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An Introduction

1.1. COMMON PRINCIPLES OF ANIMAL DEVELOPMENT

The notion that animal development is controlled by common mechanisms is not a recent idea. During the nineteenth century, French zoologist E. Geoffroy St Hilaire was the first to consider animal anatomy as a structural continuum. He hypothesised that the differences in form and function of comparable structures were merely variations on the same theme. Though he studied form, pure and simple, his ideas anticipated the importance of assigning structural homologies, both in the study of evolutionary relationships and in understanding the developmental mechanisms required in building an animal.

Empirical evidence from the last decade has indicated common sets of genes and genetic pathways are responsible for many developmental mechanisms, including the formation of the central nervous system (CNS), in triploblastic animals with an assortment of disparate body plans. A striking example of this conservation includes the homeotic or Hox gene complex (McGinnis and Krumlauf, 1992) and the cephalic gap genes (Boncinelli et al., 1993; Finkelstein and Boncinelli, 1994; Klein and Li, 1999; Patarnello et al., 1997) in vertebrates and flies. Genes of the Hox cluster encode homeodomain transcription factors that specify regional identity along the anterior-posterior (A/P) axis of insects and vertebrates alike. First identified in *Drosophila*, the 3'-5' chromosomal organisation of the genes of the Hox cluster mirrors its order of expression along the A/P axis, which is most evident during the phylotypic stage of embryogenesis. Hox clusters were also identified in other bilaterians, including vertebrates, where their conserved expression patterns formed the basis of the zootype hypothesis (Slack et al., 1993). Defined as the synapomorphy of animals, the zootype (Figure 1.1) includes the *Drosophila* Hox genes plus the *empty spiracles*, *orthodenticle* and *even-skipped* genes, all of which have regional specification roles and show conserved patterns of expression with their counterparts in other bilaterians. While the

zootype hypothesis is generally accepted in the Bilateria, there is debate over whether it can be applied to diploblastic animals (Ferrier and Holland, 2001; Schierwater and Desalle, 2001).

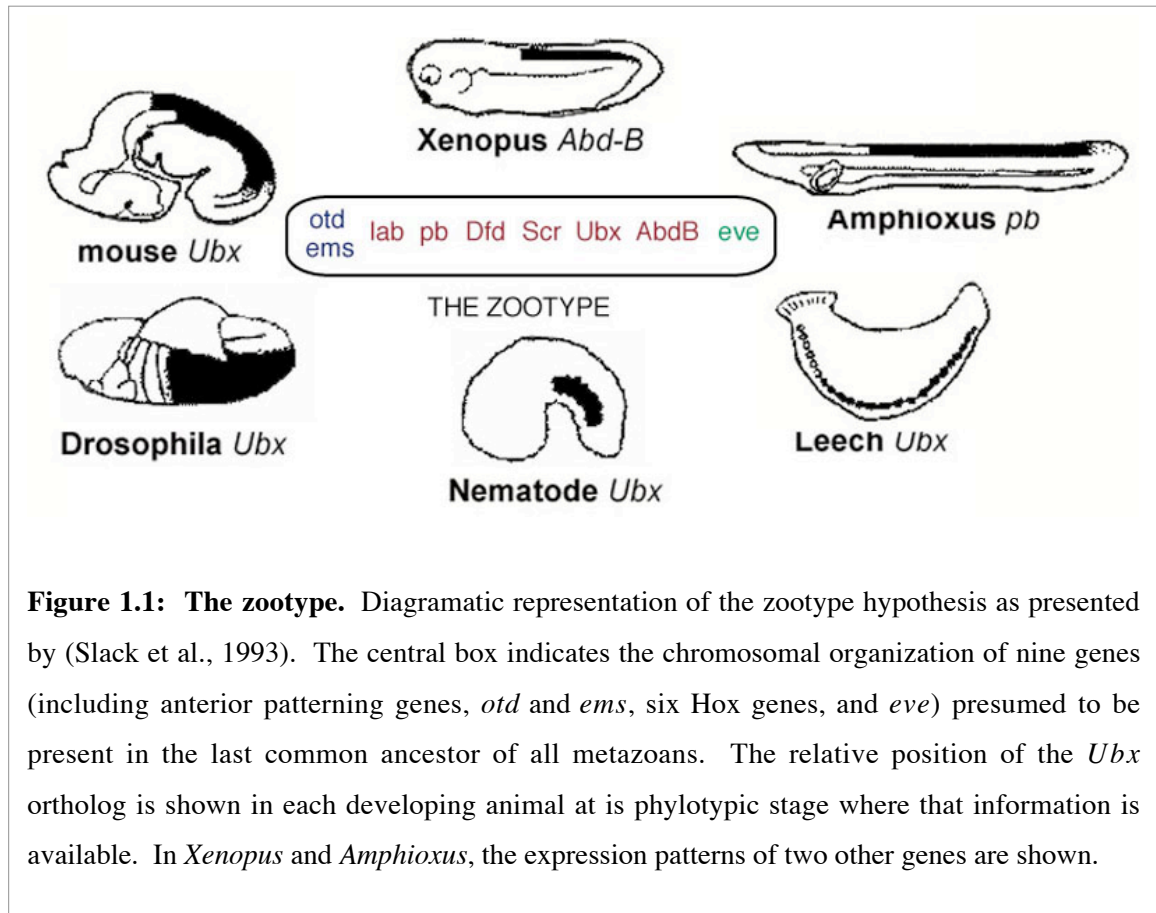
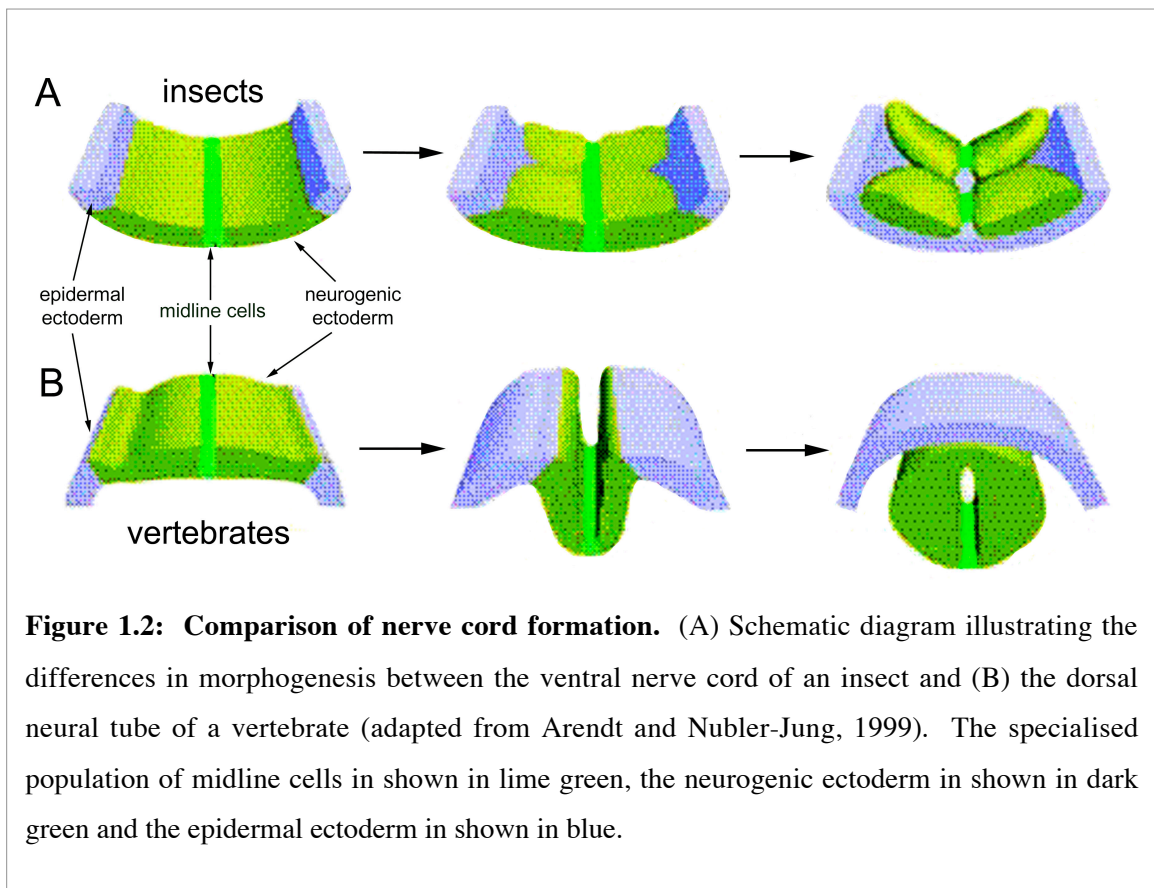


Figure 1.1: The zootype. Diagrammatic representation of the zootype hypothesis as presented by (Slack et al., 1993). The central box indicates the chromosomal organization of nine genes (including anterior patterning genes, *otd* and *ems*, six Hox genes, and *eve*) presumed to be present in the last common ancestor of all metazoans. The relative position of the *Ubx* ortholog is shown in each developing animal at its phylotypic stage where that information is available. In *Xenopus* and *Amphioxus*, the expression patterns of two other genes are shown.

The importance of Hox genes in early patterning mechanisms of higher animals is clear, yet their evolutionary origins and ancestral functions are equivocal. As a sister group to the bilaterians, the Cnidaria serve as a phylogenetic outgroup and afford an excellent opportunity to assess the commonality of these and other genes involved in embryonic development and axis specification. As diploblastic organisms, cnidarians are uncomplicated by organogenesis and intricate design. They have a simple nervous system and very few cell types. Without the complexity of higher metazoan systems, the role of early patterning genes in cnidarians can be more easily elucidated and related to the ancestral functions.

1.2. AXIS SPECIFICATION OF THE NERVOUS SYSTEM

The CNS in insects and vertebrates was originally thought to have evolved independently (Brusca and Brusca, 1990; Nielsen, 1995) but recent molecular data have provided evidence against to this theory and suggested a dorsal/ventral (D/V) axis inversion is responsible for the morphological difference in CNS development within the Bilateria. Bilateral animals are subdivided into two groups based on the location and development of the nerve cord. The gastroneurians, such as annelids and arthropods, have a ventrally positioned nerve cord, which is derived from the bilaterally symmetric ventral neurectoderm (Figure 1.2A). Neuroblasts delaminate from the neurectoderm and move inward to subsequently divide and produce neurons and glia that comprise the ladder-like CNS. In contrast, the nerve cord of notoneurians, such as in vertebrates, is derived from the bilaterally symmetric neurectoderm on the dorsal surface of the embryo (Figure 1.2B). The entire sheet of neurectoderm folds inwards to form a neural tube and eventually a brain and spinal cord.



Originally, these morphological differences were taken as evidence of an independent evolutionary origin of the CNS in each group (Brusca and Brusca, 1990; Nielsen, 1995).

Yet, recent molecular and developmental data (for example, Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998; reviewed in Arendt and Nubler-Jung, 1999) have challenged this hypothesis and lent support to an early idea (Geoffroy St Hilaire, 1822) that the ventral region of insects and the dorsal region of vertebrates are equivalent and attributable to a body axis inversion in these two groups. The assumption that the neural region on opposing body sides in insects and vertebrates are evolutionary equivalents also suggests that the central nervous systems derived from these regions are homologous.

Early molecular control of D/V axis patterning during embryonic development appears to be conserved between insects and vertebrates. Dorsoventral polarity is initially established by the signalling protein Decapentaplegic (Dpp) in *Drosophila* and one its homolog, Bone morphogenic protein (BMP), in vertebrates (reviewed in Sasai and De Robertis, 1997; Streit et al., 1999). Its activity is spatially restricted by the antagonistic action of another signalling protein known as Short gastrulation (Sog) in the fly and Chordin in vertebrates (Holley et al., 1995). In support of the body axis inversion theory, both Dpp/BMP4 and Sog/Chordin work from opposing poles in insects and vertebrates alike. However, in insects Dpp acts on dorsal cells while in vertebrates BMP4 acts ventrally. Similarly, in insects Sog activity is present in ventral cells while its vertebrate counterpart, Chordin, is found in dorsal cells. Together these data suggest a homologous relationship in the induction and specification of the CNS in both insects and vertebrates (reviewed in Streit et al., 1999).

Once the neurectoderm is specified, an additional set of genes is involved in D/V patterning within the CNS. They also appear to be evolutionarily conserved although their patterns of expression are inverted between flies and vertebrates (Chan and Jan, 1999; Cornell and Von Ohlen, 2000; Scott, 2000; von Ohlen and Doe, 2000). This gene complement is composed of three homeobox genes and is involved in the formation of D/V column domains in the ventral neurectoderm of flies and the neural plate of vertebrates. In *Drosophila*, these genes are *vnd*, *ind* and *msh* and are expressed in the ventral, intermediate and dorsal columns of the neurectoderm respectively (Figure 1.3; Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998). Each gene is expressed within a specific domain where its activity is required to induce neuroblast formation and provide D/V column identity. Within the vertebrate neural plate, homologs of these

fly genes are also expressed in D/V columns but their D/V polarity of expression relative to *Drosophila* is inverted. These genes are members of the *Nkx2* (*vnd*), *Gsh* (*ind*) and *Msx* (*msh*) gene families and are expressed in the dorsal, intermediate and ventral columns of the neural tube respectively (Figure 1.3). Functional analyses imply similar roles for these vertebrate genes in column cell fate specification, implying a degree of functional conservation with their *Drosophila* homologs (Briscoe et al., 1999). These remarkable similarities in gene structure, expression and function point toward a common evolutionary origin for the CNS of different animal phyla and imply that the differences in polarity are a consequence of a general inversion of the dorsoventral body axis.

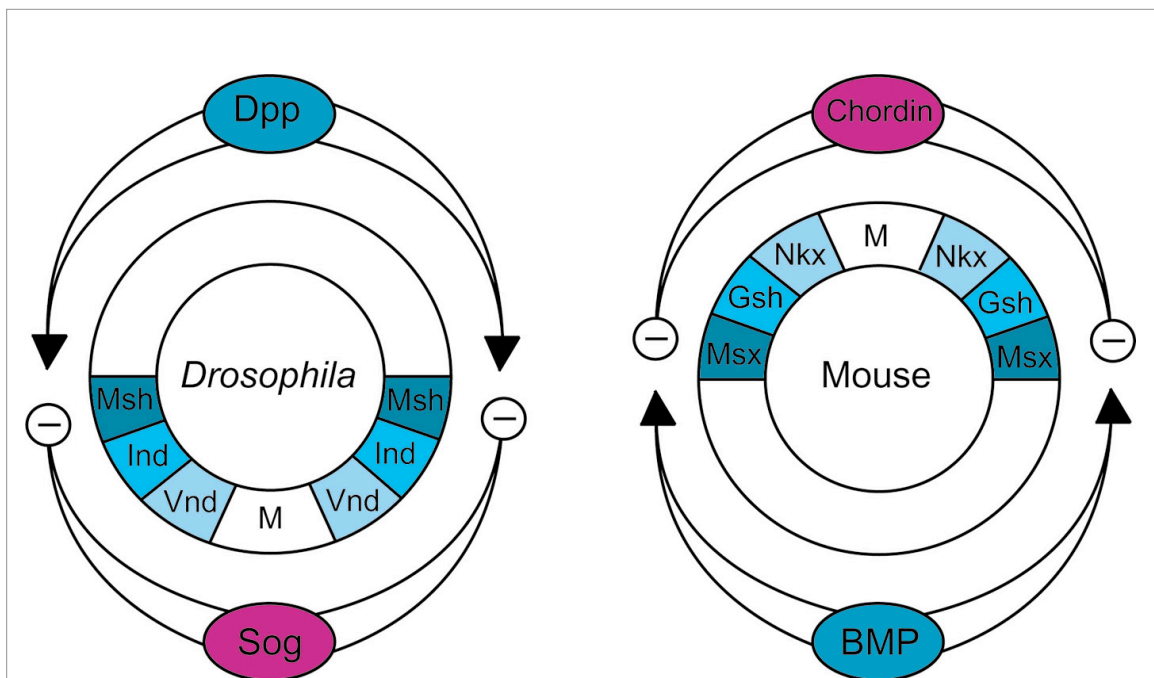


Figure 1.3: Establishing the dorsoventral axis within the CNS. Dpp/BMP and Sog/Chordin signalling pathways work from opposing poles to establish polarity across the developing nerve cord in insects and the neural tube in vertebrate embryos. The developing CNS of *Drosophila* and neural plate of vertebrates is organised into three columns along the D/V axis which are specified by the activities of homeobox genes including *Vnd/Nkx2* (expressed medially), *Ind/Gsh* (expressed in an intermediate position) and *Msh/Msx* (expressed most laterally) (adapted from Reichert and Simeone, 2001)

1.3. EVOLUTIONARY ORIGINS OF THE BODY AXES

The invention of bilateral symmetry was a major transition in animal evolution and accompanied the creation of a third germ layer, centralised nervous system and a specialised through gut (Holland, 1998). Gene duplication is thought to have played a major role in facilitating these significant changes in function and development (Lundin, 1999; Ohno, 1970; Ohta, 1988).

While general conserved mechanisms of early development are well documented in bilateral animals, it is unclear which of these mechanisms were present prior to this evolutionary step, which may have played a role in the transition from radial to bilateral symmetry. Historically, the earliest metazoans were assumed to possess radial symmetry (Brusca and Brusca, 1990; Hyman, 1940) and therefore have given rise to bilateral symmetry. There is, however, controversy surrounding the symmetrical character of the ancestral cnidarian (Holland, 2000; Martindale et al., 2002; Martindale and Henry, 1998). While zoological textbooks describe the Cnidaria as primarily possessing radial symmetry (Brusca and Brusca, 1990; Ruppert and Barnes, 1991), other authors have stated that cnidarians were likely to have been bilateral at least once in their evolution (Galliot, 2000; Gröger and Schmid, 2001; Martindale and Henry, 1998; Wilmer, 1990).

The relationship between the single cnidarian axis and the axes of bilaterians is ambiguous. Generally it is assumed that the single oral/aboral (O/A) axis of radially symmetrical cnidarians corresponds to the A/P axis of bilateral animals – the bilaterian anterior region being analagous to the cnidarian oral pore. Compelling evidence to support this theory is, however, lacking. The case for assigning homology between the two axes is further complicated by: (i) the swimming behaviour of the cnidarian planula, which swims with its aboral end directed anteriorly; (ii) the bifunctional use of the oral pore as both a mouth and anus and; (iii) an axis inversion in cnidarians between different life stages that sees the posterior pole of the planula become the mouth of the adult.

The establishment of axis polarity in cnidarians is thought to occur as early as the first cleavage, but it is unclear how this initial division can specify opposing regions (Goldstein and Freeman, 1997). In hydrozoans, the site of polar body formation and

egg fertilisation usually marks the point of cleavage initiation, gastrulation and the posterior larval region (Freeman, 1981b; Teissier, 1931). However, centrifugation of fertilised eggs can shift the first cleavage site away from the site of polar body formation, to the fertilisation site and posterior end of the larva; this suggests axis polarity is established independently of fertilisation and polar body formation (Freeman, 1990). Similarly, by briefly inhibiting cleavage following an initial fertilisation event and centrifuging an egg prior to a second fertilisation event, two gastrulation sites can be induced that lead to the formation of two posterior larval ends, suggesting that the first site of cleavage initiation introduces an early asymmetric character (Freeman, 1981a). It is possible that the first division causes a graded distribution of some factor(s) that determine the fate of posterior cells, but is neither absolute nor immediate as later stage embryos (up to early gastrulation) can still be divided to develop as two complete embryos retaining anterior-posterior polarity. This is referred to as “global polarity” (Goldstein and Freeman, 1997).

1.4. CNIDARIAN HOX-RELATED GENES

The Hox gene cluster is an essential patterning component of animal development, imparting positional information to different regions along the longitudinal axis in a spatially collinear pattern of expression, seen most clearly during the phylotypic stage of development (Slack et al., 1993). At least one Hox cluster is represented in all bilaterian animals examined implying a Hox cluster was present in the common ancestor, but little is known of the origins of this group of genes. To clarify Hox gene evolution, the Hox gene complement of the Cnidaria was examined. The first Hox-like genes, *cnox1* and *cnox2*, were reported in *Chlorohydra viridissima* (Schummer et al., 1992) and showed similarity to the group 1 and 4 Hox genes respectively. A demonstrated linkage between one group 5-7 Hox gene, *antpC*, and an *Evx* gene in *Acropora formosa*, together with data from *Chlorohydra*, hinted that these genes might represent a cnidarian Hox cluster. However, classification of cnidarian Hox genes through sequence analysis alone is difficult: Hox genes do not form a monophyletic group in phylogenetic analyses of homeobox-containing genes but are instead dispersed throughout the tree (Finnerty and Martindale, 1997).

Numerous other cnidarian Hox-related genes have since been reported (Aerne et al., 1995; Cartwright et al., 1999; Finnerty and Martindale, 1997; Kuhn et al., 1996; Kuhn

et al., 1999; Naito et al., 1993; Shenk et al., 1993a; Shenk et al., 1993b), and form at least five distinct classes (Finnerty and Martindale, 1999; Gauchat et al., 2000; Kuhn et al., 1999). Mounting homeobox sequence data have changed how these genes are interpreted and now many genes previously thought to be cnidarian Hox genes are more closely related to other gene families; for example the *cnox2* gene of *C. viridissima* more closely resembles a *Gsx* ortholog than a Hox group 4 gene as it was originally classified (Schummer et al., 1992). Embryonic expression data is limited; with the exception of *Podocoryne cnox1-Pc*, for which planula expression is absent (Aerne et al., 1995); *cnox2-Pc*, which is expressed aborally in the planula and orally in the polyp (Masuda-Nakagawa et al., 2000), and *Acropora cnox2*, which is expressed in all but the aboral planula region (and rare in the oral region) (Hayward et al., 2001), nothing is known about cnidarian Hox-like genes during early development.

No definitive Hox genes have yet been identified in cnidarians. Hox clusters in bilaterians are defined by (i) their clustered organisation along the chromosome; (ii) collinear expression along the A/P axis; and (iii) association with co-factors through the hexapeptide motif to increase DNA-binding specificity, but there is little evidence of these defining features in the Hox-like genes of the Cnidaria. To date, there is no evidence for linkage between Hox-like genes but there is one explanation for its absence; a lack of clustering in *Hydra* may be attributed to evolutionary gene loss after the divergence of the Hydrozoa (Gauchat et al., 2000). Cnidarian Hox-related genes also lack convincing hexapeptide protein motifs that facilitate an increase in DNA-binding specificity of Hox genes through interactions with Exd/Pbx cofactors (Neutoboom et al., 1995; Phelan et al., 1995). More than likely, the creation of the hexapeptide followed the Cnidaria/Eumetazoa split and enabled the diversification of true Hox genes.

1.5. ANCESTRAL HISTORY OF ANTERIOR PATTERNING

Many of the molecular mechanisms involved in patterning sophisticated bilateral organisms were originally thought to have evolved independently after the divergence of simple diploblastic animals. However, molecular evidence now suggests many of these developmental processes are ancient and share common features between diploblastic and triploblastic organisms. As previously mentioned, a common mechanism of axial patterning exists between bilaterian species, suggesting it was

present in a common ancestor of the Bilateria. While large evolutionary distances exist between cnidarian Hox-related genes and true Hox genes, clear homologs of bilaterian A/P and D/V axial patterning genes are present in cnidarians. Cnidarians possess homologs of anterior patterning genes *ems/Emx* and *otd/Otx* (Mokady et al., 1998; Muller et al., 1999; Smith et al., 1999) and three genes involved in the D/V patterning system: *vnd/NK2*, *ind/Gsx* and *msh/Msx* (Grens et al., 1996; Hayward et al., 2001; Schummer et al., 1992), which appear to play similar roles in the Cnidaria. Their presence suggests these patterning systems are part of an ancient mechanism that predates the Cnidaria/Eumetazoa split.

Recent molecular data suggest the Bilateria share developmental processes implicated in head development but whether this level of conservation extends to the Cnidaria is unknown. Bilaterian head organiser activity, which is established during early embryogenesis, is controlled by a common set of developmental genes encoding transcription factors of the *Paired*-class, *Antennapedia*-class and *Lim*-class homeobox gene families and the winged-helix and T-box gene families (Bally-Cuif and Boncinelli, 1997; Beddington and Robertson, 1998; Bouwmeester and Leyns, 1997; Hartmann and Reichert, 1998; Papaioannou, 2001). *Paired*-class genes, in particular those related to the *Drosophila aristaless* gene, are frequently involved in the development of the embryonic brain and specification of the CNS. Originally it was thought that the cnidarian ‘head’, with its mouth-like opening and surrounding tentacles, could not reflect the ancestral complexity of the vertebrate or insect head but cnidarian orthologs of bilaterian ‘head-specific’ genes have been identified recently in coral, jellyfish, *Hydra* and *Hydractinia* (Table 1.1) and are likely to provide information on ancestral gene roles and conserved molecular mechanisms. In many cases, however, data from *Hydra* can only reveal the role of these genes during the budding and regeneration processes. For more informative comparisons with *Drosophila* and vertebrate systems, cnidarian embryonic expression data is needed.

Table 1.1. Cnidarian orthologs of triploblastic genes involved in organiser activity (adapted from Galliot and Miller, 2000). (ND= not determined).

Cnidarian gene	Triploblastic gene	Animal	Cnidarian Expression Patterns		References
			Adult	Embryo	
PAIRED-CLASS					
<i>Paired-like Q₅₀</i>					
<i>Prdl-a</i>	<i>Arx</i>	<i>Hydra</i>	Head (nerve cells)	ND	Gauchat et al., 1998
<i>Prdl-b</i>	<i>Arx</i>	<i>Hydra</i>	No	ND	Gauchat et al., 1998
<i>HyAlx</i>	<i>Arx</i>	<i>Hydra</i>			Smith et al., 2000
<i>Paired-like K₅₀</i>					
<i>Otx-Pc</i>	<i>Otx</i>	<i>Podycoryne</i>	Striated muscle	None	Muller et al., 1999
<i>CnOtx</i>	<i>Otx</i>	<i>Hydra</i>	Body column	ND	Smith et al., 1999
<i>Manacle</i>	<i>Otx</i>	<i>Hydra</i>	Basal disk ectoderm	ND	Bridge et al., 2000
<i>Pax-type</i>					
<i>Pax-A</i>	<i>Paxneuro</i>	<i>Acropora</i>	ND	Oral ectoderm	Reece-Hoyes, 2001
<i>Pax-B</i>	<i>Pax-2/5/8</i>	<i>Acropora</i>	ND	Ectoderm	Reece-Hoyes, 2001
<i>Pax-C</i>	<i>Pax-4/6 Eyeless</i>	<i>Acropora</i>	ND	Ectodermal nerve cells	Miller et al., 2000
ANTP-CLASS					
<i>Cn-ems</i>	<i>Emx</i>	<i>Hydractinia</i>	Hypostome	ND	Mokady et al., 1998
<i>Cnox-1</i>	<i>Hox-1</i>	<i>Chlorohydra</i>		ND	Schummer et al., 1992
WINGED-HELIX					
<i>Budhead</i>	<i>Forkhead, Hnf-3</i>	<i>Hydra</i>	Hypostome	ND	Martinez et al., 1997
T-BOX					
<i>HyBral</i>	<i>Brachyury</i>	<i>Hydra</i>	Hypostome	ND	Technau & Bode, 1999

1.6. THE SIGNIFICANCE OF THE CNIDARIA

Cnidarians are model lower animals in which to study the ancestral functions of genes involved in developmental mechanisms and patterning processes. As one of the most ancient phyla within the Metazoa (Figure 1.4A), cnidarians are well placed to represent the ancestral traits of the last common metazoan ancestor, while their simple body plan and relatively small genome make them an ideal choice for developmental studies.

The fossil history of the Cnidaria is over 500 million years, with anthozoan-like representatives present in the Ediacara fauna (Sprigg, 1949) of the Vendian period 650-540 million years ago (mya). Mineralised coral-like fossils first appear in the fossil record during the Cambrian period 544-505 mya (for example Sorauf and Savarese, 1995; Tynan, 1983), but the first evidence of scleractinian corals is not until the Middle Triassic (Veron, 1995), around 15 million years after a mass extinction event of marine invertebrates in the Late Permian period 286-245 mya. The Cnidaria are considered to be the sister group to bilateral metazoans (Figure 1.4A).

The majority of the some 9000 species of the Phylum Cnidaria are marine dwellers (Brusca and Brusca, 1990). This phylum is subdivided into four classes: the Hydrozoa (*Hydra*); the Cubozoa (box jellyfish); the Scyphozoa (true jellyfish) and the Anthozoa (sea anemones and corals). The Anthozoa are the basal cnidarian class based on studies of mitochondrial (Bridge et al., 1992) and ribosomal DNA (Odorico and Miller, 1997, Medina, 2001 #229, Bridge, 1995 #297). Members of the Anthozoa are, therefore, arguably better positioned than *Hydra* to reflect the ancestral traits of the last common ancestor of metazoans (Figure 1.4B).

The Cnidaria are the simplest extant animals possessing true tissue layers and a nervous system (Nielsen, 1995). Their diploblastic body plan is radially symmetrical and a single body opening functions as both a mouth and anus. Their body plan is organised along a single axis, the oral-aboral (O/A) axis, suggesting the development of the second body axis occurred after the Cnidaria/Eumetazoa split.

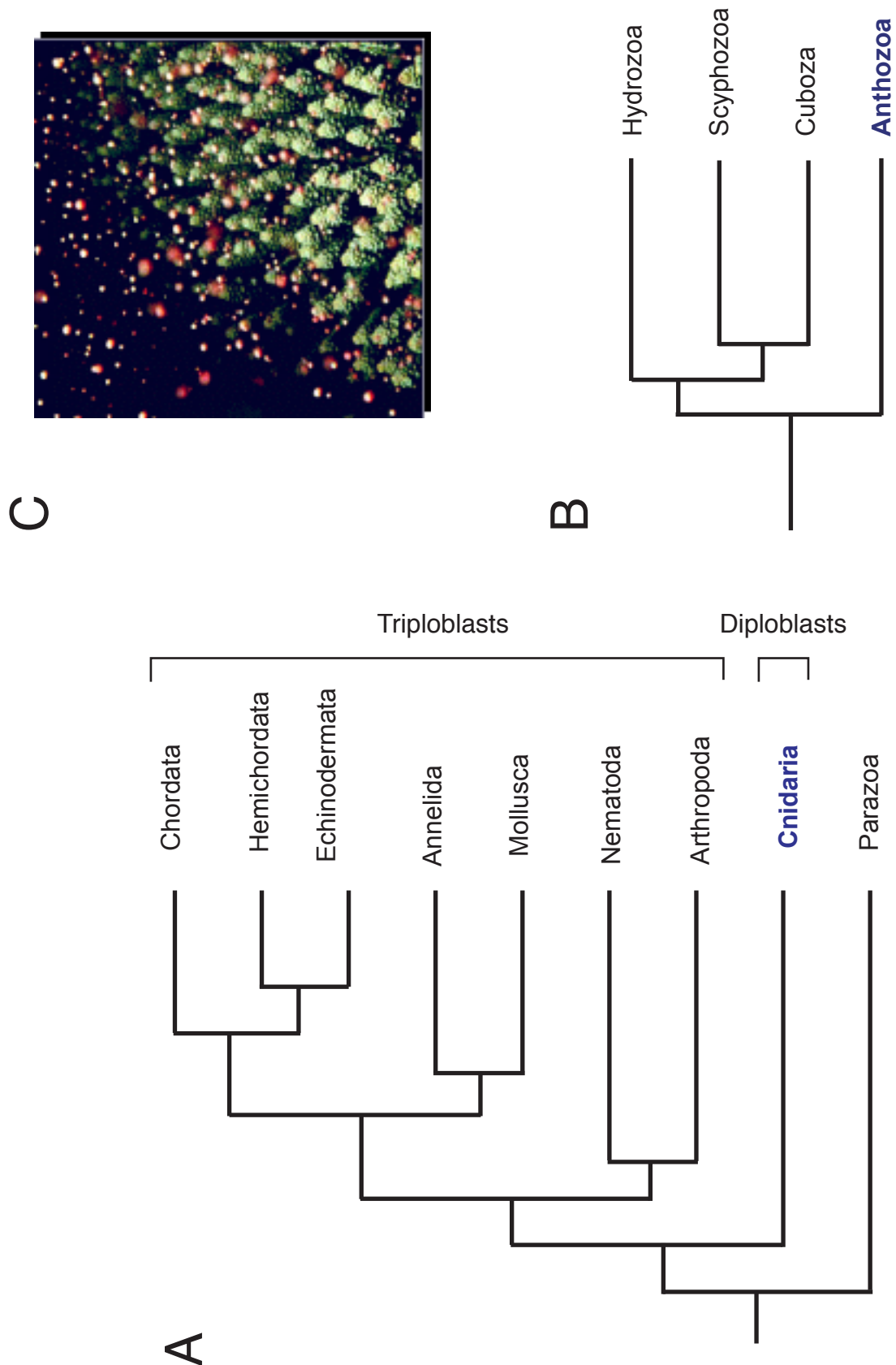


Figure 1.4: Evolutionary relationships of the Metazoa and Cnidaria.

(A) Relationships of the major metazoan phyla. The Cnidaria are the sister group to the Bilateria. (B) The Anthozoa are the basal cnidarian class, unlike the more derived Hydrozoa. (C) An *Acropora* colony dispersing egg/sperm bundles during the mass spawning events of Spring.

1.6.1. Cnidarian nervous systems

Cnidarians possess a nervous system that is anatomically simple; a diffuse two-dimensional network of cells with sensory and motor function. More than likely nervous systems first evolved in cnidarians or their closely related ancestors (Mackie, 1990). Bipolar and multipolar sensory neurons are situated in the ectoderm and are connected to a subepithelial nerve plexus via synaptic junctions, the processes of which are generally undifferentiated to allow signal transduction in any direction (Grimmelikhuijzen et al., 1996). While it appears primitive in nature, the simple organisation of cnidarian nervous systems has not necessarily impeded the evolution of a higher level of functional complexity that may resemble more sophisticated systems in other metazoans (Mackie, 1990).

In certain parts of the body of some cnidarians, the nerve net is condensed to form longitudinal nerve tracts, circular rings or bundles, thought to act as behavioural control centres in response to environmental stimuli. Given the large diameter of longitudinal nerve tracts or “giant axons”, rapid signal transmission enables fast escape reactions (Grimmelikhuijzen et al., 1996; Mackie, 1990). Circular rings are found at the bell margins of radially symmetric hydrozoan and scyphozoan medusae. They facilitate a simultaneous, symmetrical contraction of the bell to allow swimming by jet propulsion. Similarly, nerve rings located near the mouth and tentacles of hydroid polyps can fire a rapid action in the tentacles in response to a stimulus from food. Cnidarian medusae have also developed gravity sensors and photoreceptors to assist their pelagic lifestyle. Photoreceptive organs (ocelli), consisting of clusters of light-sensitive neurons, are found at the base of marginal tentacles; in some cases they may form larger organs (rhopalia) that may even be equipped with lenses and adjustable irises that resemble more sophisticated eye structures (reviewed in Grimmelikhuijzen and Westfall, 1995).

Cnidarians use peptides as neurotransmitters and neuromodulators; similar molecules were possibly used in early nervous systems (Grimmelikhuijzen et al., 1996). These neuropeptides are stored in neurosecretory vesicles that are associated with both synaptic and non-synaptic release sites. Around 40 cnidarian neuropeptides have been identified to date and contain both a C-terminal amide group and a variable N-terminal protective group that resemble similar peptides in higher organisms (Grimmelikhuijzen et al., 1996). They are first synthesized as preprohormones that undergo cleavage and

modification processing. Most likely, neuropeptides have no basic patterning function, given that nerve-free *Hydra* mutants, consisting solely of epithelial cells, have normal form and regenerative properties (Sugiyama and Fujisawa, 1978). They may, however, be involved in the differentiation of stem cells (also known as interstitial or I-cells) into progeny cells such as nerve cells or gametes (Grimmelikhuijzen et al., 1996; Takahashi et al., 2000).

The nervous system can be visualised by staining animals with an antiserum against cnidarian neuropeptides. First described in *Hydra* using antisera against molluscan neuropeptide Phe-Met-Arg-Phe-NH₂ (FMRFamide), this method gave a much clearer view of nervous system organisation than previously seen with other histochemical techniques (reviewed in Grimmelikhuijzen et al., 1996). Before these experiments, the nervous system was thought to be a diffuse nerve net with no centralisation (Barnes, 1987; Brusca and Brusca, 1990), but is now known to have a more specific organisation.

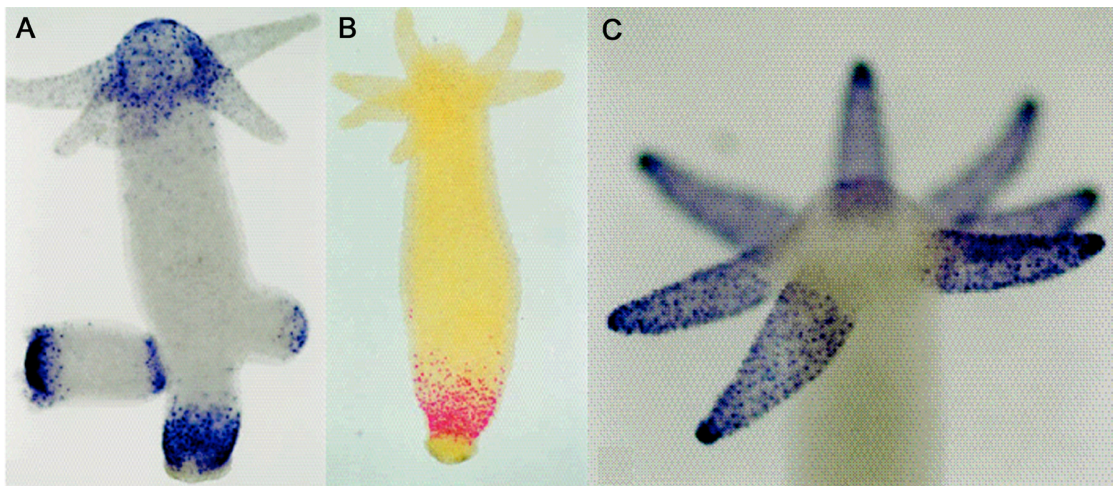


Figure 1.5. Whole-mount in situ hybridisation of *Hydra* with probes coding for Hydra RFamide preprohormones. (A) Hybridisation with preprohormone-A shows a concentration of neurons in the tentacles, hypostome and peduncle region with a similar pattern observed in developing buds. (B) Hybridisation with pre-prohormone-B shows a dense population of neurons at the base of the body column above the peduncle. (C) Hybridisation with preprohormone-C. Intense expression is only observed in the tentacles with a sharp boundary before the hypostome. (Adapted from Hansen et al., 2000)

More recently, studies with a variety of neuropeptides have revealed an even more complex picture in *Hydra* with region-specific expression patterns and possibly different functional roles of each neuropeptide (For example, Darmer et al., 1998; Hansen et al., 2000; Hansen et al., 2002; Mitgutsch et al., 1999). Figure 1.5 demonstrates three examples of the different locations and possible functions of subsets of the RFamide-expressing neuron population in *Hydra*.

1.7. A MODEL CNIDARIAN - *Acropora millepora*

Until recently, the cnidarians of choice have been *Hydra* and *Chlorohydra*. Due to a wealth of research carried out on these textbook cnidarians, their cell biology and developmental mechanisms are well understood. However, their mode of sexual reproduction is unpredictable and inaccessible, occurring within a thick, opaque cuticle (Martin et al., 1997), which makes these animals unsuitable models for comparative molecular embryological studies. The anthozoan coral, *Acropora millepora*, has recently been proposed as a more suitable candidate for cnidarian developmental studies (Miller and Ball, 2000). As a member of the basal cnidarian class, the Anthozoa (Bridge et al., 1995; Bridge et al., 1992; Medina et al., 2001; Odorico and Miller, 1997), *A. millepora* is more likely to display characteristics that better reflect the ancestral metazoan than are the more derived hydrozoans. Acroporid corals participate in the annual mass spawning events (Harrison et al., 1984), providing access to large quantities of synchronous embryonic material (Figure 1.4C). The *A. millepora* genome contains less of a nucleotide bias (~61% A+T rich; Miller, unpublished) than does the *Hydra* genome (~71% A+T-rich; Fisher and Bode, 1989). For this reason it should be less unstable in bacteria and recombinant protein expression should be less problematic due to rare codon usage. The derived character of *Hydra* is also evident in the way it forms the endodermal layer; gastrulation is replaced by the multipotential immigration of cells to form the second germ layer (Brauer, 1891).

1.7.1. The life cycle of *Acropora millepora*

Preliminary studies of the life cycle of *A. millepora* begin to explain the dynamic embryonic and larval development of this species (Miller and Ball, 2000; Miller and Harrison, 1990). Development is divided into two main stages including the dominant, sessile polyp stage and the motile planula stage. The medusa stage observed in other cnidarian classes is absent in anthozoans. *A. millepora* coral colonies consist of

hermaphroditic polyps that grow and divide through asexual reproduction, but form genetically novel individuals through sexual reproduction. During the mass spawning events, egg and sperm bundles are released from each polyp into the water column and participate in fertilisation with gametes from other colonies. A new individual moves rapidly through the morphological stages of embryonic development to the planula stage, finally settling to form a new polyp.

Figure 1.6 shows the development of *A. millepora*. The fertilised egg first undergoes rapid cell division to become an irregular ball of cells (9 h) that flattens into a cellular bilayer, colloquially known as the prawn chip stage due to its resemblance to a fried prawn cracker (13 h). The edges of the embryo then appear to thicken and fold inwards and upwards to form a depression on one side of the flattened sphere (22 h). The cellular details of this process are still unclear but it leads to the formation of a second inner tissue layer known as the endoderm or gastroderm. A schematic diagram of endoderm formation is shown in Figure 1.7. The edges of the outer ectoderm continue to move inward toward the central pore or depression until this pore is completely closed and the inner endoderm is pinched off and internalised.

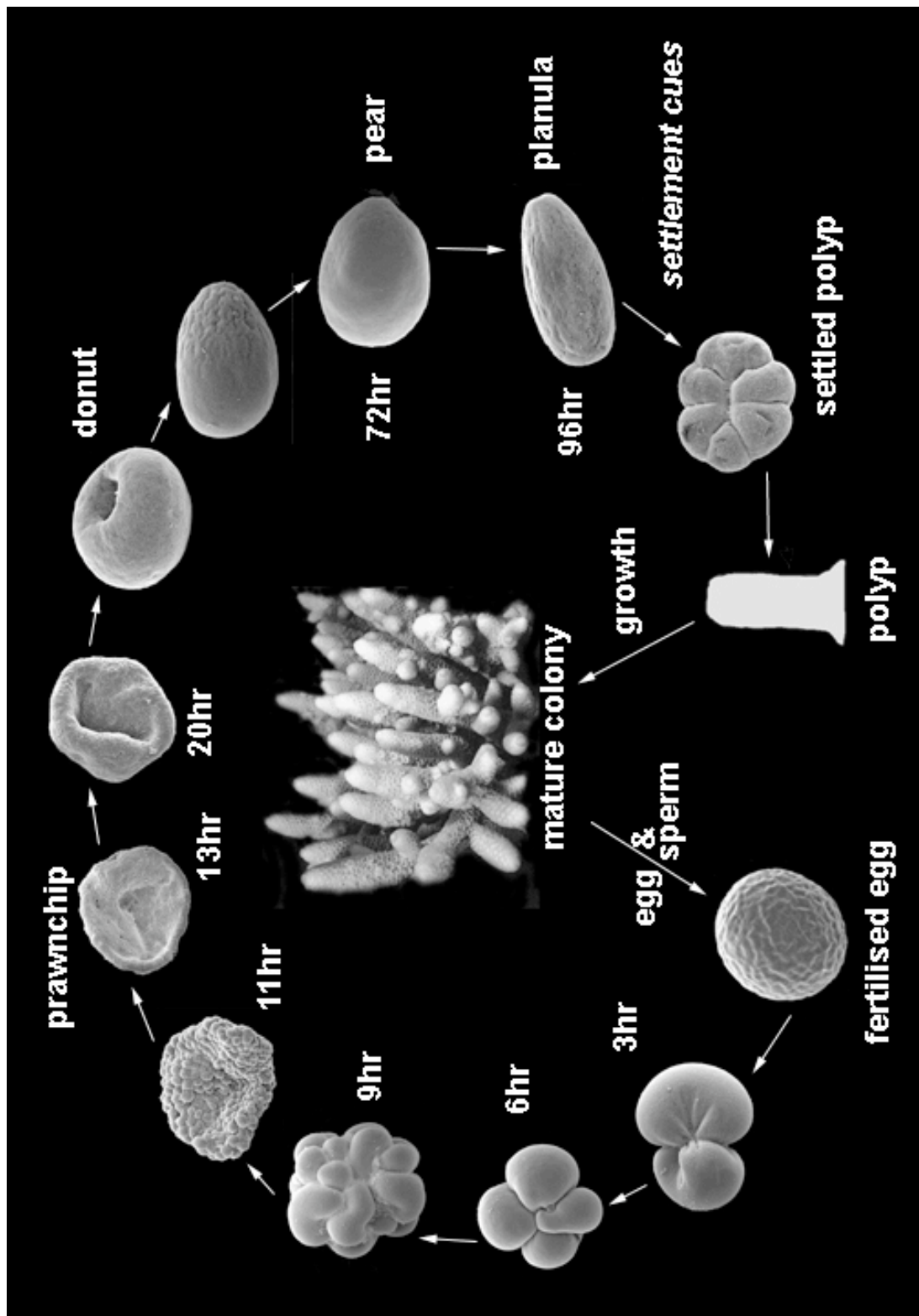


Figure 1.6. Micrographs of the embryonic development of *Acropora millepora*.

The stages are not to scale. Times shown start at fertilization and are representative only, as temporal development is variable depending on several conditions including external temperature. The morphology of late stage embryos (around 96 h) is somewhat dynamic but older planulae are more likely to have an elongated spindle shape. (SEM images courtesy of Dr. Eldon Ball, RSBS, ANU)

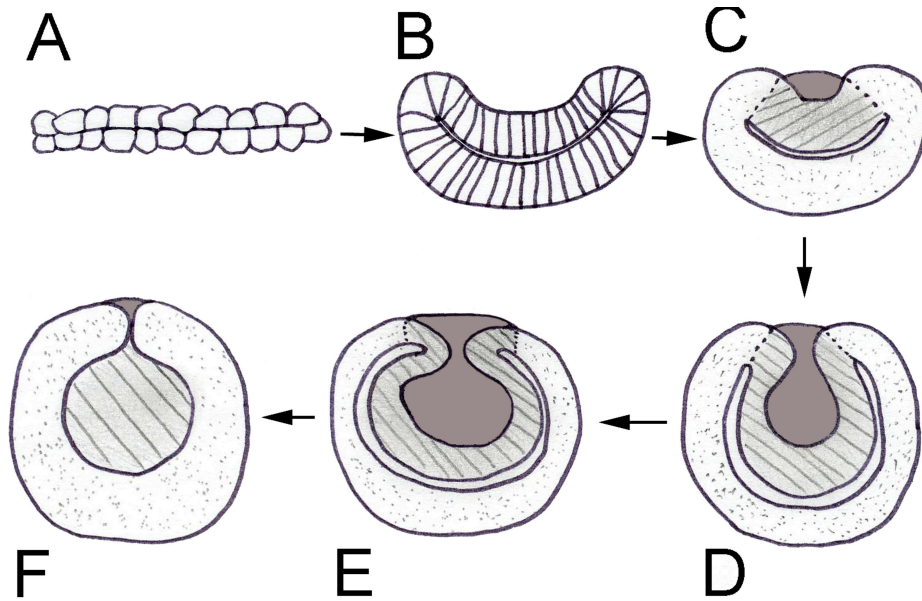


Figure 1.7: Formation of the two tissue layers in *Acropora*. Schematic representation (A) The cellular bilayer of the prawn chip stage. (B) The cellular bilayer begins to contract and thicken and the outer edges appear to fold upwards. (C) The early donut stage. The edges continue to fold upwards and inwards. (D-E) The sphere stage. A deep gastral pore is present before (F) the outer edges make contact with each other. The presumptive ectoderm is speckled, the presumptive endoderm is hatched and the hollow area created by the folding tissue is darkly shaded.

With a more spherical shape, the embryo develops external cilia and over time develops a more pear-shaped morphology, with an oral pore appearing from an invagination of the ectoderm at the posterior end, as defined by the direction of swimming (72 h). The spheroid planula larva persists near the water surface and begins to spin slowly around its central axis as cilia develop over the epidermis. High magnification videography (Ball et al., unpublished) has shown that these cilia beat synchronously to cause locomotion and local currents capable of transporting small particles towards the oral pore. Cilia are also present on the cells lining the oral pore, but unlike the external ectoderm, these cells are exclusively glandular in appearance and may play a role in extracellular digestion (Ball et al., 2002). These cells may form an active feeding mechanism, which may supplement the energy provided by the yolk-containing cells of the endoderm.

As growth continues, the planula larva becomes pear-shaped and then elongated showing an increasing range of swimming behaviour throughout the water column. Its swimming motion is such that the aboral end is foremost while the larva rotates around its longitudinal axis. The motion is dynamic and can be straight, rapid, spiralling or spinning. Once the larvae is competent to settle, it initiates intensive exploratory behaviour, seeking an appropriate place on which to attach and settle. During this behaviour, the aboral epidermis is placed in close proximity to the substratum, while the planula spins on its axis or sways side to side. Sensory components within the aboral epidermis may have an important role in co-ordinating and interpreting information received during this searching motion, but what information is extracted from the surrounding environment is unknown. In the absence of any larval settlement cues, such as coralline algae (Morse et al., 1996), the planula larva can persist in the plankton, as inferred from laboratory studies, for several months (van Oppen, pers. comm.).

Upon receipt of the appropriate settlement cues, the process of metamorphosis into a juvenile polyp begins. The planula larva attaches to the substratum at its aboral end and contracts along the oral-aboral axis to form a flattened disc. Radially symmetrical mesenteries, or septa, appear within the flatten disc as calcification begins. The area surrounding the central mouth then begins to rise with developing tentacles forming on the upper surface. Tentacle development continues as the new polyp continues its vertical development. As the colony grows, it eventually takes on its species-specific morphology.

1.7.2. Coral cell biology

Before the appearance of the oral pore, little cellular differentiation occurs. Early embryos consist of only two cell layers, the endoderm and ectoderm, which are separated by an acellular mesoglea (Figure 1.8 A-C). Recognisable cell types are first detected in the pear-shaped larval stage (around 50 h post fertilisation), largely in the ectoderm. At this stage, neurons can first be visualised with an antibody to the cnidarian neurotransmitter RFamide. These neurons form a nerve net (Figure 1.8F) lying parallel and adjacent to the basement membrane in the ectoderm and form synapses with neurons that span the ectoderm, extending perpendicularly to the surface.

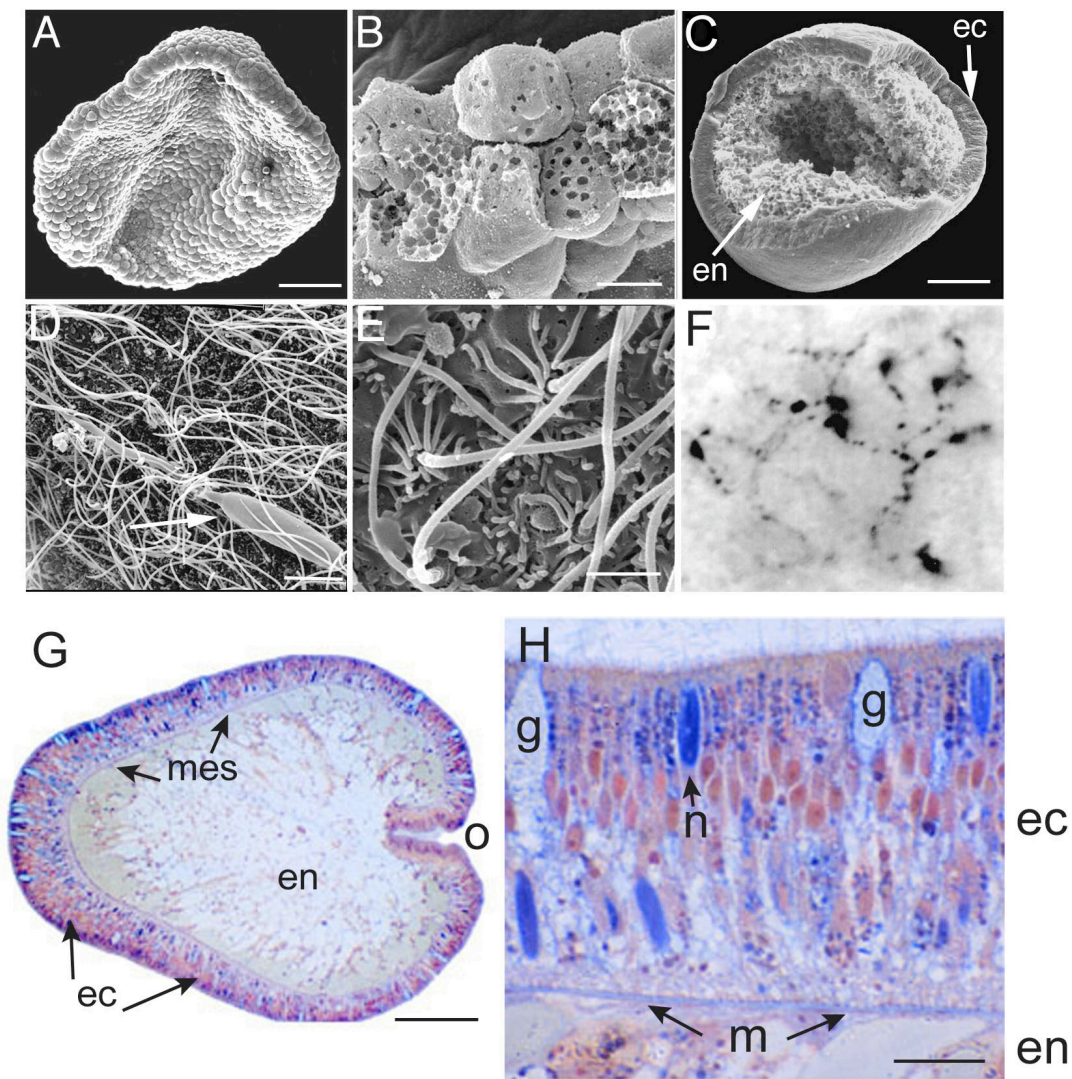


Figure 1.8: Morphology and anatomy of developing embryos of *Acropora millepora*.

(A) The prawn chip stage consists of a bilayer of cells (close up shown in B). (C) By the pear stage of development, the two organised tissue layers are apparent (en = endoderm, ec = ectoderm). (D) By the spindle stage, nematocysts (arrow indicates discharged hook and thread lying on the surface ectoderm), (E) locomotory cilia and (F) an RFamide-containing nerve net can be identified (A-F; Miller and Ball, 2000). (G,H) Trichrome staining reveals the internal structure of a planula larva (Ball et al., 2002). As seen in G, on the outside of the larva there is a highly differentiated ectoderm (ec) separated from the internal endoderm (en) by a thin layer of mesoglea (mes). The majority of endodermal cells contain yolk, but other cell types are scattered amongst them. This section passes through the oral pore (o), which is lined with cilia. Gland cells (g) and a nematocyst (n) are labeled in H. Scale bars: A, C, G = 100 μ m; B = 25 μ m; D = 2.5 μ m; E = 1 μ m; H = 10 μ m

In later larval stages, a number of cell types differentiate and are clearly identified in trichrome-stained specimens (Figure 1.8 G,H). There are also marked differences between the ectoderm inside and outside of the oral pore as previously mentioned. The ectoderm lining the pore consists of a uniform layer of ciliated gland cells, with the cilia densely packed within the oral cavity (Figure 1.8G). The ectoderm covering the majority of the planula outside the pore contains a range of cell types similar to those reported in other coral species (Harrison and Wallace, 1990) including ciliated cells, nematocysts and a number of secretory cells (Figure 1.8 D,E,H).

1.8. PROJECT OBJECTIVES

As indicated above, the relationship between the single body axis of cnidarians and the two perpendicular axes of bilateral animals is unclear. Traditionally, the O/A axis of cnidarians is assumed to correspond to the bilateral A/P axis. However true Hox genes, which are central to A/P patterning in bilaterians, have not yet been isolated in cnidarians. Furthermore, cnidarians have unambiguous orthologs of *vnd/Nkx2*, *ind/Gsx*, and *msh/Msx* - genes that are known to pattern the D/V axis of the CNS in *Drosophila* and vertebrates. In *Acropora*, the *ind* homolog *cnox2-Am* is expressed in a restricted pattern along the O/A axis that strongly reflects the D/V expression pattern of the *ind/Gsx* genes in *Drosophila* and vertebrates. Together these data suggest that the O/A axis is more likely to correspond to the D/V axis of bilaterians, rather than the A/P axis as was original thought.

While true Hox genes are most likely lacking in cnidarians, orthologs of other key components of the A/P patterning system are known in these metazoans and some of these have been cloned in our laboratory; the *Acropora Emx* gene had been previously cloned by Dr David Hayward and Ms Heather Dodd, and a *Tlx* gene was isolated by Ms Laretta Grasso. The main objective of this project was the characterisation of these genes in order to better understand both the relationship between ‘radial’ and bilaterian axes, and the evolution of nervous system patterning processes. During the course of this project, *Acropora* orthologs of several other genes playing important roles in nervous system patterning were also cloned and characterised.