setulosum*), the Mopalioidae having near-parallel origins, and the Lepidopleurida and more plesiomorphic Chitonida having the origins of the lateral nerve cords significantly anterior to those of the ventral nerve cords (this also holds true for *Leptochiton rugatus* (Sigwart et al., 2014)). The major nerve cords are laterally broadly distributed in *Lepidochitona cinerea*; this appears to also be the case in the anterior region of *Schizoplax*

564 *brandtii* but due to the incomplete nature of the specimen, this cannot be determined.
565 *Acanthochitona fascicularis* and *Hemiarthrum setulosum*, though both members of
566 Cryptoplacoidea, are strikingly different in their neuroanatomy, and they are not resolved as
567 sister taxa in our analysis. However, *A. fascicularis* shares several features with other
568 congeneric species studied by other authors. The distinctive overall shape of the oesophageal
569 nerve ring, which is heavily lateralised, is apparent in both illustrations and confocal images
570 (Faller et al., 2012; Pelseneer, 1898; Plate, 1899), so it is possible that this is a synapomorphy
571 of the genus. *Hemiarthrum* is also highly unusual morphologically (Sigwart et al., 2013), so it
572 may also be the case that it does not reflect the typical state of Cryptoplacoidea, or that this
573 group is characterised by a higher degree of variability in this regard than other chiton clades.
574 Of course, some neuroanatomical features may be the result of adaptation in body size or
575 form, such as the lateral distribution of the ventral nerve cords, which is, of course, heavily
576 dependent on the overall body plan and shape of the foot. But we recommend that the
577 characters identified here are suitable for inclusion in future phylogenetic analyses, and
578 suggest that further examination of nervous systems (central and peripheral) and increased
579 taxonomic coverage will contribute to resolving the longstanding questions surrounding
580 chiton relationships. Finally, similar studies in fresh material may of course cast light on the
581 robustness and phylogenetic utility of these characters, and comparative ultrastructural,
582 immunohistological and developmental studies will doubtless expand on this character set in
583 the future. The characters identified here are restricted to overall nervous architecture that
584 can be determined from historic slides more than a century old. However, the data we present
585 are a credit to the quality of both the original histological material and its subsequent
586 curation. Slide collections such as Thiele's offer an as yet underexploited resource for modern
587 morphological research through tomographic reconstruction; the technical expertise and

comprehensive taxonomic coverage of our predecessors provide a great asset and an efficient starting point for comparative studies such as this, and we encourage further use of historic slide collections in this way, in parallel with the increased recognition of wet material as a resource for computed tomography (Sumner-Rooney & Sigwart, 2017).

Conclusions

The definitions of a brain as an anterior concentration of nervous tissue or a concentrated multipartite neural mass are useful and (almost) universally applicable to identify brains in individual taxa (Richter et al., 2010), and the chiton nervous system meets both of these definitions. However, these structures may be difficult or inappropriate to compare among distantly related groups. In this context, identifying homologous structures as ‘brains’ in different taxa is much more valuable and informative from a both an evolutionary and a functional perspective. This is a significant challenge in molluscs, as although the ganglia are homologous, the brains may not be. In cephalopods, the brain comprises homologues of not only the cerebral ganglia, but also the pedal and pleural ganglia, which together form the circumoesophageal ring. However, in scaphopods, bivalves, caudofoveates and solenogastres, it is only the cerebral or fused cerebropleural ganglia that are interpreted to form the brain, if they are attributed one at all (Faller et al., 2012; Sigwart & Sumner-Rooney, 2015). So, these are potentially competing definitions for what comprises the “brain” in molluscs: one, or two, or three pairs of ganglia, which may or may not be fused. In chitons it is impossible to delineate homologues of the three typically discrete pairs of ganglia. Chitons evidentially have a brain, but its fused structure would appear to support the more expansive definition of a brain as also seen in cephalopods. Importantly, this is not to imply any inherent similarity between chiton and cephalopod brains, but is merely a test of the ontology of

“brain” among molluscs. Indeed, in most cases this would also complement Richter and colleagues’ definition (Richter et al., 2010), but in some taxa, such as scaphopods and bivalves, an expansion of the “brain” to encompass the pedal, pleural, and cerebral ganglia would imply that it is quite spatially disparate, with a distended nerve ring due to the displacement of the foot (Sumner-Rooney et al., 2015). Conversely, if this is not the case, it implies either that brain structures are highly plastic throughout the phylum, or that brains (not ganglia) have evolved multiple times. There are distinctly different apomorphic brain characters in several molluscan classes, such as the frontal swellings of the cerebral ganglia in caudofoveates and solenogastres (Sigwart & Sumner-Rooney, 2015), which could be a result of multiple independent origins of the brain. All of these possibilities remain to be investigated, but objective comparisons and a clear ontology are a necessary first step. Thus, the combined identification of the circumoesophageal nerve ring in chitons as both organised and centralised (i.e., a brain) has substantial implications for the assessment of the central nervous system and brain in other molluscs with recognised ganglionic organisation.

Three-dimensional visualisation of anatomy is a powerful tool to clarify the true extent and variability of key structures. The interpretation of chiton anatomy may be historically stymied by circular logic: if we assume that chitons are primitive, then we see their nervous system as primitive, and the nervous systems is seen as “proof” that the animals retain plesiomorphic features. Instead, the chiton nervous system shows an unappreciated level of complexity, and a brain.

Availability of data and materials

The models produced during the current study are available online from the corresponding author on request. The original histological sections from the collection of Johannes Thiele, and a digitised set of these sections, remain the property of the Museum für Naturkunde, Berlin; original slides of *Leptochiton asellus* are the property of the Bavarian State Collection of Zoology, Munich; all are available on request from the relevant malacological collection.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Both authors conceived and designed the project. LSR digitised slides, reconstructed tomographic models, prepared figures and wrote the manuscript. JDS performed phylogenetic analyses, prepared figures and wrote sections of the manuscript. Both authors read and approved the final manuscript.

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Figure 1. Tomographic models of the body and nervous systems of chitons from the slide collection of Johannes Thiele. **A–C**, *Acanthochitona fascicularis*. **D–F**, *Callochiton septemvalvis*. **G–I**, *Chiton olivaceus*. **J–L**, *Hemiarthrum setulosum*. **A, D, G, J**: Whole body, ventral view. Scale bar 500 μ m. **B, E, H, K**: Circumoesophageal nerve ring, dorsal view. Scale bar 250 μ m. **C, F, I, L**: Animal *in vivo*. Purple: Lateral nerve cords and tracts. Green: Ventral nerve cords and tracts. Pink: Cerebral tracts. Yellow: Buccal ganglia and nerves. Teal: Subradular ganglia and nerves.

Figure 2. Tomographic models of the body and nervous systems of chitons from the slide collection of Johannes Thiele. **A–C**, *Lepidochitona cinerea*. **D–F**, *Lepidopleurus cajetanus*. **G–I**, *Leptochiton asellus*. **J–L**, *Schizoplax brandtii* (anterior only). **A, D, G, J**: Whole body, ventral view. Scale bar 500 μ m (J: 250 μ m). **B, E, H, K**: Circumoesophageal nerve ring, dorsal view. Scale bar 250 μ m. **C, F, I**: Animal *in vivo*, dorsal view. **L**: Historic and updated views on the chiton anterior nervous system, dorsal view. Left, *Lepidochitona monterosatoi* redrawn from Gantner (1987); right, generalised plan combining aspects of the taxa used herein. Note the difference in relative thicknesses of the nerve ring, lateral nerve cords (lnc) and ventral nerve cords (vnc) between the original and updated figures, marked in black. Purple: Lateral nerve cords and tracts. Green: Ventral nerve cords and tracts. Pink: Cerebral tracts. Yellow: Buccal ganglia and nerves. Teal: Subradular ganglia and nerves.

Figure 3. Subdivision of the oesophageal nerve ring. The central neuropil of the nerve ring is broadly divided into three distinct regions by interspersed veins of cell bodies. This was originally illustrated by Plate (1895) in *Acanthopleura echinata* (left), and is clearly visible in both historic (centre, *Hemiarthrum setulosum* from Thiele's material ZMB/Moll 230880-230999) and recent semi-thin (right, *Leptochiton asellus*) histological sections. The three regions correspond to the lateral nerve cords (lnc), ventral nerve cords (vnc) and a presumed cerebral tract homologous to the cerebral ganglia (ct). All three images were taken from the posterolateral part of the oesophageal nerve ring. Scales adjusted to facilitate comparison.

Figure 4. Proposed development of the chiton nervous system. Schematics drawn from descriptions and data in Voronezhskaya et al. (2002), Friedrich et al. (2002) and the current study. Ventral view, anterior at the top. The precursor to the cerebral cord appears at the anterior of the developing larva, followed by the precursors to the ventral cords and then the lateral cords, which extend posteriorly and then anteriorly in turn (c, cerebral region; l, lateral region and nerve cord; v, ventral region and nerve cord).