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Source: Zoological Science, 23(5) : 403-418

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.23.403

ZOOLOGICAL SCIENCE **23**: 403–418 (2006)

[REVIEW]

The History of Scientific Endeavors Towards Understanding Hagfish Embryology

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Due to their curious phylogenetic position and anatomy, hagfishes have attracted the interest of zoologists, especially in the context of vertebrate evolution. Embryological information on these animals is now also needed in the field of evolutionary developmental biology (Evo-Devo), as it is expected to provide hints about the origin of vertebrate traits, whether the hagfishes are an in- or outgroup of vertebrates. This review summarizes the importance of hagfish embryology from a phylogenetic perspective, and the history of attempts to obtain hagfish eggs and embryos. Clearly, the main difficulty associated with these animals is their deep-sea habitat. To circumvent this problem, this review also discusses the future prospects for obtaining embryological material, both from the wild and in the laboratory.

Key words: hagfish, embryo, evolution, phylogeny, development

Significance of hagfish embryology in the vertebrate Evo-Devo study

Hagfishes have been, and still are, recognized as among the most important animals for understanding vertebrate evolutionary history (Fig. 1; reviewed by Janvier, 1996; Hall, 1998, 1999). One reason is that their ontogenetic development is poorly known, and the other is that their phylogenetic position remains enigmatic. A number of authors have investigated hagfishes in various ways in various biological fields, including physiology, endocrinology, immunology, and molecular biology (Brodal and Fänge, 1963; Gorbman, 1997; Jørgensen *et al*., 1998; Kubota *et al*., 2001; Pancer *et al*., 2005). Recent studies involving large-scale sequencing analyses have also elucidated intriguing characteristics of this group (Suzuki *et al*., 2004, 2005; Pancer *et al*., 2005). Thus, it appeared to us that it would be useful to summarize and review the few embryological studies and the previous attempts made to collect embryos of this animal group, especially with the aim of obtaining embryological information for future projects.

As to the question of phylogeny, there are two major hypotheses, the cyclostome theory and the craniate theory (Fig. 2; Løvtrup, 1977; Yalden, 1985; Forey and Janvier, 1993; Janvier, 1996; Hall, 1999; Kuratani *et al.,* 2002; Kuratani, 2005). The craniate theory defines hagfishes as invertebrates. This is based on the large number of adult morphological characters shared by gnathostomes and lampreys, and a group consisting of lampreys and gnathostomes has been recognized as a natural taxon by several authors. In particular, the fact that hagfish do not possess vertebrae has promoted this theory, removing hagfishes from the clade of vertebrates that includes only gnathostomes and lampreys. Technically, by definition, the hagfishes are regarded as an outgroup of vertebrates. The group consisting of hagfishes and vertebrates is now called the Craniata (meaning animals with clearly defined heads), which are assumed to have split after the acraniate protochordate, amphioxus, which has no overt head. Support for this idea has come mainly from recent morphological and paleontological studies (Løvtrup, 1977; Forey and Janvier, 1993; Hall, 1998; reviewed by Janvier, 1996; Colbert, 2001).

The craniate theory implies that hagfishes represent an evolutionarily intermediate lineage that split from vertebrates following the acquisition of the head. In this context, the concept of a new head, which describes vertebrates as animals possessing a head based on newly acquired cell lineages (neural crest and placodes; Gans and Northcutt, 1983), makes hagfishes even more important than ever for Evo-Devo understanding of chordate phylogeny in its entirety (see Hall, 1999). According to the limited literature, the neural crest in hagfishes does not seem to de-epithelialize as crest cells, a process seen in lampreys and gnathostomes, but only grows ventrolaterally to form a preganglionic nodule (Fig. 3; Conel, 1942; reviewed by Hall, 1999; for information on crest cell development in lampreys, refer to Horigome *et al*., 1999, and Bronner-Fraser *et al*., 2002, 2003). Therefore, the craniate theory might fit, at least for the scenario of graded evolution of the neural crest, if it really followed sequentially the states of neurepithelium, epithelial pocket,

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doi:10.2108/zsj.23.403

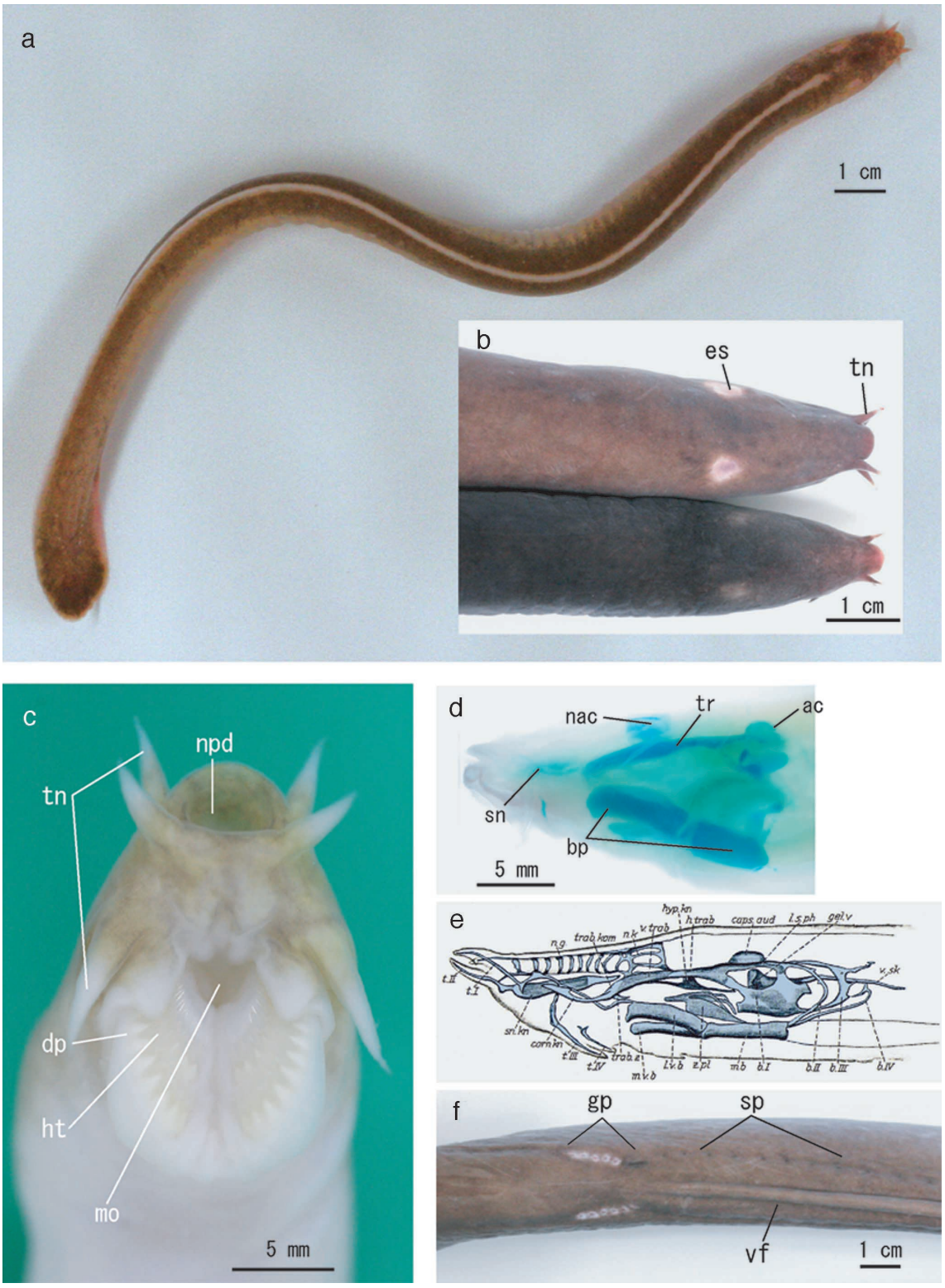

Fig. 1. Hagfishes. **a)** Dorsal view of an adult *Eptatretus burgeri*. **b)** Head region of *Paramyxine okinoseanus* (top) and *P. atami sp.* (bottom) viewed from the dorsal aspect. **c)** Ventral view of the head of *E. burgeri* with horny teeth (ht) opened. **d)** Whole-mount specimen of *E. burgeri*, stained with Alcian blue. For the cartilage matrix of this animal, see Hall (1999, 2005). Some skeletal elements are labeled. **e)** Anatomy of the cranium of *Myxine glutinosa* by Holmgren and Stensiö (1936), for comparison with (d). **f)** Ventral view of *Paramyxine atami sp.*, showing gill pores (gp) and mucous pores (sp). Abbreviations: ac, auditory capsule; bp, basal plates; dp, dental plate; es, eye spot; mo, mouth; nac, nasal capsule; npd, opening for nasopharyngeal duct; sn, subnasal cartilage; tn, tentacles; tr, trabecula; vf, ventral fin fold.

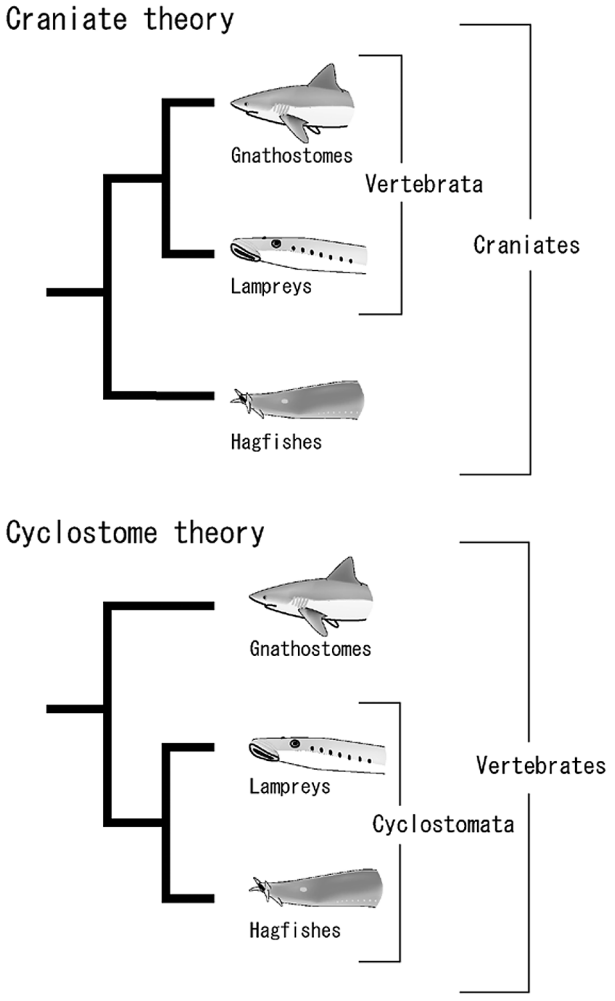

Fig. 2. Two alternative hypotheses on the phylogenetic relationships among gnathostomes, lampreys, and hagfishes. In the craniate theory (top), gnathostomes and lampreys are assumed to form the monophyletic group Vertebrata, and hagfishes are placed as an outgroup of vertebrates. In the cyclostome theory (below), hagfishes and lampreys are clustered as sister groups to form the group Cyclostomata, with gnathostomes as an outgroup (see text).

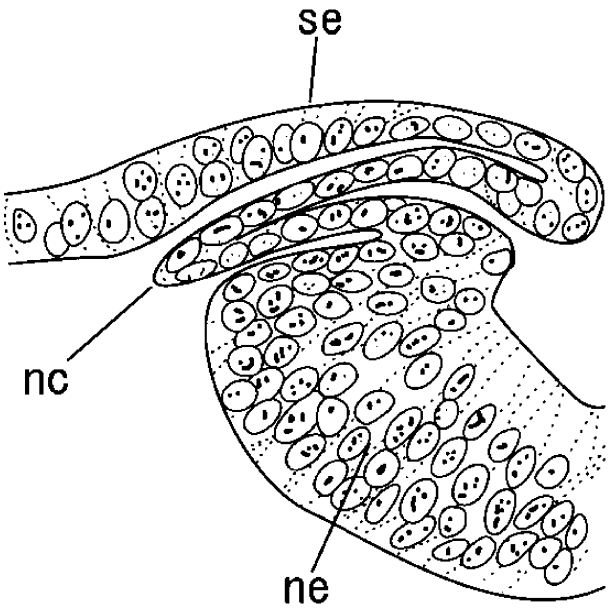

Fig. 3. The neural crest in the hagfishes. A diagrammatic illustration of the cephalic ganglionic primordium in an early embryo of *Bdellostoma stouti* by Conel (1942). Based on the embryonic material obtained by Dean (1898, 1899). In this animal, the neural crest (nc) does not appear to be de-epithelialized to migrate, but grows as an epithelial pocket arising between the surface ectoderm (se) and the neural tube (ne).

and finally, migrating mesenchymal cells. However, it is equally plausible that the condition in hagfishes may represent a secondarily introduced heterochrony in the development of the neural crest, in which the epithelialization of the crest is delayed. Similarly, the morphologically diverse vertebrate spinal nerves, which are found intersegmentally in hagfishes, are partially intrasegmental in lampreys and totally intrasegmental in gnathostomes; this may also favor the position of hagfishes as basal to vertebrates (see Goodrich, 1930; Jefferies, 1986).

An alternative hypothesis proposes that lampreys and hagfishes represent sister groups, forming the clade Cyclostomata. This hypothesis, the cyclostome theory, was a classical standard among paleontologists and morphologists (Jarvik, 1968, 1980; Yalden, 1985; reviewed by Kuratani *et al.,* 2001, 2002). Especially due to anatomical similarities, including features of the nasopharyngeal region, hagfishes have been classified as heterostracans, and lampreys as osteostracans (Jarvik, 1959, 1960; reviewed by Heintz, 1963). Recent molecular phylogenetic data (mitochondrial and nuclear DNA sequences) also generally support this hypothesis (Stock and Whitt, 1992; reviewed by Janvier, 1996, 2001; Mallatt and Sullivan, 1998; Kuraku *et al.,* 1999; Mallatt *et al.,* 2001; Delarbre *et al.,* 2002; reviewed by Kuratani *et al.,* 2002; Takezaki *et al.,* 2003). Under this scenario, the term Craniata is no longer valid, and the group Vertebrata again includes hagfishes, lampreys, and gnathostomes. Although even now the fossil record from the Cambrian era tends to interprested on the basis of the craniate theory (Forey and Janvier, 1993; Xiang-guang *et al*., 2002; Shu *et al*., 2003), it could also be reinterpreted as a cyclostome version. In other words, Cyclostoma as a natural taxon appears equally plausible.

Morphologically, Yalden's precise anatomical analysis of the oral apparatus in hagfishes and lampreys provides some of the best evidence for cyclostome monophyly. The oral apparatus of hagfishes consists of retractable horny teeth and associated skeletal and muscle elements that are anatomically comparable to those found in the tongue of lampreys: homology can be established for every structural element (Fig. 4; Yalden, 1985). A highly organized configuration as found in these organs is unlikely to represent a synplesiomorphy of craniates. Rather, it is more likely to support cyclostomes as a synapomorphic trait. As for the absence of vertebrae, those in the lamprey only appear in adults as poorly developed, irregular, neural arch-like cartilage nodules, and are totally absent from the ammocoete larva (reviewed by Hardisty, 1982); the true vertebra with a centrum is likely to represent a gnathostome synapomorphy.

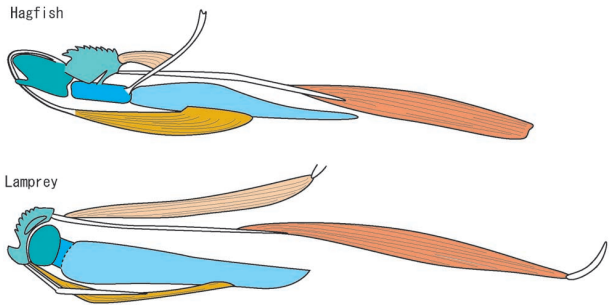

Fig. 4. Homology in the feeding apparatus. Diagrammatic representations of the mandibular arch-derived feeding organs in hagfishes (upper) and lampreys (lower). Shown from left lateral views. Homologous muscles and skeletal elements are indicated with the same colors. Modified from Yalden (1985).

Embryonic traits can also serve as clues to the phylogeny of animals, but robust phylogenetic information in turn is necessary for the understanding of evolutionary scenarios for changes in developmental programs (see Janvier, 2001). Therefore, the arguments tend to become circular, as shown above. However, embryological data may at least eliminate some of the apparent plesiomorphy in hagfishes, by showing a secondary loss was involved (see below). In the same context, based on a comparison between lamprey and gnathostome embryos, the following conserved developmental patterns have been observed: the distribution pattern of cephalic crest cells, oropharyngeal membrane, architecture of the mesoderm, extrinsic eye muscles, pharyngeal arches, rhombomeres and cranial nerve patterns, and gene expression patterns including *Otx* and the Hox code (see Shigetani *et al*., 2002, for conserved gene cascades in oral patterning; see Kuratani, 2005, and references therein; also see Murakami *et al*., 2005, for gene expression and vertebrate brain patterning). If the cyclostome theory is correct, all of these developmental processes are expected to be shared by hagfish embryos, unless some of them have been secondarily modified in the hagfish lineage, or *vice versa* (see discussion of the neural crest above). In another example, the absence of a lateral line in hagfishes can be explained by secondary loss, as a lateral-line placode-like structure has been identified in their embryos (Braun and Northcutt, 1995). Thus, some of the traits that have been recognized as shared only by gnathostomes and lampreys (along the line of craniate theory) may actually represent vertebrate plesiomorphies that were likely lost in hagfishes.

The above line of argument leads to the assumption that many gnathostome-specific embryonic traits can be regarded as gnathostome synapomorphies, or novel changes in the developmental programs leading to gnathostomes. For such traits, seven major characteristics related to the body plan have been assumed, especially in the context of jaw evolution, including the expression patterns of some regulator genes such as the Dlx code (Kuratani, 2005; see Depew *et al*., 2002, for the Dlx code).

Other features in common in developmental processes and patterns between hagfishes and lampreys support the cyclostome clade. Here, it is crucial to determine whether such traits are symplesiomorphic or synapomorphic. For example, monorhiny (the state of having a single nostril; see Janvier, 1996, for further description and definition), which is

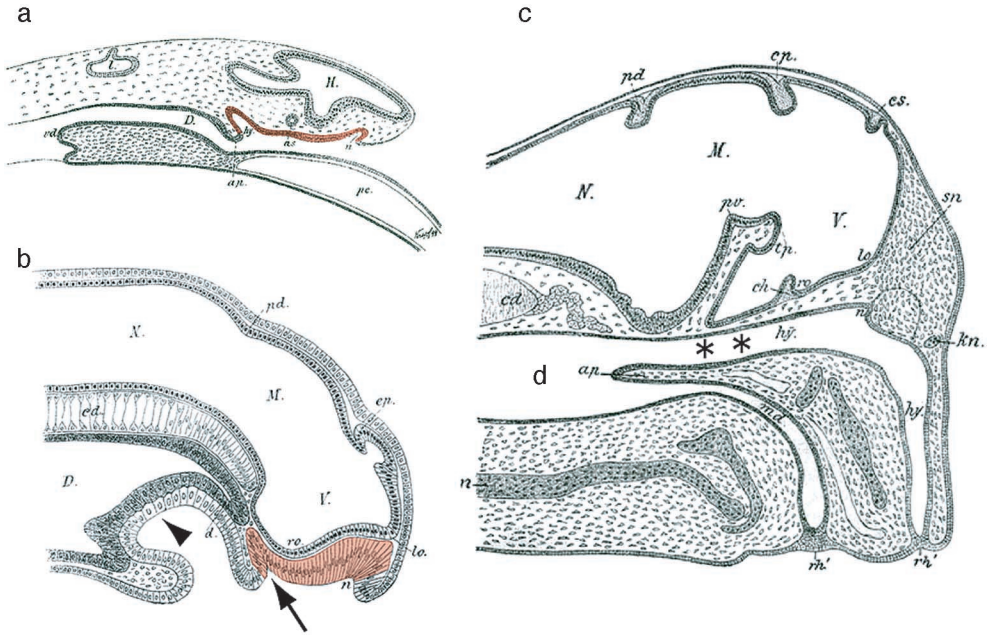

Fig. 5. Embryos of hagfishes and lampreys. **a)** Early embryonic head of the hagfish *Bdellostoma*. **b)** Head of early pharyngula of *Petromyzon*. **c)** Late embryonic head of a hagfish. From von Kupffer (1899). Nasohypophysial placodes are colored red in (a) and (b). Overall morphological patterns are very similar between early hagfish and lamprey embryos, although the oropharyngeal membrane remains in the lamprey (arrowhead in (b)). A hagfish-specific nasopharyngeal duct has not formed. In (c), the basic anatomy of the adult hagfish has been established, as seen in the development of the nasopharyngeal duct (asterisks). This canal corresponds to the hypophysial pit in the lamprey (arrow in (b)), which, however, does not open into the pharynx in the lamprey. The hypophysis of the hagfish differentiates from the dorsal wall of this canal. For details, see von Kupffer (1899).

shared by cyclostomes and is associated with the cyclostome-specific position of the adenohypophysis rostral to the oral ectoderm (Fig. 5), appears to be plesiomorphic to the apparently more complicated diplorhinic (double nostril) state. Thus, monorhiny *per se* will not support the monophyly of the two agnathans, although it is important for the evaluation of gnathostome-specific traits.

More problematic are the differences occurring between the two agnathan animals, as seen in their adenohypophyses and life histories. It has been assumed that the adenohypophysis arises uniquely from the oral endoderm in hagfishes. This was first illustrated by von Kupffer (1899) and has been cited many times (Fig. 5; Gorbman, 1983; Gorbman and Tamarin, 1985; Jefferies, 1986; Gorbman, 1997; also see Kuratani, 2004, 2005). This is quite an unusual condition and provides another example to shake our concept of homology. Detailed study of early head patterning in hagfishes will be necessary to analyze this, especially as the early embryonic head morphology of hagfishes and lampreys is very similar (Fig. 5). Endoderm/ectoderm interfaces tend to blur embryologists' eyes, because of the rapid and complicated reorganization during embryogenesis (see, for example, Kastschenko, 1887).

Comparing life histories, hagfishes develop directly with no larvae, whereas larval lampreys, called ammocoetes, undergo a metamorphosis from the larval state to the adult state (Dean, 1899; Koltzoff, 1901; Damas, 1944; Pivavis, 1960; Tahara, 1988; Kuratani *et al*., 1997; see Richardson and Wright, 2003). To explain these differences, two mutually exclusive hypotheses are possible: (1) the common ancestor of cyclostomes underwent metamorphosis, and metamorphosis became abbreviated in the hagfish lineage (Fig. 6a), or (2) the common ancestor of cyclostomes developed directly, as for extant hagfishes, while in the lamprey lineage the ammocoete stage was secondarily inserted in the developmental process (Fig. 6b).

Some authors have discussed which hypothesis is correct (Hardisty, 1982). For example, Dean (1899), the founder of hagfish embryology (see the next part), considered the ammocoete stage as an adaptation that evolved in the lamprey lineage. Regarding the amount of yolk in eggs, opinions are divided between those who maintain that the large, yolky, shelled hagfish eggs are a primitive character (Løvtrup, 1997), and others who believe that the absence of a larval stage is a secondary condition, resulting from an evolutionary increase in the size of the egg and its yolk content (Schreiner, 1955). Some paleontologists have assumed that a similar larval form was present among fossil agnathans (Watson, 1954; Strahan, 1958; Whiting, 1972; see Hardisty, 1982), while other authors contend, on the basis of the fossil record of Carboniferous lampreys (see Janvier, 1996), that fossil lampreys did not undergo larval development and metamorphosis. To resolve this argument, a detailed comparison will be required of the developmental processes of hagfishes and lampreys.

As we mentioned above, hagfish embryology provides intriguing issues, and new data are expected to be relevant to the Evo-Devo study of vertebrates. Unfortunately, for over a century, there has been almost no major progress in hagfish embryology, due mainly to the difficulties involved in obtaining fertilized eggs. In this report, we will review the previous studies of hagfish reproduction, focusing primarily on why we have been unable to find hagfish embryos or fertilized eggs. We will also offer a proposal on how to collect eggs for future studies.

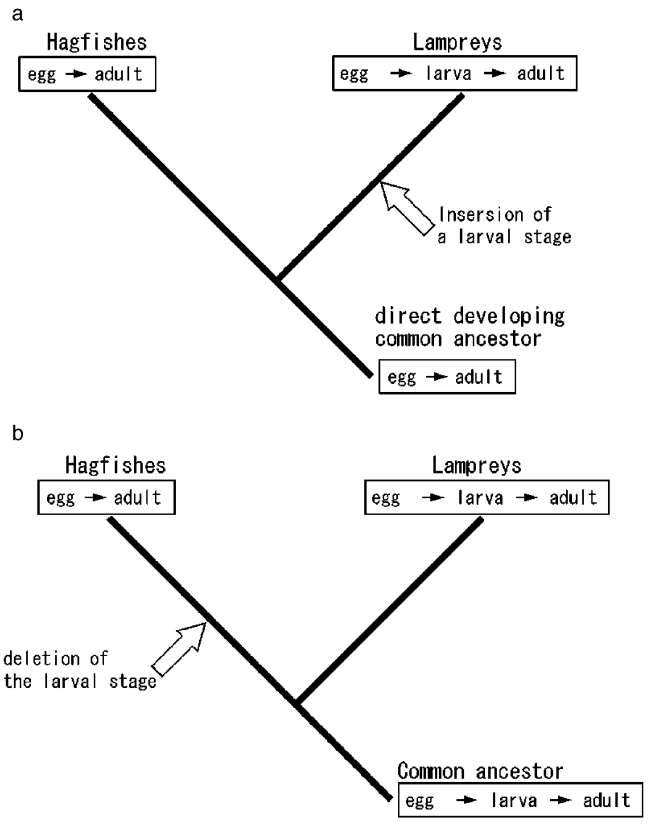

Fig. 6. Two alternative scenarios for the evolution of the life history in two cyclostomes. **a)** The insertion of an ammocoete stage is assumed to have occurred in the lamprey lineage. **b)** The ammocoete state is hypothesized to have been lost in the hagfish lineage.

A brief history of hagfish embryological studies

Since the 1870s, when the scientific importance of hagfishes was already well recognized, many embryologists have endeavored to sample hagfish embryos at various locations in the North Sea and off the Californian coast (Dean *et al.,* 1896). In 1864 and 1865, the Royal Academy of Copenhagen offered an award to anyone who successfully elucidated the reproduction and development of an Atlantic hagfish species (*Myxine glutinosa*), although the award was officially withdrawn in the 1980s (Martini and Flescher, 2002).

Of those scientists who searched for hagfish embryos at that time, only George C. Price, Franz Doflein, and Bashford Dean collected embryos. Price obtained three different developmental stages of *Eptatretus stouti* (then known as *Bdellostoma stouti*) at Monterey, California (Price, 1896a, b, 1897, 1904). Before he published the third report (Price, 1897), he attempted to obtain additional material, but was unable to collect more embryos. Doflein obtained a few embryos of *E. stouti* from the same location; he gave these to Carl von Kupffer, who described histologically for the first time the cranial development of this species (von Kupffer,

1899).

Unlike Price and Doflein, Dean was very successful in covering almost all the developmental stages of embryos of *E. stouti* from the same area (Dean, 1897, 1898, 1899). Dean obtained about 800 eggs in total, of which about 150 contained developing embryos (Dean, 1899). To collect them, he employed the services of local fishermen skilled at longline fishing. On the basis of these specimens, he made more than 130 drawings in his 1899 report (Fig. 7; Dean, 1899). He then transferred these embryos to his coworkers to be investigated with the histological methods available at the time (von Kupffer, 1899, 1906; Stockard, 1906a, b; Neumayr, 1938; Gorbman, 1997). Derived from the eggs collected by Dean, these reports are still cited in recent textbooks and scientific papers as standards for hagfish embryology (Fig. 7; Gorbman and Tamarin, 1985; Janvier, 1996; Gorbman, 1997; Jørgensen *et al*., 1998; Hall, 1999; Kuratani, 2004).

In 1946, Nils Holmgren of Sweden (Holmgren, 1946) reported the fourth study on hagfish embryos. Holmgren distributed jars of Bouin's fixative to local fishermen, promising a liberal reward for each egg. Unfortunately, the Second World War interrupted his efforts. Still, he obtained no fewer than 113 deposited eggs from 1939 to 1944 (Holmgren, 1946), and among these samples were two fertilized *Myxine* eggs. Although the eggs were poorly preserved, Holmgren was the first person to photograph *Myxine* embryos. Using these embryos, he also studied histologically the development of the skull, brain, and cranial muscles, and compared them with Dean's *Eptatretus* embryos.

Bo Fernholm, another Swedish scientist, published the fifth study on hagfish embryos (Fernholm, 1969). He obtained only a single *Myxine* specimen from one of his collaborators, Gustafson, who had also collaborated with Holmgren (Fernholm, 1969). In this 4.5-cm-long embryo, apparently at the stage just before hatching, Fernholm described the development of the adenohypophysis at the histological level. Gustafson had obtained this egg at a depth of 70 m off the Swedish west coast. Gustafson is known for having observed hagfish behavior in an aquarium tank at the Kristineberg Zoological Station, and he also kept *Myxine* species alive for a long time in a tank (Gustafson, 1935). The animals even deposited an egg, which was unfertilized, apparently due to the absence of mature males (Holmgren, 1946).

Our current understanding of hagfish embryology still relies largely on Dean's description and his coworkers' histological observations (Dean, 1898; Doflein, 1898, 1899; von Kupffer, 1899, 1906; Stockard, 1906a, b; Neumayr, 1938). Although a few reports have been published on hagfish reproduction since the last report by Fernholm in 1974 (Fernholm, 1974; Tsuneki *et al*., 1983; Koch *et al*., 1993; Patzner *et al*., 1998; Morisawa, 1999a, b; Hong, 2003; Yuuki *et al*., 2003; Chen, 2004; Powell *et al*., 2004, 2005; Kavanaugh *et al*., 2005), no fertilized eggs have been reported. It is unknown why only Dean could collect such a large number of fertilized eggs, while all recent efforts to collect eggs have failed. To clarify this problem, the techniques used by subsequent scientists to obtain hagfish embryos are reviewed below.

Recent efforts by scientists to collect hagfish embryos

The major difficulty associated with research on hagfish embryology derives from the animals' habitat: hagfishes generally inhabit the deep sea, and the depth differs for each species. *Myxine* species tend to live in deeper areas (around 200–1,000 m) than *Eptatretus* and *Paramyxine* (Fernholm, 1998). Exceptionally, *E. burgeri* can live close to the coast (Kobayashi *et al*., 1972; Patzner, 1998; Tsuneki *et al*., 1983; Fernholm, 1998). To observe hagfishes in the wild, a submersible or remotely operated vehicle (ROV) is required.

In Monterey Bay, where Dean obtained the fertilized eggs, a project to use a submersible to study hagfish reproduction was undertaken by John Wourms in 1991 (Reynolds *et al*., 2001). At the same location, other submersible investigations have also been undertaken to observe other animal species (Greene *et al*., 1999; Reynolds *et al*., 2001). By using similar submersible technology, the behavior of hagfishes has been observed in other areas (Wakefield, 1990; Johnson, 1994; Collins *et al*., 1999). Although a number of adult hagfishes have been observed during these studies, their reproductive behavior has never been observed. Despite these efforts using modern technology, no new data have been obtained to elucidate hagfish reproduction. These unsuccessful studies suggest the possibility that hagfishes may deposit their eggs in cryptic spots on the seafloor, such as in gaps between rocks or under the sand, places that cannot be easily investigated using submersibles or ROVs, which are more suitable for surface observations of flat-bottom areas.

At the Misaki Marine Biological Station in Kanagawa, Japan, several attempts have made by Japanese endocrinologists and Fernholm to collect hagfish embryos (Fernholm, 1975; Kobayashi, 1979; Ichikawa *et al.,* 2000; Nozaki *et al.,* 2000). To find fertilized eggs, they dredged and trawled the sea floor near the station at depths of 30 to 110 m, with no success. They also kept several Japanese hagfish (*E. burgeri)* in an aquarium tank in the spawning season (October), and have even sunk several cages, each containing males and females, 50 m below the sea for the entire spawning season. Despite these efforts, they could not obtain any fertilized eggs (Fernholm, 1975; Kobayashi, 1979; Ichikawa *et al.,* 2000; Nozaki *et al.,* 2000).

Another strategy would be to use artificial fertilization. A number of researchers in the field of endocrinology have used hagfishes as an experimental model (Fernholm and Olsson, 1969; Fernholm, 1972; Rurak and Perks, 1974; Tsuneki *et al*., 1974, 1976; Casley-Smith and Casley-Smith, 1975; Matty *et al*., 1976; Dickhoff and Gorbman, 1977, Dickhoff *et al*., 1978; Schultz *et al*., 1979; Patzner, 1998; Nozaki and Gorbman, 1983; Jirikowski *et al*., 1984; Buckingham *et al*., 1985; Tsukahara *et al*., 1986; Braun *et al*., 1995; Sower *et al*., 1995; Nishioka and Bern, 1996; Candiani *et al*., 2000; Sower and Kawauchi, 2001; Powell *et al*., 2005; Nozaki *et al.,* 2005; Kavanaugh *et al.,* 2006). Although they did not always report it, some of these scientists tried to control hagfish reproduction using hormonal treatment, as has been done with many gnathostome species. Powell *et al*. (2005) applied a hormonal treatment to the Atlantic hagfish (*M. glutinosa*) using lamprey gonadotropin-releasing hormone-III (GnRH-III). Although the lamprey GnRH-III can stimulate the reproductive organs in

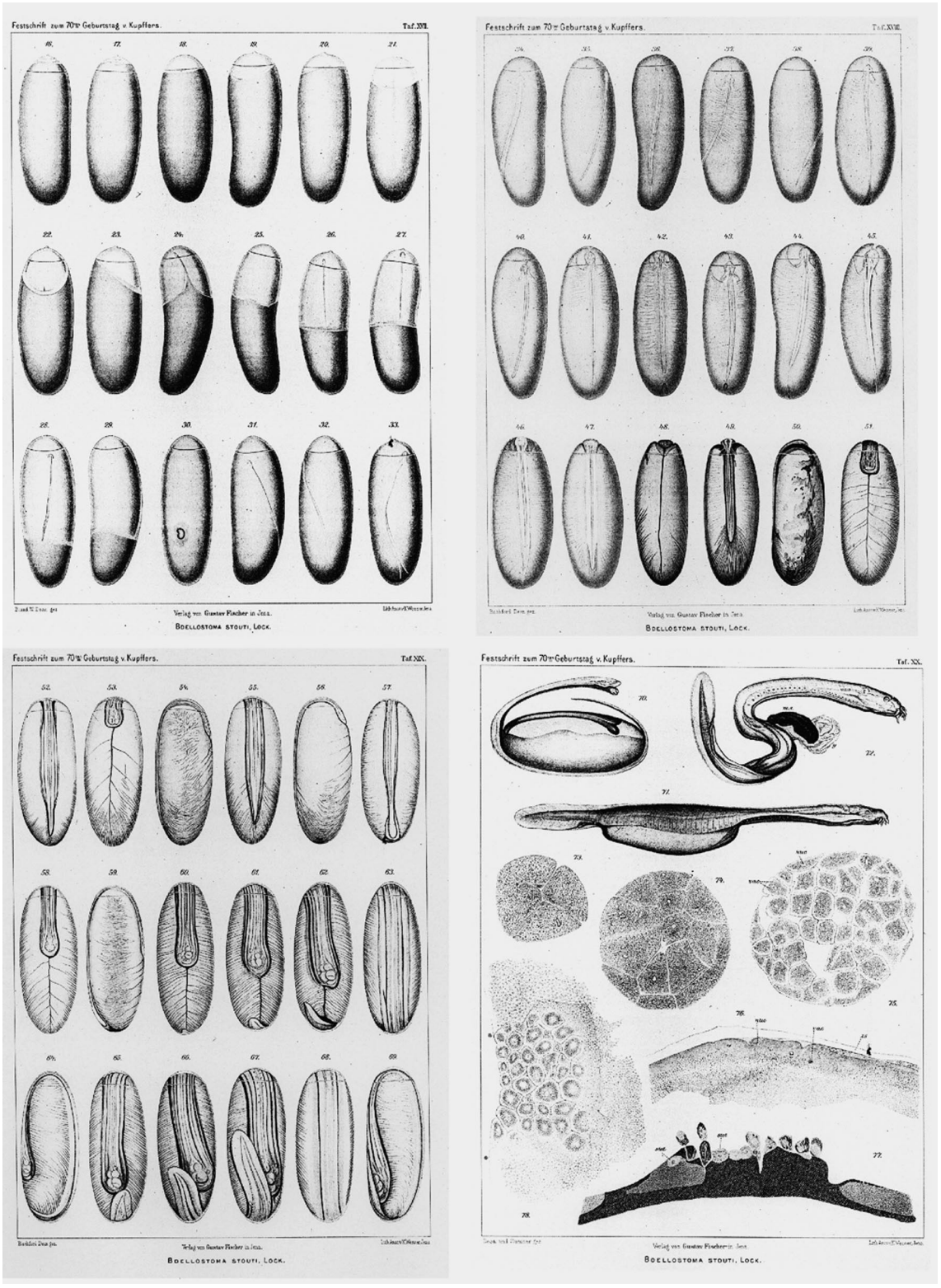

Fig. 7. Embryos of *Bdellostoma stouti* from Dean (1899): early (upper left), middle (upper right), and late (lower left) larvae, and segmentation stages (lower right). Dean drew these figures based on fixed and live embryos.

this hagfish, no one has so far succeeded in obtaining fertilized eggs. Some other hormones have also been tested at various dosages, with no significant results (Fernhorm 1975; Morisawa, personal communication).

Although a number of recent studies have utilized modern technology that was unavailable in Dean's era, none has been successful. To clarify why, it will be useful to examine how Dean actually obtained the eggs in Monterey Bay. In

Dean's (1896) report, the eggs appear to have been obtained purely by accident when mature hagfish were captured by a hook on a longline. They had also secreted an enormous quantity of mucus. Therefore, this suggests a specific feature of the local environment, with a higher abundance of hagfish occurring in the area at the time Dean was sampling. It is possible that the local fishermen could have easily sampled a particular area of the bay where mature hagfish were gathering in the spawning season. Unfortunately, it is not known where mature hagfish deposit their fertilized eggs. If we are to use other strategies such as artificial fertilization, hormonal control, breeding in aquarium tanks and so on, several biological features of hagfishes will have to be considered, including their taxonomy, ecology, distribution, environmental requirements, and behavior.

Molecular approaches to systematics and ecology

It is crucial to accurately identify the species to be used, especially for the investigation of animals collected from their natural habitat. To avoid misidentification of species, the taxonomy and systematics must be well documented. Hagfishes occur in all oceans, except for the polar seas. Living hagfishes are classified into six genera, *Myxine, Eptatretus, Paramyxine, Nemamyxine, Neomyxine,* and *Notomyxine*, although their taxonomy is still confused and the number of genera recognized tends to differ between researchers (see Nelson, 1994; Fernholm, 1998; Wisner, 1999; McMillan and Wisner, 2004). This confusion is due mainly to the limitation of available morphological characters: all the species have an eel-like body shape without any distinct and stable color patterns (Fernholm, 1998; McMillan and Wisner, 2004; see Figs. 1a, b). Thus, the systematics and taxonomy of this group depend on limited morphological characters such as the number of gill pouches, mucous pores, and teeth cusps (Figs. 1c, f).

As an example of taxonomic confusion, Wisner (1999) proposed the genus *Quadratus* for certain hagfish species on the basis of detailed morphological characters, including the position of the gill pouches and the length of the branchial ducts. However, a recent molecular phylogenetic analysis based on a partial sequence of mitochondrial DNA has suggested that this genus should be synonymized with *Paramyxine* (Kuo *et al*., 2003). What becomes clear from this example is that the morphological characters mentioned do not necessarily carry any phylogenetic significance, but rather tend to change rapidly during evolution.

Molecular evolutionary techniques need to be more rigorously applied in order to refine the systematics of hagfishes. Mitochondiral 16S rDNA has been used for the reconstruction of phylogenetic trees of hagfishes (Kuo *et al.,* 2003; Chen, 2004; Chen *et al.,* 2005). However, the resolution of these trees is not high enough to address the phylogenetic relationships among closely related species. In particular, the phylogenetic relationship among most *Eptatretus* and *Paramyxine* species remains unclear. To reconstruct a robust phylogenetic tree among these closely related species, highly diverged DNA sequences will have to be identified. As an example of a recent promising advance in this field, complete mitochondrial sequences have been published for two hagfish species, *E. burgeri* and *M. glutinosa* (Delarbre *et al*., 2001, 2002). In addition, more than 20,000 expressed sequence tags (ESTs) have been collected (Suzuki *et al*., 2004). With these DNA sequences, genes that are more informative, such as the mitochondrial D-loop region and noncoding regions of the nuclear genome, will become available for future analyses.

Molecular methods have advantages for the identification of species, especially in distinguishing sympatric species. Current morphological methods require dissection to observe the teeth cusps of the animal (McMillan and Wisdner, 2004), and therefore cannot be applied to animals that must be kept alive, for example, as for a breeding program in an aquarium. A few hagfish species are sympatric in the sea close to Japan (McMillan and Wisner, 2004). Different species are commonly captured from the same site, which requires an improvement in the methods used for species identification. To avoid misidentification, mitochondrial DNA sequences can be applied.

Molecular evolutionary methods can also be applied to investigate the ecology of hagfishes, as gene flow among local populations may be used to identify potential sites where egg deposition occurs. Although *E. burgeri* occurs in coastal areas in both the Pacific Ocean and the Japan Sea, it is unknown whether the two populations are genetically separate. In addition, it is still unclear whether hagfishes undertake a large-scale spawning migration. To investigate gene flow, high-resolution molecular markers such as restriction fragment length polymorphism (RFLP), microsatellites, and single nucleotide polymorphisms (SNPs) will be needed. Although there have been no suitable molecular markers so far, it is worth trying to analyze the mitochondrial sequences, and suitable microsatellite sequences are expected to be found by screening the bacterial artificial chromosome (BAC) libraries for these animals (Suzuki *et al*., 2004; Pancer *et al.,* 2005).

Hagfishes in the laboratory

To maintain deep-sea animals in aquarium tanks, their environmental requirements must be considered. For hagfishes, salinity and temperature have been suggested as important environmental factors. Below, we discuss some technical problems associated with the maintenance of hagfishes in artificial conditions, based on previous reports as well as on our own experience.

Because no hagfish species so far investigated tolerates low salinities, rapid changes in salinity seem to be detrimental to their survival. For example, although *M. glutinosa* were maintained for weeks at salinities of 29–31 ppt, they could not survive at 20–25 ppt (Gustafson, 1935; Adam and Strahan, 1963; see Martini, 1998). For *P. atami*, it has been reported that after 30 hours of exposure to 27 ppt, all of the animals died or were moribund (Martini, 1998). This sensitivity to low salinity can be a major problem in field sampling using traps or trawl nets. Hagfishes in a trap or trawl net should not be exposed to low-salinity water at the surface, such as occurs especially after rains or due to fresh-water influence from rivers. Hagfishes must be returned to water of suitable salinity as soon as possible after capture. Salinity must also be carefully controlled in an aquarium facility.

Although water temperature may not be as critical as salinity, immediate changes in temperature can have lethal effects. The tolerance appears to vary for each species. For

example, *M. glutinosa* can withstand a change in temperature from 10 to 15°C (Gustafson, 1935; Holmgren, 1946; see Martini, 1998). This species can also be kept at extremely low temperatures (0–4°C) for a long period. *Eptatretus hexatrem* has been kept for over two years at 14–17°C (Kench, 1989). *Eptatretus stouti* has been maintained for a month in seawater at 22°C, and it could survive even at 30°C (Worthington, 1905). *Eptatretus burgeri* feeds in shallow sea areas from November to May, when the water temperature becomes cool (less than 21°C), but migrates into deeper waters from June to October, when the water temperature rises above 20°C (Fernholm, 1974), implying that for this species the optimum temperature is lower than 20°C.

Among commercial traders of hagfishes, it is generally known that *E. burgeri* has a wider range of temperature tolerance than the other Japanese species. We observed that *E. burgeri* could tolerate a change from 12 to 24°C that occurred within 16 hours. Twenty-five individuals accidently exposed to this change in temperature remained at 24°C for more than 24 hours. Nonetheless, they survived and even resumed feeding soon after the water temperature was lowered to the previous level.

In general, there is an apparent correlation between the range of temperature tolerance and the depth of the habitat of hagfish species (see the section on hagfish taxonomy): *Eptatretus* and *Paramyxine* tend to have a wider range of tolerance than *Myxine* species. Thus, to collect fertilized hagfish eggs in an aquarium tank, species of the shallow-water genera *Eptatretus* and/or *Paramyxine* seem to be a better choice than *Myxine* species.

Light conditions, nocturnal habit, and degenerated eyes

Hagfishes in aquaria and in shallow waters have been reported to have nocturnal patterns of activity (Worthington, 1905; Gustafson, 1935; Strahan, 1963; Fernholm, 1974; Ooka-Souda *et al*., 1991; Martini, 1998). An obvious circadian rhythm has been observed in *E. burgeri* by using an automatic recording system (Kabasawa and Ooka-Souda, 1989; Ooka-Souda *et al*., 1993, 1995, 2000), and the existence of dermal photoreceptor cells has been indicated (Newth and Ross, 1954; Kabasawa and Ooka-Souda, 1989; Ooka-Souda *et al*., 1991, 1993, 2000). The relationship between the degenerated eyes, dermal photoreceptors, and the circadian rhythm has also been investigated (Kabasawa and Ooka-Souda, 1989; Ooka-Souda *et al.,* 1991, 1993, 2000). It is now believed that the light perceived through the degenerated eyes is more important for the circadian rhythm than light perceived via the dermal photoreceptors (Ooka-Souda *et al*., 1991, 1993, 2000).

Light sensitivity has been well investigated in hagfishes, while the correlation between light and reproduction is still unclear (Powell *et al*., 2004; Kavanaugh *et al*., 2005). In the case of *E. burgeri*, which is one of the better known shallow-water hagfishes, it is assumed that not only light but also water temperature is an important environmental factor in reproduction. Indeed, seasonal changes in the size of the reproductive organs have been observed in this species: the size of the gonadal tissues is greatest from summer to autumn (Kobayashi *et al*., 1972; Tsuneki *et al*., 1983; Yuuki *et al*., 2003).

In contrast to *E. burgeri*, most other hagfishes live in the deep sea, where light and temperature are nearly constant. It is thus hard to imagine how light conditions can influence reproduction. However, in *M. glutinosa*, which occurs at depths of 150–200 m, seasonal changes have been observed in the level of reproductive steroids (Powell *et al*., 2004; Kavanaugh *et al*., 2005). Furthermore, according to an oceanographic report (Clerke, 1970), blue light is detectable near a depth of 1,000 m in the clearest ocean water (see also Marshall, 1979). In addition, evidence exists for the seasonal breeding of some deep-sea fishes, echinoderms, mollusks, and crustaceans (Harrison, 1988). Therefore, it may not be so surprising if deep-sea hagfishes detect subtle changes in light intensity. Dean (1899) found large numbers of hagfish embryos during the summer and autumn. Additionally, the gonads are largest in summer and autumn in *E. burgeri,* as noted above (Kobayashi *et al*., 1972; Tsuneki *et al*., 1983; Yuuki *et al*., 2003). Considering both lines of evidence, it is possibile that the spawning season of some hagfishes is synchronized by seasonal changes in ambient light. However, we have no clear answer as to whether the light level is the main factor affecting hagfish reproduction. Nevertheless, to control the reproduction of hagfishes, the light conditions (for example, seasonal changes in day length) should be adjusted to reflect changes in their natural environment.

Manner of fertilization and the site of egg deposition

The reproductive behavior of hagfishes must also be examined. Unfortunately, no one has ever observed their reproductive behavior or the deposition of fertilized eggs in the sea or in aquarium tanks. Therefore, there is currently little to discuss concerning their reproductive behavior, except for some circumstantial observations. For example, the characteristic features of their reproductive organs have been described.

Hagfish eggs are larger than those of most oceanic vertebrates, exceeded in size only by the eggs of some chondrichthyans. A mature hagfish egg is ellipsoid in shape, with a long axis of about 3 cm (Fig. 8); there are anchor filaments, similar to the spike hooks of the Velcro® hook-and-loop 'magic tape' fastener, on both the animal and the vegetal poles (Dean, 1899; Kock *et al*., 1993; Morisawa, 1999b; Fig. 8a). The micropylar funnel and micropylar are located on the side of the animal pole (Kosmath *et al*., 1981; Morisawa, 1999b; Fig. 8b).

Female hagfishes tend to have a small number of eggs, although the number produced depends on the species. In *M. glutinosa*, mean egg number is 18 (range 6–32); in *E. burgeri*, the mean number is 32 (range 13–67) (Patzner, 1998). In *E. stouti*, 12–45 large oocytes have been observed in a single gonad (Kock *et al*., 1993). The small number of eggs implies that the strategy of egg production is analogous to that of some elasmobranches that produce a few eggs with a huge amount of yolk, rather than that of teleosts that tend to produce huge numbers of eggs.

The small size of the hagfish testis also offers a clue to reproductive behavior (Cunningham, 1886; Schreiner, 1955). It has been suggested that hagfish produce insufficient sperm for fertilization to occur in open water (Cunningham, 1886). Thus, the possibility of internal fertilization has

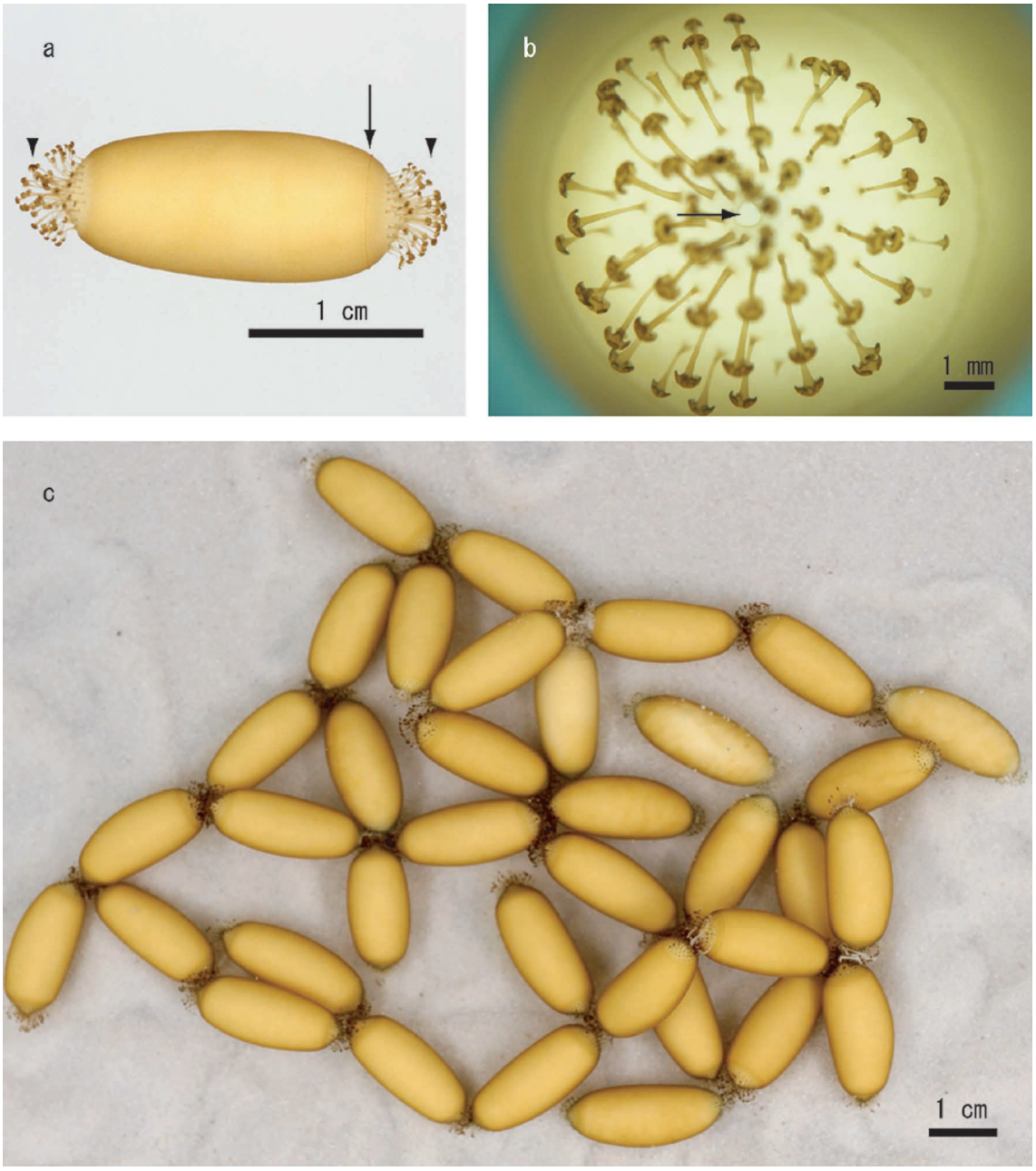

Fig. 8. Deposited eggs of *Eptatretus burgeri*. All the eggs were obtained in November 2005 from a hagfish maintained in our aquarium tank. **a)** Each egg has anchor filaments at both ends of the long axis (arrow) and an opercular ring near the animal pole (arrowhead), showing the morphology typical for hagfish eggs. **b)** Outer opening of the micropylar funnel (white arrow) at the animal pole, surrounded by anchor filaments. **c)** The hagfish eggs stick together at their ends with the anchor filaments to form a cluster.

been suggested (reviewed by Gorbman, 1997). However, due to the absence of external genital organs, it is now assumed that hagfish have external fertilization (Kosmath *et al*., 1981; Gorbman, 1997). Some electron microscopic analyses have shown that hagfishes are more similar to some invertebrates, which depend mostly on external copulation, than to vertebrates in the acrosomal reaction of their spermatozoa (Morisawa, 1999a; Morisawa and Cherr, 2002). This similarity may be related to the efficiency of external fertilization.

Sex ratios are another clue to reproductive behavior and the manner of fertilization. In the sea, female hagfishes are more frequently found than males for all species that have been investigated, although the ratio can differ for each species. For example, in *M. glutinosa* (Nansen, 1887; Conel, 1931) and *E. stouti* (Gorbman and Dickhoff, 1978; Gorbman, 1983), females greatly outnumber males, whereas in *E. burgeri*, the ratio is nearly equal, or females are only slightly more abundant (Tsuneki *et al*., 1983; Patzner, 1998). Surprisingly, mature males are rarely found in their natural habitat; among 1,000 individuals of *M. glutinosa*, only one animal contained motile sperm (Jespersen, 1975). In *M.*

glutinosa and in *E. burgeri*, none among more than 2,000 males had a mature testis (Patzner, 1998). Taken together, these observations likely indicate that the number of available sperm is incredibly limited in hagfish populations. To efficiently fertilize eggs with a limited number of sperm, it has been thought that hagfishes would choose sheltered deposition sites, where the sperm would not be dissipated by strong water currents.

For the above reasons, previous researchers, including Dean himself, have suggested three hypotheses about the manner of fertilization and the site of egg deposition in hagfishes: (1) the eggs are laid in pockets or interstices among rocks (Fig. 9a: Dean, 1899); (2) hagfishes burrow into the sand to deposit their eggs (Fig. 9b: Holmgren, 1974); and (3) the eggs are laid in the mass of slime the animals secrete (Fig. 9c: Gorbman, 1997). However, there is no direct evidence to support any of these hypotheses.

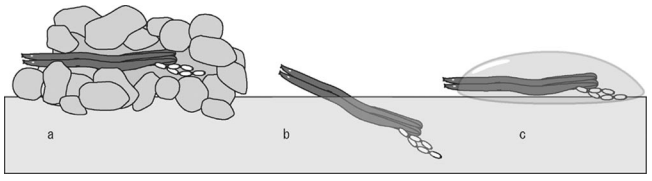

Fig. 9. Hypothetical places for hagfish egg deposition. It has been speculated that hagfishes deposit their eggs either **a)** in the spaces between rocks, **b)** under the sand, or **c)** in a mass of their own slime.

Dean (1898) proposed the first hypothesis on the basis of his own observations. He pointed out that the spawning ground generally has a shelly or rocky bottom. This is also inferred from the rock fragments brought up on the fishing lines that yield hagfishes and eggs simultaneously, from the uniform cleanness of the egg membranes, and from the bits of bryozoans and fragments of mollusk shells entangled with the egg filaments.

In the second hypothesis, Holmgren and others proposed soft bottoms as one of several possible substrata for egg deposition (Holmgren, 1946; Gorbman, 1997). This seems reasonable, taking into account the burrowing behavior and natural habitats of these animals. However, Gorbman (1997) also pointed out that eggs deposited in mud can suffer from anoxic conditions, and thus he suggested the third hypothesis (Gorbman, 1997).

At present, there is no knowledge of where the eggs are deposited. Nevertheless, unsuccessful surveys made using ROVs and submersibles may suggest that hagfishes deposit their eggs in rocky areas, as Dean speculated (Dean, 1899; Fig. 9a), or at least in areas of the seafloor that cannot be easily observed by ROVs and submersibles.

Further studies and problems

To obtain a large number of fertilized eggs, we propose to follow three strategies in our research: (1) to collect fertilized eggs directly from natural habitats, as has been done during all successful studies conducted in the past; (2) to maintain hagfishes and have them breed in aquarium tanks; and (3) to improve the methods of artificial fertilization. However, as mentioned above, there are many uncertain factors and unsolved problems concerning hagfish behavior, habitat, taxonomy, reproduction, etc. We next discuss the problems possibly associated with each strategy, and how these can be solved. We also introduce our ongoing studies on hagfish embryology.

Collection of eggs directly from the sea

Dean (1899) was the only person who was able to collect a reasonable number of hagfish embryos using this strategy. Due to the lack of information on sites of egg deposition, it is extremely difficult to choose an area of the sea to sample, without the services of local fishermen. Indeed, Dean's success was due not only to his luck and the abundance of hagfish in Monterey Bay in those days, but also to the knowledge and skill of the local fishermen, who were familiar with the bay (Dean, 1897). Thus, it is of primary importance to obtain local information about potential locations for hagfish sampling. We have had contact with a local fisherman who is extremely familiar with the seasonal behavior of hagfish in the area around Shimane Prefecture, Japan. With the help of this fisherman, we obtained a single deposited egg of *E. burgeri* from this area. Unfortunately, this egg was not fertilized. Still, we could possibly sample the hagfish habitat throughout the year, and hopefully locate potential spawning sites in the future.

Maintenance and breeding of hagfishes in aquarium tanks

Some researchers (Worthon, 1905; Holmgren, 1946; Kobayashi, 1979) have attempted this strategy, but none has been successful. Although eggs have often been observed in aquarium tanks, these have not been fertilized (Fernholm, 1947; Kobayashi, 1979; Powell, 2005), probably due to a limited number of mature males or sperm, as noted above. Therefore, to increase the possibility of obtaining mature males, we are now focusing on *E. burgeri* as a target species for breeding in tanks, as the species has a nearly equal sex ratio (see above).

Another factor behind the previously unsuccessful studies could be related to the bottom condition of the tank. To mimic the hypothetical natural environment, we have prepared fine-grained sand, oyster shells, and various lengths of plastic tubes as bottom materials (Fig. 10). A total of 13 males and 12 females have been maintained in an aquarium from October 2005 until February 2006 (the time of writing this paper), as *E. burgeri* has the advantages of being easily handled and having a wide temperature tolerance. We actually observed about 50 deposited eggs in the aquarium tank in November 2005, although none seemed to have been fertilized (Fig. 8c; a full description of these eggs and the females will be given elsewhere), implying that the conditions in our tank largely replicate the ideal conditions of the natural habitat.

Improvement of methods of artificial fertilization

Artificial fertilization has not been successfully applied to hagfish species, although it has been used for a number of gnathostomes. For this strategy, the limited number of mature males is again a major problem, as noted above. To obtain mature males, hormonal treatments may possibly be effective. Although no researchers have obtained fertilized

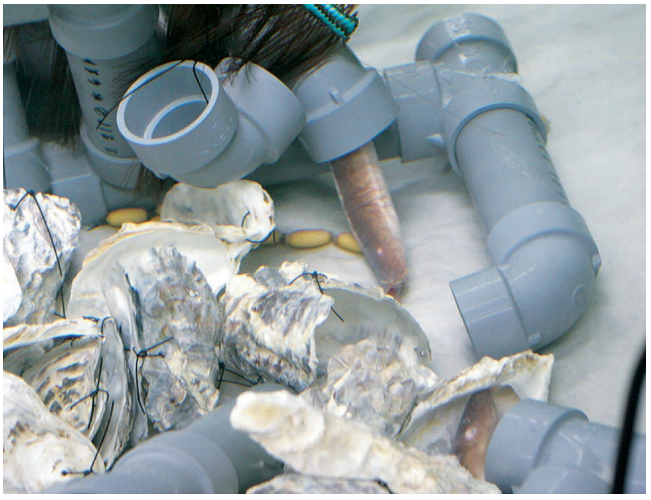

Fig. 10. The hagfish aquarium tank. Plastic tubes and oyster shells were placed on the fine-grained sand at the bottom, so that animals could hide themselves. A string of eggs is deposited on the sand. This condition has been maintained since October 2005.

eggs by artificial fertilization, significant changes have been detected in the animals at the histological and endocrinological levels (Powell *et al*., 2005).

To confirm whether hormonal treatment with gnathostome gonadotropin affects the reproductive organs of hagfishes, we gave mature *E. burgeri* large doses of mammalian gonadotropin. Specifically, we treated six females with premature eggs, caught from September to October 2005, each with 10,000 units of human gonadotropin per day for a week. However, we did not detect any significant changes in the morphology of the eggs (data not shown). Considering the phylogenetic relationships among vertebrates and the indeterminate results of the hormonal treatment, we speculate that the endocrinological mechanism changed in the cyclostome or hagfish lineages, after the divergence between gnathostomes and cyclostomes. A molecular project is currently underway to clone hagfish pituitary hormone genes (Nozaki, 2005). We expect that this endocrinological work will eventually provide a breakthrough in the hormonal control of hagfish reproduction.

Conclusions and perspectives

Since Dean (1899) published his monumental report on the eggs and embryos of a hagfish, many scientists have tried to investigate hagfish development and reproduction. However, no further progress has been made, even though adult animals are frequently observed in their natural habitat. Indeed, modern submersible vehicles and hormonal treatments have been used to investigate hagfish reproduction, but no scientist has obtained successful results. This indicates that elucidation of these life-cycle stages will be achieved with great difficulty. As a reason for undertaking this difficult challenge, we introduced the significance of hagfish embryos in understanding the evolution of the developmental processes of characteristic features, including the neural crest cells, placodes, adenohypophysis, expression patterns of morphogenesis-related genes, and life history (see above). However, to study the evolutionary history of hagfishes, some sophisticated experimental work must be conducted on their embryos, such as electron microscopic observations, molecular studies including gene expression, and detailed comparisons between hagfishes and other vertebrate embryos at various developmental stages. To use hagfishes for these comparative and experimental studies, it will be necessary to secure a sufficient amount of embryonic material. To realize the potential of the hagfishes for Evo-Devo studies, their reproduction has to be well understood.

In this review article, we have mainly emphasized the significance of hagfish embryos for these Evo-Devo issues. However, not only for the Evo-Devo studies, but also for other biological fields, hagfish embryos will deepen our understanding of vertebrates or, more generally, chordates. For example, it has been reported for all hagfish investigated that predetermined chromosomes and DNA fragments are eliminated during a stage of cell division in somatic cells, but not in germ-line cells. The phenomenon, called chromosomal elimination, has not been observed in any other vertebrates (Kohno *et al*., 1986; Kubota *et al*., 1992, 1993, 1997, 2001; Kohno, 1998; Nabeyama *et al*., 2000; Kloc and Zagrodzinska, 2001). The functions, roles, mechanisms, and evolutionary origin of this strange phenomenon are unclear.

Hopefully, whole-genome sequences of hagfishes and lampreys will be available in the near future (Miyake and Amemiya, 2004; Suzuki *et al*., 2005; http://www.genome.gov/12511858). We expect that a combination of genomic and embryonic approaches in hagfish and lamprey studies will allow further progress in Evo-Devo and the other fields of the evolutionary biology of vertebrates.

ACKNOWLEDGMENTS

We are grateful to Masaaki Morisawa, Sachiko Morisawa, and Shigehiro Kuraku for their critical reading of the manuscript, and to Yoshiki Yamamoto for his useful suggestions for aquarium design. We extend our sincere gratitude to Capt. Osamu Kakitani, members of the fishery association in Gotsu city, and Capt. Kouji Yamada in Heda village for their aid in hagfish sampling. This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

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(Received February 10, 2006 / Accepted March 30, 2006)