

Environmental factors affecting reproduction and development in ascidians and other protochordates¹

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Abstract: Protochordate reproduction and development are influenced by many kinds of environmental factors. For example, spawning, sexual and asexual reproduction, larval behaviour, and life-cycle transitions (metamorphosis) are key processes known to be affected by environmental factors. This review must be restricted primarily to only one group of protochordates, the ascidians or “sea squirts”, because information on the reproductive ecology of hemichordates and cephalochordates is limited to only a few studies. Topics discussed in the present review include (i) environmental factors that regulate larval settlement, (ii) how pelagic embryos avoid damage to DNA caused by UV radiation, (iii) the effect of water temperature and food availability on sexual reproduction in colonial ascidians, (iv) environmental regulation of asexual budding, (v) environmental regulation of metamorphosis, and (vi) the possible role of the environment in the evolution of direct-developing ascidians. A novel role for HSP90 and nitric oxide signaling in the integration of environmental factors with cell signaling pathways in ascidians is discussed near the end of this review. Throughout this review, the multiple roles of environmental stress on ascidian reproduction and development are emphasized.

Résumé : La reproduction et le développement des protochordés sont influencés par diverses sortes de facteurs environnementaux. Par exemple, la fraye, la reproduction sexuelle et asexuelle, le comportement larvaire et les transitions du cycle biologique (métamorphose) sont des processus clés que l'on sait être affectés par les facteurs du milieu. Cette rétrospective est centrée nécessairement surtout sur un seul groupe de protochordés, les ascidiens ou « les tuniciers », parce que l'information disponible sur l'écologie de la reproduction des hémichordés et des céphalochordés se résume en un petit nombre d'études. Les sujets que nous discutons incluent (i) les facteurs environnementaux qui contrôlent la fixation des larves, (ii) l'évitement des dommages à l'ADN par la radiation UV chez les embryons pélagiques, (iii) les effets de la température de l'eau et de la disponibilité de la nourriture sur la reproduction des ascidiens coloniaux, (iv) le contrôle environnemental du bourgeonnement asexué, (v) le contrôle environnemental de la métamorphose et (vi) le rôle possible du milieu dans l'évolution du développement direct chez les ascidiens. En fin d'article, nous discutons d'un rôle nouveau pour la signalisation par HSP90 et par oxyde nitrique dans l'intégration des facteurs environnementaux dans les voies de signalisation cellulaire. Dans toute cette synthèse, l'emphasis porte sur les rôles multiples du stress environnemental dans la reproduction et dans le développement des ascidiens.

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Introduction

Protochordates have emerged as an important group of invertebrates to address basic questions pertaining to how ecological factors affect reproduction and development. One group of protochordates in particular, the ascidians or “sea squirts”, have received the most attention by researchers to date. Ascidians are a group of marine invertebrate chordates

that display a wide variety of reproductive mechanisms and life-history strategies.

Many different types of environmental factors are known to affect reproductive and developmental processes in protochordates, including photoperiod, temperature, food quality and availability, ocean hydrodynamics, biomechanics, and proximity to co-specifics (for an overview see Lambert 2005a and Lambert 2005b). Seasonal changes in photoperiod have

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been shown to regulate protochordate spawning and the development of gonads is influenced by ocean temperature. The development of a specific type of blood cell in ascidians requires the uptake of vanadium from the ocean (Michibata et al. 2001). The accumulation of vanadium to four million times the concentration of seawater in ascidian vanadocytes illustrates the role of an environmental factor (in this case, vanadium) in the development of a specific type of ascidian blood cell (namely, the vanadocyte).

Light has been shown to trigger the release of ascidian larvae (Whittingham 1967) and changes in ocean temperatures have been shown to affect not only gametogenesis and embryonic development (Bingham 1997), but also larval recruitment of exotic ascidian species, but not native ascidian species (Stachowicz et al. 2002). In this study, three non-native species (*Botrylloides violaceus* Oka, 1927, *Diplosoma listerianum* (Milne-Edwards, 1841), and *Asciidiella aspersa* (Müller, 1776)) were compared with three native species (*Ciona intestinalis* (L., 1767), *Molgula manhattensis* (Dekay, 1843), and *Botryllus schlosseri* (Pallas, 1766)). Their results suggest that elevated seawater temperatures, presumably caused by global warming, are changing the distribution patterns of protochordate populations. Clearly, this form of environmental stress is having a significant role in protochordate larval recruitment worldwide and is directly responsible for the invasion of "alien" species.

Most protochordates produce short-lived larvae that interact with a highly complex three-dimensional, physical-chemical matrix of ecological factors that either promote, delay, or inhibit larval settlement and subsequent metamorphosis. Therefore, environmental factors play a significant role in regulating the life history of a protochordate, which in part, involves life-cycle-dependent changes in gene expression and cell signaling. Therefore, in asking the question how is reproduction regulated in protochordates, we must examine how environmental factors regulate gene expression. The ecological regulation of gene expression in protochordates is just beginning to be explored. A recent example of how specific environmental cues can inhibit a critical life-cycle transition in a protochordate has come from laboratory studies of an ascidian that lives on the Heron Reef, which is part of the Great Barrier Reef of Australia. Three species of non-geniculate coralline algae (NCA) were shown to inhibit the settlement and metamorphosis of *Herdmania curvata* Kott, 1952 larvae (Degnan and Johnson 1999). Their results contrast with many other studies showing that NCA induce settlement and metamorphosis in echinoderms, mollusks, annelids, and coelenterates (reviewed by Degnan and Johnson 1999), and many kinds of marine larvae. The three types of NCA examined by Degnan and Johnson (1999) were *Neogoniolithon brassica-florida* (Harvey) Setchell and Mason, 1943, *Hydrolithon onkodes* (Heydrich) Penrose and Woelkerling, 1992, and *Lithothamnium prolifer*. Each of these NCAs was tested with competent ascidian larvae in laboratory experiments. The suppression of the life cycle was shown to be dose-dependent and depended on which species of NCA the ascidian larvae interacted with. Depending on the type of NCA used in laboratory experiments, larvae that settled onto NCA biofilms sometimes underwent necrosis or in some cases they survived and could subsequently be artificially induced to undergo metamorphosis

using KCl-elevated seawater. In some cases, the anterior chemosensory papillae that interacted with NCA died and were subsequently sloughed off in the NCA-treated larvae, demonstrating that NCA can function as a factor that triggers a cell stress response (that is, cell death) in protochordate larvae.

Future field studies are needed to determine if NCA-mediated inhibition of the life cycle, including cell death, actually occurs on coral reefs. Furthermore, it will be important to determine the identity of the molecules that are responsible for the suppression of the life cycle and triggering cell death. These results demonstrate how a complex benthic landscape such as a tropical coral reef can negatively influence larval settlement and thereby increase population mortality rates. Their results also support a central theme which runs throughout this review that many components of the marine environment function as cell stress factors that play critical roles in protochordate reproduction and development. Let us now look at another environmental factor, UV, in the life of a protochordate.

UV radiation, development, and reproduction

UV can affect protochordate development and reproduction. To date, most of the information on the effects of UV has been obtained from several solitary ascidians. Tunicates have developed sunscreens to prevent damage of UV-sensitive targets (Epel et al. 1999; Epel 2003). One of the best-studied targets of UV is DNA. Many studies have shown that DNA is damaged by UV irradiation. Epel (2003) has shown that mycosporines, which function as natural sunscreens, can help to limit DNA damage caused by UV radiation. Accessory cells attached to the chorion of the egg were shown to absorb most of the UV-A and UV-B. As it has been shown that *C. intestinalis* sheds its gametes at dawn (Lambert and Brandt 1967), their gametes and early stages of development would be exposed to harmful UV. Exposure to harmful UV would be increased in eggs that float, such as in *A. aspersa*. *Corella willmeriana* Herdman, 1898 follicle cells use ammonium ions for flotation (Lambert and Lambert 1978). Other species produce eggs that sink and as UV is quickly attenuated in water, UV damage would not be a problem unless the spawning adults reside in shallow tide pools.

What are the "sunscreens" that protect the developing embryos? In *Ascidia ceratodes* (Huntsman, 1912), the extra ovarian cells contain UV-absorbing materials that are different than in the egg cytoplasm. Egg components peak at 312 nm, whereas outer cells peak at 342 nm. The concentrations of these components change with the seasons: lower concentrations in the winter and higher concentrations in the summer. HPLC shows that mycosporine-like amino acids (MAAs) are important in blocking out UV-A and B. Furthermore, the egg cytoplasm contained different types of MAAs compared with the outer accessory cells. In eggs, they are mycosporine-glycine and palythine, whereas in follicle and test cells they are shinorine and palythine. From where are the sunscreens derived? Quite possibly they are derived from phytoplankton and bacteria filter-fed by the adults. Epel et al. (1999) has shown that ascidians obtain their sunscreens

from the environment and, therefore, the amount of sunscreen in an egg can vary with seasonal changes in food, which itself is correlated with ambient UV intensity. Therefore, coincident with the rhythmic cycles of the seasons and changing patterns of food availability, the amount of sunscreen available to an ascidian would presumably fluctuate and, therefore, so would the capacity of embryos to ameliorate the effects of UV-mediated cell stress.

In a related study, Bingham and Reynolds (1999) showed that *Corella inflata* Huntsman, 1912 is absent in areas with direct sunlight. In the laboratory, UV irradiation damaged all developmental stages; however, UV-absorbing compounds were not detected in their study of *C. inflata*. Their results suggest that exposure to high levels of UV are avoided in *C. inflata*, not by using sunscreens, but by optimizing the timing of spawning, the brooding of embryos within adults, and the length of larval life. Maruyama et al. (2003) showed that UV light was absorbed exclusively by adult tunic bladder cells in two colonial didemnid ascidians (*Lissoclinum patella* (Gottschaldt, 1898) and species of *Diplosoma* Macdonald, 1859). Their studies suggest that bladder cells help to protect the adult from UV exposure. Furthermore, they showed that live tissues absorbed UV at 320 or 330 nm, corresponding to wavelengths known to be absorbed by MAAs.

Environmental regulation of phenotypic plasticity of sexual reproductive effort in colonial ascidians

Colonial ascidians have recently emerged as an exciting model system to study protochordate reproductive ecology. Detailed information on how the environment regulates reproduction has been obtained primarily from *Botryllus schlosseri* (Pallas, 1774). It is a colonial ascidian in which individual zooids reside within a common extracellular tunic and share the same circulatory system. These animals display a modular type of organization and they can reproduce either sexually or asexually. Before discussing how environmental factors affect reproduction in this group of protochordates, it is important to first examine how reproduction is related to modular organization. Colonial animals have three basic levels of organization: the zooid, the colony, and the multicolony genet. A "genet" is defined as a genetic individual. Each of these levels has its own life cycle and mode of reproduction. Wasson and Newberry (1997) have recently proposed a classification of reproduction in colonial animals that is helpful in understanding the diversity of reproductive patterns in protochordates. In their survey of invertebrates, 10 different modes of modular sexual reproduction were identified including gonochoric (either male or female), 3 modes of sequential hermaphroditism, and 6 modes of simultaneous hermaphroditism. As Wasson and Newberry (1997) indicate, modular animals can have either clonal or colonial forms. In their definition of a clonal form, newly formed modules do not remain in close association with the parent after their formation and they can survive on their own. In contrast, colonial modular animals (among which they include colonial ascidians) produce modules that remain connected to parent and therefore contribute to the overall growth of the whole colony. They further point out that for strictly clonal animals the levels that are important for un-

derstanding reproduction are the genet and module levels, whereas for colonial animals it is the genet, colony, and module levels. They devised a letter code to designate the various modes of reproduction: G for gonochoric; H for simultaneous hermaphroditic; S for sequential hermaphroditic. They identified 6 modes of sex in clonal animals represented by a two letter code and 10 colonial modes of sex represented by a three letter code.

This classification allows us to more accurately compare general reproduction patterns displayed by the various groups of protochordates. Colonial hemichordates (pterobranchs) display HhG and HhH modes (reviewed by Wasson and Newberry 1997). In HhG colonial mode, the colonies are simultaneously hermaphroditic. In HhH mode, simultaneous hermaphroditism is displayed at all levels of organization. Cephalochordates are restricted to solitary forms and display only gonochorism (Wickstead 1975). Colonial urochordates, including many ascidians and thaliaceans, predominantly display the HhH mode of sex (Berrill 1975 as cited by Wasson and Newberry 1997). However, some urochordates use the GgG mode (that is, they display gonochoric modules, colonies, and genets), the SsS mode (in which the genets and modules are sequentially hermaphroditic; for example, *B. schlosseri*, which will be discussed in more detail later in this paper), the HhS mode (in which the genets are simultaneously hermaphroditic and modules are sequentially hermaphroditic), the clonal HH mode (genets and modules simultaneously hermaphroditic), or many ascidians display the HhH mode (in which genets are simultaneously hermaphroditic and the modules are sequentially hermaphroditic; for example, in *Perophora viridis* Verrill, 1871, which will be discussed in more detail later in this paper).

Natural populations of *B. schlosseri* show two different life-history morphs (Grosberg 1988, 1991). Semelparous colonies undergo rapid death after their first cycle of reproduction, become reproductive at an early age, grow rapidly to first reproduction, and show a high reproductive output. By contrast, in iteroparous colonies more than three clutches of eggs are made before death; they delay reproduction until the colonies are approximately twice the age of semelparous colonies; their growth rates are about one-half those of semelparous colonies; their reproductive effort is approximately 75% of semelparous colonies. Grosberg's field studies demonstrated that these two morphs exhibit significant seasonal variations in that semelparous colonies dominate the population through midsummer and then iteroparous colonies dominate the population in late summer. This seasonally repeating polymorphism in colony type suggests that the switch in colony morphs evolved as an adaptive response to the changing seasons.

Food availability has been shown to be an important factor that affects egg production in *B. schlosseri* (Grosberg 1988). Seasonal changes in the amount and quality of food is directly correlated with seasonal changes in environmental stress to which animals must respond. Furthermore, field studies of *B. schlosseri* have shown that different environments cause variations in sperm and egg production in individuals that have the same genotype (Stewart-Savage et al. 1999, 2001).

A recent study by Newlon et al. (2003) examined how

environmental factors regulate reproductive effort in *B. schlosseri*. Reproductive effort can be defined as the proportion of an organism's total energy, which is directed towards reproduction. Their field studies, which were conducted in an estuary, showed that environmental factors determine male versus female SsS mode of modular reproduction. Field sites located up an estuary that had higher food availability and warmer temperatures (that is, a less stressful environment) favoured female reproduction, whereas environmental stress in the form of low food availability and cooler temperatures favoured male reproduction. These results support a growing body of evidence suggesting that environmental stress plays an important role in protochordate reproduction, particularly in modular species, which will be discussed in detail later in this review. Furthermore, their results support predictions from the important sex allocation theory proposed by Charnov (1982).

Proximity to conspecifics is another ecological factor that has been shown to be important in colonial ascidian reproduction. Water-borne sperm from conspecifics function to trigger vitellogenic egg growth (Bishop et al. 2000a). In reproductively isolated animals, the vitellogenic egg growth does not occur. Therefore, the local hydrodynamic conditions responsible for the passive dispersal of sperm play an important role in egg development in this species. The triggering of vitellogenesis would dramatically shift the reproductive effort of the organism, as egg production is said to be "expensive" in terms of the organisms total energy budget. The only sperm able to stimulate egg development are those which "condition" the seawater, and then are taken up by a colony and are stored next to the developing eggs. How these sperm are able to trigger egg development is a project for future studies.

Sperm precedence has been shown in the compound ascidian *D. listerianum* that has internal fertilization and broadcast shedding of sperm (Bishop et al. 2000b). The sperm from the first male that sheds is stored next to the ovary followed by the sperm from the second male and so on; hence, the phenomenon of "sperm precedence". This reproductive strategy allows the animal to produce brooded embryos over a lengthy period of time and at the same time have only a brief period for mating.

Environmental regulation of asexual reproduction

In contrast to cephalochordates (Wickstead 1975), asexual reproduction occurs in many ascidians: all the Aplousobranchiata, some Phlebobranchiata, and Stolidobranchiata (Berrill 1935, 1951, 1975; Brien 1948; Barth and Barth 1966; Nakauchi and Kawamura 1982; Nakauchi and Kawamura 1986; Sabbadin et al. 1992; and others see Satoh 1994). Many studies have shown that the sexual cycle is linked to the asexual cycle and that the degree of integration of zooids which make up a colony directly affects these reproductive modes.

For example, *B. schlosseri* zooids are highly integrated within a colony and as a result three generations (adults, buds, budlets) co-exist and cyclic regression of adults triggers the asexual reproduction of buds to replace adult zooids at the end of their life cycle. The sexual cycle is linked to

the asexual cycle. In most species, the asexual buds stay close to the adult from which they came, but in a few species the asexual buds are released into the water column and promote the dispersal of the species including *Perophora japonica* Oka, 1927 (Mukai et al. 1983) and *Polyzoa vesiculiphora* Tokioka, 1951 (Fujimoto and Watanabe 1976).

Studies on asexual reproduction in species of enterogonid ovoviviparous *Perophora* go back to classic studies by Kowalevsky (1874), Ritter (1896), Lefevre (1898), and others. In a broad definition of a "bud", there are three types of buds: for colony growth (production of new zooids); to make new colonies; and to survive adverse environmental conditions. Mukai et al. (1983) have provided a definition of a "bud": budding equals blastogenesis. A blastozooid in *Perophora* always forms by stolonial budding. In contrast, in *Botryllus* and *Botrylloides*, blastozooids develop in two ways: by pallean and by vascular budding (Oka and Watanabe 1957, 1959). Furthermore, Freeman (1965) showed that blastogenesis is initiated by lymphocytes. *Perophora japonica* exists as a colony of zooids that are connected to each other basally and arise from a common stolon by terminal asexual budding, as first described by Tokioka (1953). Sub-terminal budding produces most of the buds, but buds that are liberated from the stolon also arise at the terminus of the stolon. In *Perophora*, each bud is a double vesicle made up of an outer vesicle derived from the epidermis of the stolon and an inner vesicle derived from the stolonial septum (Mukai et al. 1983). The adult epidermis develops from the outer layer, whereas all the other adult tissues develop from the septum. This is called stolonial budding, and there is terminal growth of the stolon and lateral branching of the stolon. In *P. japonica*, when stolons from the same colony meet each other they fuse and form a stolonial web.

Terminal budding is suppressed by sexual reproduction. Terminal buds attract many granular amoebocytes, then precise morphogenetic processes produce a discoidal stellate body that lifts off the substrate, and is followed by the production of a contractile wave similar to the waves observed in ascidian ampullae (Torrence and Cloney 1981; Bates 1991) to release the stellate bud into the water column as a dispersal mechanism. Buds are transported to new sites and form new colonies. Buds are sticky (Mukai et al. 1983), thereby facilitating their attachment to the substrate. Attached buds immediately produce a long stolon without making zooids and subsequently develop buds that form adult zooids. Mukai et al. (1983) have observed that this development is directly affected by the rate at which the water is flowing: fast growth occurs in running seawater and slow growth occurs in standing seawater. These were laboratory studies, but they suggest the possibility that hydrodynamic input is an important factor in the regulation of blastogenesis.

In *Polyzoa vesiculiphora*, two types of pallean buds are formed: stolonial and planktonic buds. Planktonic buds are packed with granular amoebocytes and they can form new colonies (Watanabe and Tokioka 1972; Fujimoto and Watanabe 1976). But there are significant differences in these two species: in *P. vesiculiphora* the buds are zooid primordia that develop adult structures, but in *P. japonica* the planktonic bud must first grow stolons and then make zooids.

Perophora japonica zooids are protandrous (Mukai et al. 1983) and they brood their embryos in a tubular organ lined with peribranchial epithelial cells formed by part of the branchial wall. Colonies of *P. japonica* that did not show sexual reproduction continued to produce terminal buds. In sexually reproducing colonies, very few terminal buds were observed, suggesting that sexual reproduction suppresses terminal budding. No terminal budding occurs in closely related *Perophora formosana* (Oka, 1931) and *Perophora sagamiensis* Tokioka, 1953. The latter showed a seasonal “replacement budding” in which a bud appeared on the stolon at the site of origin of the degenerating zooid (Mukai et al. 1983). Replacement budding is commonly observed during the sexual breeding season.

Seasonal variations in budding

Mukai et al. (1983) described in Shimoda, Japan, the seasonal hibernation of *P. formosana* and *Perophora orientalis* Tokioka, 1953. In late autumn, zooids and buds regress and throughout winter only the stolons survive. Stolon development is inhibited during this time of stress; that is, in response to lower temperatures and food availability and less light. Every late April to early May, this population makes new blastozooids from the dormant subterminal buds as the environment becomes less stressful. *Perophora sagamiensis* Tokioka, 1953 showed a different link to seasonal climate changes in that in some years dormant stolons were produced during the winter, while in other winters the zooids survived the winter. The reason for this variation is not known at present. *Perophora japonica* colonies produced zooids that survived all year, but there is evidence for seasonal influences. For example, in August zooids sometimes disappeared with stolons surviving.

According to Sabbadin et al. (1992) when *B. schlosseri* is under environmental stress, sexual reproduction stops and budding is reduced until there is only one bud per zooid as the lone surviving zooid, thereby arresting colony growth. If predation or other factors cause the loss of all zooids, and only the vascular ampullae in the matrix survive, then vascular budding in this species can generate a new colony when environmental conditions improve (Milkman 1967). Therefore, some ascidians have evolved mechanisms to survive periods of extreme environmental stress and delay reproduction.

Metamorphosis and environmental stress

The regulation of metamorphosis is another dramatic example of how environmental factors regulate reproduction and development. Most of the recent studies have focused on ascidians. Many recent studies have focused on a variety of topics ranging from larval competence, larval behaviour, larval settlement, and cell signaling events involved in triggering metamorphosis. Many animals, including ascidians, have a biphasic life cycle: the zygote develops into a re-reproductive larva and then undergoes metamorphosis into the juvenile that is eventually capable of sexual reproduction.

What are the factors that induce metamorphosis in ascidians?

Many unrelated forms of cell stress, such as treatment with dyes and physical shaking, can trigger ascidian metamorphosis (Cloney 1961, Cloney 1982; Lynch 1961; Burke 1983; Jackson et al. 2002). Natural inducers, such as biofilm, have been shown to induce metamorphosis. Another inducer is lumichrome that induces metamorphosis in *Halocynthia roretzi* (von Drasche, 1884) but not in other species tested (Tsukamoto et al. 1999). This is a natural inducer thought to be derived from riboflavin (vitamin B2). It is localized in the adhesive organ and posterior region of larvae.

Larval competence and cell signaling mechanisms

Degnan et al. (1997) showed in *Herdmania momus* (Savigny, 1816) that settlement and metamorphosis require larvae to be in what is called a “competent state”. Elevated levels of KCl in seawater will trigger metamorphosis if larvae are competent. They also showed that an anterior signaling center releases a factor which induces metamorphosis. This was shown by separating larval trunks and tails: tails do undergo metamorphosis after KCl treatment. Competence is a physiological state in which larvae can respond to environmental cues. Cations including K^+ , Ca^{2+} , and NH_4^+ induce metamorphosis in ascidians as well as in a wide variety of other marine invertebrates. Competence in *H. momus* occurs 3 h after hatching of larvae at 24 °C. Metamorphosis is linked to apoptosis and is evident by the disruption of myofibrils 24–30 h after KCl treatment in *H. momus*. Degnan et al. (1997) suggest that papillae respond to environmental cues by the activation of sensory neurons that release a signaling factor which is transmitted along the anterior–posterior axis of the larva to trigger cell death and activate the morphogenetic program responsible for adult development.

Kimura et al. (2003) have shown that treatment of swimming larvae with noradrenaline or adrenaline triggers tail resorption in *Ciona savignyi* Herdman, 1882. B-adrenergic receptor antagonists propranolol and B1-adrenergic receptor antagonists metoprolol that inhibit noradrenaline signaling blocked tail resorption. These two neurotransmitters were shown to be localized near the brain vesicle at the time of metamorphosis. These results point toward a role for noradrenalin or adrenaline interactions with B1-adrenergic receptor signaling in the metamorphosis of the genus *Ciona*. Another pathway has been suggested that play a role in ascidian metamorphosis. Davidson and Swalla (2001) showed that a homolog of cornichon which has a role in epidermal growth factor (EGF) signaling is expressed in the anterior papillary region of *Boltenia villosa* (Stimpson, 1864) larvae as they acquire competence. Therefore, their results suggest a role for EGF signaling in the competence of *Boltenia*.

Eri et al. (1999) identified in *H. curvata* a novel protein called Hems, which has four EGF-like repeats. Hems is localized in larval papillae and the anterior epidermis of larvae. Settlement results in the release of Hems, which spreads in a posterior direction along the long axis of the larva during metamorphosis. Metamorphosis of competent larva is blocked by treatment with anti-Hems antiserum. Future studies with other species will be required to see if this mechanism operates in other ascidians.

Patricolo et al. (2001) suggest that *C. intestinalis* metamorphosis requires thyroxine signaling. This is an intriguing result given the role of thyroxine in amphibian metamorphosis. Treatment of larvae with L-thyroxine triggers metamorphosis and larval thyroxine was detected using HPLC. Carosa et al. (1998) reported that *C. intestinalis* has a nuclear receptor belonging to the steroid/thyroid hormone receptor family. In situ hybridization experiments showed that endoderm cells from the neurula to the larval stages expressed this receptor. Based on these results, they suggested a role for this type of signaling in ascidians. However, this ascidian receptor, which by sequence is similar to vertebrate thyroid receptors, cannot bind thyroid hormone, despite the fact that ascidian larvae have thyroid hormone. Additional experiments are needed to determine the role of thyroxine signaling in ascidians and in other protochordate groups.

Gelsolin has been suggested to be involved in metamorphosis of *H. roretzi* (Ohtasuka et al. 2001). Gelsolin is an actin-binding protein involved in regulating modifications of the actin cytoskeleton. Immunostaining indicates gelsolin is enriched in the anterior adhesive papillae, motor neurons, and epidermal sensory neurons in larvae, and then this expression pattern changes during metamorphosis. It is reduced in neurons, but highly expressed in mesenchyme cells and later in tunic cells.

Together, these results suggest that ascidians use various signaling pathways in the transformation of a nonreproductive larva into a sexually reproductive adult. At present, the extent of species-specific signaling pathways involved in the transition in biphasic life cycles can not be properly evaluated until more species have been investigated. As signaling pathways are commonly part of a more complex genetic circuit, it is likely that each research group is describing one aspect of a complex web of interacting signaling processes.

Evolution of direct-developing ascidians and Berrill's "substrate hypothesis"

Most ascidian species have indirect development in which the zygote develops into a free-swimming larva that undergoes habitat selection, settles onto the benthos, and undergoes metamorphosis followed by adult development. However, there are about a dozen or so species that have direct development or incomplete indirect development (Lacaze-Duthiers 1874, 1877; Berrill 1931; Whittaker 1979; Young et al. 1988; Bates 1991, 1993, 1994, 1995, 2001, 2002; Bates and Mallett 1991a, 1991b; Jeffery and Swalla 1992; Jeffery 1997; Tagawa et al. 1997).

Protostyela longicauda Monniot, Vazquez, and White, 1995 is a good example of an ascidian having what can be called "incomplete indirect development". Larval development in this species is modified so that the tail is nonfunctional (Turon and Vazquez 1996). In *P. longicauda*, the sticky tail is a long strand of tunic without muscle cells, nerve cord, or a notochord. The development of a sticky tail is thought to facilitate the highly aggregated, philopatric pattern of distribution shown by this species.

Anural species are restricted to 2 of 14 families of ascidians: Styelidae and Molgulidae (Jeffery and Swalla 1992). Depending on which species is examined, fertilized eggs de-

velop into tailless anural larvae or in some species eggs develop directly into juveniles without the development of any larval structures such as the notochord and ocellus. *Molgula oculata* Forbes, 1848 is an example of the former case in which nonfunctional larval structures partially develop. In this species, a larval notochord placode develops, but notochord cells do not undergo convergence and extension, resulting in the absence of an elongated tail (Jeffery 1997). In the case of *Molgula pacifica* (Huntsman, 1912), it appears that all the features typically associated with larval development are absent. This being the case, this species displays what can be termed "maximum direct development" in contrast to anural (tailless) larval development. *Molgula pacifica* embryogenesis is very similar to well-studied urodele (tailed, indirect) species, except that the embryos develop directly into juveniles. These animals are simultaneous hermaphrodites that can self-fertilize and they are broadcast spawners. The pelagic phase of their life cycle is restricted to the period between spawning and when the sticky zygotes adhere to the substratum. This species is most commonly found in high-energy subtidal habitats where there is a lot of wave action. Their sticky coats are produced by the secretion of mucus from follicle cells that surround the egg and is thought to be an adaptation to this kind of environment (Young et al. 1988; W.R. Bates, personal observations).

In contrast to indirect developers that can select a habitat for settlement and subsequent metamorphosis, *M. pacifica* embryos passively stick to the substrate without any selection and their development is mostly restricted to the benthos and occurs within an environment that is largely insulated from environmental cues. The sticky mucous coat is thick and, therefore, it is highly unlikely that there is any environmental input into their development and reproduction. In contrast to indirect-developing larvae that must develop competence which allows them to respond to environmental cues before they can progress to the next phase of their life cycle, direct developers do not need to develop competence or respond to specific environmental cues. Therefore, the failure to develop competence or interact with environmental factors would increase mortality of indirect developers, but not direct developers.

Insight into how direct- versus indirect-developing ascidians may have evolved has been the focus of many recent studies. For details pertaining to protochordate phylogeny that are relevant to this review, refer to Zeng and Swalla (2005). Based on comparisons of 18S rDNA sequences, these results suggest that direct-developing ascidians may have evolved from indirect developers (Huber et al. 2000). As it has recently been shown that sequence information from different genes can generate different trees (Rokas et al. 2003), the robustness of this 18S rDNA tree must await the future sequencing of other genes. Putting this possible caveat aside for the moment, the question arises as to what may have been the factors responsible for the evolution of direct development?

In 1931, the outstanding Canadian biologist N.J. Berrill presented an intriguing evolutionary hypothesis that is based on environmental factors which modify specific developmental genetic processes. Specifically, he suggested that features associated with the benthic landscape may have been important in the evolution of direct-developing ascidians.

His hypothesis, sometimes termed the “substrate hypothesis”, was based on field studies of two overlapping molgulid populations that display either indirect or direct development. Both species, *Molgula occulta* Kupffer, 1875 and *M. oculata*, live partially buried in the relatively uniform sand flats near Roscoff, France. The development of these animals was first described by Lacaze-Duthiers (1877).

Berrill’s substrate hypothesis can be summarized as follows: direct development first evolved in a relatively uniform sandy habitat in which the complexity of the substrate is highly reduced compared with rocky types of habitats. In uniform benthic habitats, natural selection would favour the spread of genetic changes within the population that would gradually eliminate the developmental processes responsible for generating a free-swimming larva capable of choosing a suitable microhabitat mediated by complex larval behaviors, which include swimming and interactions of the larval ocellus/otolith with light and gravity. Berrill’s hypothesis suggests that there would be no selective advantage in maintaining a complex larval dispersal stage within the life cycle in habitats which are homogenous. Berrill’s hypothesis is supported by recent field observations at Roscoff in that the direct developer is significantly more frequent than the indirect developer (W. Jeffery, personal communication; W.R. Bates, personal observations).

However, Berrill’s so-called “substrate hypothesis” is difficult to reconcile with field studies of other direct-developing ascidians that live in nonuniform habitats. *Molgula pacifica* (Young et al. 1988; Bates and Mallett 1991a), *Molgula tectiformis* Nishikawa, 1991 (Tagawa et al. 1997), *Molgula retortiformis* (Verrill, 1871) (Bates 1995), *Molgula bleizi* (Lacaze-duthiers, 1877) (Berrill 1931), and *Molgula provisionalis* Van Name, 1945 (Bates 1995) are direct developers that attach to rocky substrates in complex microhabitats. However, Berrill’s hypothesis remains valid if one assumes that rafting may have secondarily introduced species to rocky types of substrates, as he suggested in his 1931 paper.

HSP90 and NO: Mechanisms that may link the environment to protochordate reproduction and development

In contrast to other protochordate groups, there is a vast body of ecological, embryological, developmental genetic, and most recently whole genomic information that has been accumulated for ascidians for more than 130 years. Therefore, this group of protochordates is now ripe for ecological developmental studies which will tackle the big question of cellular molecular mechanisms that interact with specific environmental factors to regulate development and reproduction.

Heat-shock proteins (HSPs) are excellent candidate proteins that may link environmental changes with cell signaling. Many recent papers suggest that this ancient and highly conserved superfamily of proteins was present during the formation of the first cells during times of extreme environmental stress (Sangster et al. 2004). HSPs can best be described as “environmental stress proteins” because many different kinds of stress (temperature, osmotic shock, heavy metals, etc) induce the expression of HSP genes. Ascidians

were shown to express HSP60 and HSP70 at all developmental stages examined (Bates and Bishop 1996). Furthermore, HSP70 has been shown to have a role in fertilization of ascidians (Marino et al. 1998), raising the possible role of HSPs in mediating the changing effects of environmental stress on fertilization success. Furthermore, heat shock has been shown to trigger ascidian metamorphosis (Kroiher et al. 1992).

Bishop et al. (2001) have shown that ascidian metamorphosis may involve NO/cGMP signaling and HSP90. Treatment of *B. villosa* and *Cnemidocarpa finmarkiensis* (Kiaer, 1893) with drugs disrupt the wild-type function of HSP90-triggered metamorphosis. In these studies, two specific inhibitors of HSP90 activity, geldanamycin and radicicol, were used. HSP90 is a key regulatory chaperone which has been shown to physically interact with many “client” proteins that have important roles in signal transduction; therefore, HSP90 may function to help to transduce environmental changes into cell signaling pathways that are important in reproduction and development. One of the many molecules known to interact with HSP90 is nitric oxide (NO). NO production depends on the activity of nitric oxide synthase (NOS). Treatments with inhibitors of NOS, for example, L-NAME (L-nitroarginine methyl ester), have been shown to increase the frequency of metamorphosis. Guanylyl cyclase (EC 4.6.1.2) is often the downstream target of NO signaling. When this enzyme was inhibited using ODQ (1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one), the frequency in metamorphosis of competent larvae increased. Anti-NOS immunostaining and NOS histochemistry showed that tail muscle cells expressed NOS. Bisection of tails results in the initiation of metamorphosis of heads. These results suggest that HSP90-dependent NO-based signaling systems repress ascidian metamorphosis. Bishop and Brandhorst (2001) showed that a similar signaling system regulates the metamorphosis of a sea urchin larva. It was also shown that HSP90 plays an important role in ascidian morphogenesis (Bishop et al. 2002).

Bishop et al. (2001) proposed that environmental cues function to release larvae from a repressed state. The inhibitor that the environment acts upon is NO. A reduced level of NO initiates the transition from a pre-reproductive to a reproductive state. Methylene blue has long been known to induce metamorphosis (Lynch 1961) and it has been shown that Methylene blue inhibits NOS and guanylyl cyclase (Grozdanovic and Baumgarten 1999). Crowding of larvae can also lower the activity of NOS, as discussed by Bishop et al. 2001. It has been shown that K channels are inhibited by NO, as discussed in Bishop et al. (2001). Copper and zinc also induce metamorphosis and they too have been shown to inhibit NOS. Thus, the induction of metamorphosis by a variety of agents and treatments may be linked to NO signaling. It remains to be seen if there are species-specific mechanisms that link environmental cues to cell signaling pathways which are involved in the transition to the reproductive phase of the ascidian life cycle. The connection between HSPs and NO/cGMP signaling must await future experiments. We have proposed that a reduction of NO/cGMP signaling may de-repress the release of HSPs, which is mimicked by elevated levels of K ions. Or HSPs may reduce NO/cGMP signaling from tail muscle cells. At least now we have a clear direction for future studies of how

the environment regulates metamorphosis. Future studies should look at the whole picture in a wide variety of species and also extend research to other protochordate groups, because much less is known about these groups compared with ascidians.

Protochordate reproduction and evolution

Bishop and Brandhorst (2003) have argued that the results obtained using ascidians may be relevant to the evolution of biphasic life cycle which most invertebrates display. Most protochordates begin life as embryos that develop into pre-reproductive larvae, as do most other animal groups. The environment triggers the rapid transition to a phenotypically distinct reproductive stage. They argue that biphasy regulated by the environment is ancient. NO signaling is involved in the regulation of life-history transitions not only in at least some ascidians, echinoids, and mollusks, but also in the transition from single cellular to mycelial growth in *Candida albicans* (Robin) Berkhout, and the transition from single cellular growth to fruiting body formation in the slime mold *Dictyostelium discoideum*, and the transition from the pre-reproductive plasmodium to reproductive sporangium in the genus *Physarum*. Thus, many organisms use NO to regulate their transition from pre-reproductive to reproductive phases.

Although the details of the evolutionary implications are beyond the scope of this review, they are relevant to the "big picture" of how the environment and reproduction and development are inseparably linked together. It has been widely suggested that atmospheric oxygen is one abiotic factor which caused the Cambrian explosion when most phyla, including protochordates, suddenly appeared. In their paper, Bishop and Brandhorst (2003) point out that NO signaling is linked to nitrogen metabolism and aerobic respiration, which is linked to the nutritional and metabolic state of the organism. As a water and lipid soluble molecule with low stability, NO is an ideal switch controlling life-cycle transitions in response to environmental circumstances. Furthermore, it has been shown that NOS activity requires an interaction with HSP90. During times of environmental stress, HSP90 chaperones would be diverted to ameliorate the effects of misfolded proteins and therefore would not be available to interact with NOS; this would reduce NO, triggering a transition in life-cycle phase.

Based on molecular genetic and pharmacological evidence, Rutherford and Lindquist (1998) suggested that HSP90 may act as a capacitor of evolution by buffering cryptic mutations, which would be unmasked during periods of environmental stress. Indeed, it has been shown that ascidian eggs and cells contain these highly conserved, constitutive HSPs, derived from a family of genes of ancient origin (Bates and Bishop 1996). HSP90 is present in all cells and constitutes about 1%–2% of the total protein of many cells. This likely accounts for why Kroiher et al. (1992) observed that heat stress induces ascidian metamorphosis if HSP90 is diverted from its role as a chaperone for NOS. These results, only briefly discussed in this review, point to an exciting new direction for understanding how environmental stress regulates reproduction and development in protochordates and possibly other groups of organisms. The

environmentally dependent phenotypic plasticity in colonial ascidian reproduction may involve an HSP90-dependent, NO-based regulatory mechanism.

Reproduction in other protochordate groups

Phylogenetic analysis based on 18S rDNA sequences of hemichordates, cephalochordates, and urochordates has been presented by Cameron et al. (2000). There are three classes of hemichordates: Enteropneusta (acorn worms), Pterobranchia (tube dwellers), and Planctosphaeroidea (planktonic animals). An excellent review of hemichordate reproduction and development can be found in Hadfield (1975). Enteropneusta are solitary animals that reproduce sexually. Both indirect developers (in which the life cycle includes a pelagic tornaria larva) and direct developers (in which the tornarian stage is absent) are present in this group. Asexual reproduction has been described in detail in the following Enteropneusta species: *Balanoglossus capensis* (Gilchrist, 1908), *Balanoglossus australiensis* (Hill, 1894), and *Glossobalanus crozieri* (van der Horst, 1924). The mechanism is similar for these three worms. Asexual reproduction is by fragmentation of the adult. A limited number of field studies suggest that asexual reproduction is seasonal in that summer triggers asexual reproduction. However, *Ptychodera flava* Eschscholtz, 1825, studied in detail by Hadfield (1975) in Hawaii, does not display seasonal asexual reproduction. Hadfield (1975) reports that Enteropneusta do not display hermaphroditism and he also provides details on the reproductive anatomy of hemichordates. Burdon-Jones (1951) provides information on enteropneust spawning in England. He observed seasonal breeding of *Saccoglossus horsti* Brambell and Goodhart, 1941 in early summer. Spawning in the field always happened approximately 30 min after a low-tide exposure. He noted that water currents which enter the burrows of *S. horsti* in the sand triggered spawning. The animals moved towards the opening of the burrow, produced large quantities of mucus, and then released gametes.

Pterobranchia are the second major group of hemichordates and again readers are asked to refer to Hadfield's (1975) review. Unlike the worm-like morphology displayed by Enteropneusta, two of the three genera of Pterobranchs are colonial. Both sexual reproduction, in which planula-like larvae metamorphose into juveniles, and asexual reproduction are present in this group. Well-studied colonies of the genus *Cephalodiscus* M'Intosh, 1882 propagate asexually by budding along a stolon similar in some ways to *Perophora*. The genus *Rhabdopleura* Allman, 1869 is another colonial pterobranch that can reproduce asexually. Similar to the stolonial budding in *Cephalodiscus*, *Rhabdopleura* zooids send out stolons from which buds are formed.

Hadfield (1975) reported that sexual reproduction in *Cephalodiscus* and *Rhabdopleura* is not deterministic given that hermaphrodites and neuters are sometimes observed. Field studies by Stebbing (1970) near Plymouth, England, on *Rhabdopleura compacta* Hincks, 1880 showed that it produces brooded embryos throughout the year. In contrast, it has been reported that sexual reproduction is restricted to a specific season in *Cephalodiscus*. Hadfield (1975) reported that spawning has not been observed in any pterobranch.

Cephalochordates are sometimes called Acrania, amphioxus, or lancelets (see review by Wickstead 1975). To my knowledge, asexual reproduction including regeneration of adults has not been reported in this group. Lancelets do not clearly display sexual dimorphism (Wickstead 1975; Kubokawa et al. 2003; Yamaguchi et al. 2003a, 2003b). Hermaphrodites have been described in several species of lancelets, as well as how temperature, tides, light, and parasites can affect lancelet gametogenesis in Wickstead (1975). Willey (1894), Conklin (1932), and Hatschek (1893) all observed that spawning always occurred at sundown in *Branchiostoma* Costa, 1834. Hatschek (1893) observed that poor weather conditions inhibited spawning.

Conclusion

This review has presented only a small glimpse into the importance of environmental factors in the regulation of protochordate reproduction and development. It will be of great interest to extend the developmental ecology approach to other protochordate groups in future studies. Detailed comparative information on the reproductive ecology of hemichordates and cephalochordates, along with tunicates, will further demonstrate the extensive diversity of reproductive mechanisms exhibited by our vertebrate ancestors and thereby provide fundamental new insights as to how these mechanisms may have evolved.

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