# How gemmules become sponges: known facts and open questions

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ABSTRACT. Freshwater sponges (Demospongiae: Spongillida) have achieved their global distribution largely through gemmules — specialized asexual reproductive structures. Covered with a robust and thick coat, gemmules protect several hundred binucleated pluripotent cells, called thesocytes, from harsh environmental conditions. Gemmules can withstand low temperatures, desiccation, hypoxia, and high salinity, ensuring the survival and dispersal of freshwater sponges. Despite over a hundred years of research, many aspects of gemmule hatching and subsequent sponge development remain unclear. Most studies addressing morphogenetic events during gemmule hatching were conducted in the last century and often do not include microscopic images to support their descriptions. Recently, however, developing gemmules have gained recognition as a model system for cell and developmental biology, representing the entire Porifera phylum. This review compiles existing knowledge about the developmental processes involved in gemmule hatching. We discuss gemmule structure and dormancy, environmental cues triggering germination, morphogenetic processes of hatching and early development, genetic networks underlying sponge body formation, and critical gaps in current knowledge.
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KEY WORDS: freshwater sponges, Spongillida, gemmules, dormancy, hatching, early development.

# Развитие губок из геммул: существующие знания и перспективы исследования

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РЕЗЮМЕ. Своим широким географическим распространением пресноводные губки (Demospongiae: Spongillida) обязаны геммулам — специализированным структурам бесполого размножения. Плотная оболочка геммул защищает несколько сотен двуядерных плюрипотентных клеток, тезоцитов, от суровых условий окружающей среды. Способные выдерживать промерзание, высыхание, гипоксию и высокую солёность воды, геммулы играют ключевую роль в выживании и распространении пресноводных губок. Несмотря на вековую историю исследований, многие аспекты прорастания геммул остаются слабо изученными. Большинство работ, описывающих

морфогенетические события, происходящие при развитии из геммул, датируются прошлым столетием и зачастую не содержат микроскопических снимков, подтверждающих описания. Несмотря на это, в последние годы развивающиеся геммулы стали модельной системой в клеточной биологии и биологии развития, представляя весь тип Porifera. В данной статье обобщены накопленные знания о процессах, задействованных в прорастании геммул. Обсуждается физиология состояния покоя геммул и факторы окружающей среды, вызывающие их активацию, рассматривается организация геммул и морфогенетические события, обуславливающие прорастание и дальнейшее развитие молодых губок, приводятся сведения о генетических сетях, участвующих в формировании тела, и, наконец, подчеркиваются ключевые пробелы в понимании процесса развития губок.
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КЛЮЧЕВЫЕ СЛОВА: пресноводные губки, Spongillida, геммулы, диапауза, прорастание, раннее развитие.

## Introduction

Freshwater sponges, a small lineage within the Demospongiae class, successfully colonized inland waters on every continent except Antarctica. Their widespread distribution is largely attributed to gemmules — peculiar structures enabling clonal reproduction (Fell, 1993; Manconi, Pronzato, 2016). These small, spherical bodies, typically 250–1000 μm in diameter, are dispersed throughout the sponge body or accumulate at its base. Each gemmule features a tough protective layer that encases a cluster of pluripotent cells.

While some marine Demospongiae can also produce gemmules (Simpson, Fell, 1974; Ereskovsky, 2024), those of freshwater sponges must withstand particularly harsh and unstable conditions, requiring exceptional adaptations. For instance, spongillid gemmules can survive exposure to the scorching sun while persisting on high tree branches during the dry season in Brazil (Manconi, Pronzato, 2016), and can tolerate temperatures as low as –45 °C during winter in Alaska (Holmquist, 1973). Moreover, gemmules were shown to endure –80 °C without losing their ability to hatch (Barbeau *et al*., 1989). Gemmules are also known to hatch after several months of hypoxia (Reiswig, Miller, 1998) and a month of sea water exposure (Fell, 1992).

Research on freshwater sponge gemmules began more than 100 years ago, when Zykoff published a study on their hatching and development in 1892 (Zykoff, 1892). Cellular and morphogenetic processes were later described by Wierzejski (1915). Since then, numerous studies have been published on various aspects of gemmule development including biochemical, molecular, cellular, histological, and immunological processes. Young sponges originating from gemmules — primarily *Ephydatia muelleri* (Lieberkühn, 1856), *Ephydatia fluviatilis* (Linnaeus, 1759), and *Spongilla lacustris* (Linnaeus, 1759) — have served as model systems in cell and developmental biology. These models provided new valuable data on cytoskeleton structure in epithelial-like cells of the young sponges (Pavans de Ceccaty, 1986; Wachtmann *et al*., 1990; Wachtmann, Stockem, 1992; Schippers, Nichols, 2018; Mitchell, Nichols, 2019), contractile capacity of pinacoderm (Ruperti *et al*., 2024; Colgren, Nichols, 2022), stem cell establishment (Funayama *et al.,* 2010; Okamoto *et al*., 2012; Alie *et al*., 2015; Ereskovsky *et al*., 2024), early skeleton formation (Kishimoto *et al*., 2019), cell differentiation (Mohri *et al.,* 2008; Funayama *et al*., 2005, 2010), allorecognition (Curtis, Van de Vyver, 1971; Van de Vyver, 1979, 1983), and the role of Wnt signaling during sponge development (Windsor, Leys, 2010; Windsor *et al*., 2018). Moreover, the complete genome of *E. muelleri* was recently sequenced (Kenny *et al*., 2020), and the single-cell transcriptome of *S. lacustris* was generated based on gemmule-derived specimens (Musser *et al*., 2021).

However, despite these impressive achievements, the development of young sponges from gemmules remains surprisingly unclear. Most studies describing morphogenetic events during

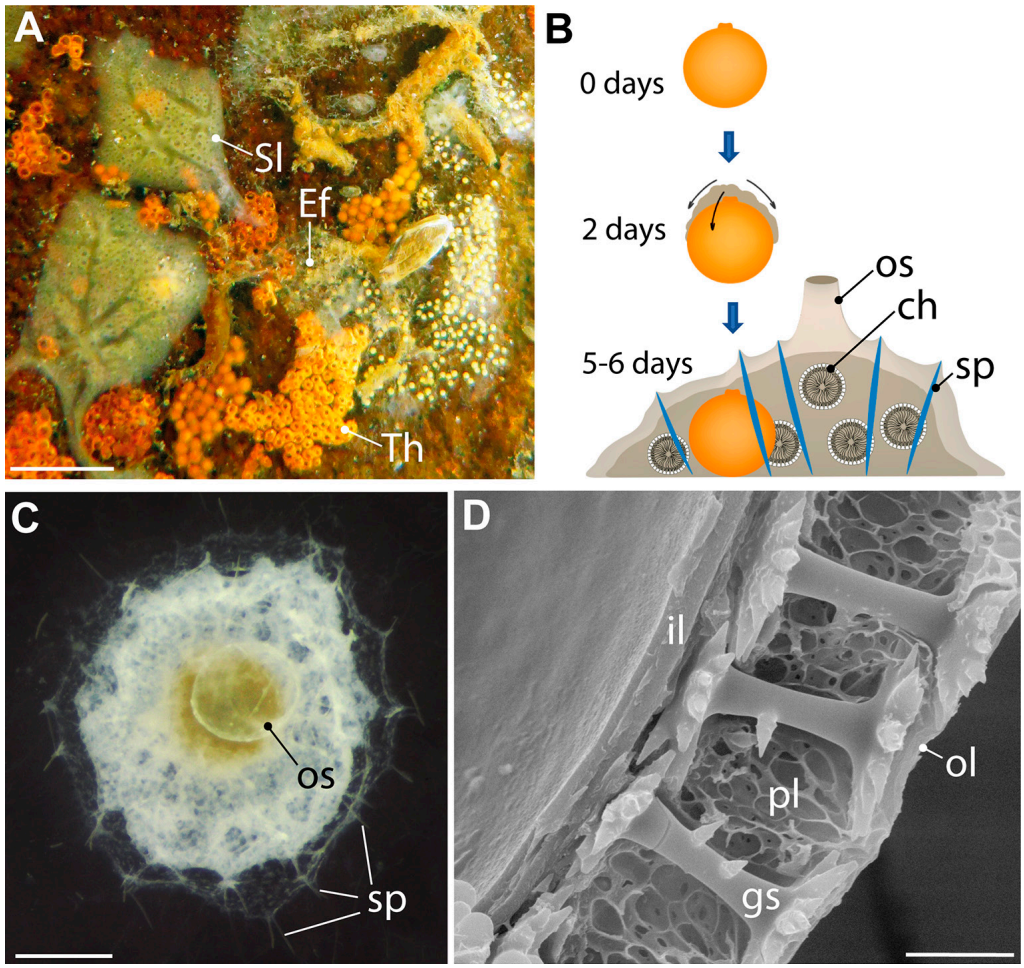


Fig. 1. Freshwater sponges gemmulation. A — september gemmules of three different sponge species, *Spongilla lacustris* (Sl), *Ephydatia fluviatilis* (Ef), and *Tubella horrida* (Weltner, 1893) (Th), *in situ*. Note different stage of tissue degradation among species; B — schematic representation of the young sponge development from a single gemmule; C — juvenile sponge grown in laboratory, top view; D — gemmular coat of *Ephydatia fluviatilis* (thesocytes are removed), SEM image.
Abbreviations: ch — choanocyte chamber, gs — gemmulosclere, il — inner layer, ol — outer layer, os — osculum, sp — spicule, pl — pneumatic (middle) layer. Scale bars: A — 0.5 cm, C — 200 µm, D — 20 µm.

gemmule hatching date back to the last century and often lack microscopic images to support their findings. There is still no comprehensive and integrated description of the processes that occur after gemmules exit dormancy and develop into young sponges. In this review, we aim to: 1) compile disparate data to gradually reconstruct the developmental process during gemmule hatching, 2) align the terminology used by different authors, and 3) highlight persisting questions that require further investigation.

## Gemmules as a key element in freshwater sponge survival and dispersal

The primary function of gemmules is to withstand environmental stress. Inland water systems are often subject to dramatic seasonal fluctuations, such as winter freezes and summer droughts. To survive these challenges, sponges use an approach commonly observed in various freshwater invertebrates. Gemmules in sponges, cryptobiosis in rotifers, resting eggs

in branchiopods, and statoblasts in bryozoans all represent variations of a common survival strategy — dormancy. This strategy not only ensures the survival of the individual/colony but also enhances the long-term sustainability of species with shared ecological niche (Cáceres, 1997). For instance, although different freshwater sponges occupy the same niche, they often differ in the timing of gemmulation, tissue degradation, and hatching, which helps reduce interspecific competition (Fig. 1A).

The dormancy stage in aquatic invertebrates can take various forms, involving the entire organism, eggs, or asexually produced structures. Gemmules belong to the latter category and function as a clonal dispersal mechanism, with each gemmule capable of developing into an independent individual (Fig. 1B). Researchers actively take advantages of this feature, as gemmules from a single sponge can be reared under laboratory conditions to produce multiple genetically identical young sponges.

In nature, cell masses hatched from different gemmules typically merge and colonize the vacant skeleton left by the 'maternal' sponge, if it is still available (Simpson, Gilbert, 1973; Gilbert, 1975). However, if gemmules are scattered — by birds or fishes feeding on sponges — they may float away and establish new genetically identical individuals in nearby areas. Additionally, gemmules can disperse over long distances via ingestion by birds (McAuley, Longcore, 1988; van Leeuwen *et al*., 2017) or, potentially, via adhesion to mud on beaks and feathers (Bass, Volkmeir-Ribeiro, 1998; Fell, 1992; Manconi, Pronzato, 2016; Økland, Økland, 1989). It was also suggested that gemmules may float to the surface and drift downstream thanks to the air trapped in the pneumatic layer of the coat (Manconi, Pronzato, 2007). Thus, gemmules enable freshwater sponges to withstand harsh conditions and successfully colonize new areas. The following section discusses the adaptive features of dormant gemmules that support these abilities.

## Structure of resting gemmule

The coat of gemmules (= gemmular theca/shell) is a firm, highly specialized protective envelope that encases the dormant cells. In many spongillids, it consists of three layers: laminated inner layer, pneumatic (= alveolar) middle layer, and simple outer layer (Simpson, Fell, 1974; Simpson *et al*., 1985; Masuda, 1998; Manconi, Pronzato, 2002) (Fig. 1D). The inner layer comprises electron-dense dark bands of striated spongin fibrils (De Vos, 1977). The inner border of this layer contains a distinctive chitinous band (Jeuniaux, 1963; Masunari, 1982; Simpson *et al*., 1985). The structure of the pneumatic layer varies among species, ranging from minimal or absent to large and complicated, with alveolar or orthogonal mesh patterns (Simpson, Fell, 1974; Manconi, Pronzato, 2002). Spicules of various shapes pierce this layer in a more or less radial manner, protruding from the gemmule surface or lying tangentially. The shape of gemmular spicules is an essential diagnostic feature for species and genera delimitation (Manconi, Pronzato, 2002). The third, outer layer of the gemmular coat is similar to the inner one but has less regularly arranged spongin fibrils and lacks the chitinous layer (De Vos, 1977; Simpson *et al.*, 1985).

The most remarkable structure of the gemmule is the micropyle (= pore, aperture), an opening that can be single or multiple (usually 1–2). Its walls are formed by the outer and inner layers of the coat, with the pneumatic layer significantly reduced up to absent. The micropyle may be located in a small depression, slightly elevated with a narrow collar around it, or concealed inside a long foraminal tube (Masunari, 1982). In resting gemmules, the micropyle is sealed with one or two thin chitinous membranes (Brien, 1932; Masunari, 1982).

The complex, thick coat of the gemmule is not the only feature that helps sponges survive. The enclosed cells themselves are highly adapted to harsh conditions. According to Ruthmann (1965), gemmules contain about 500 cells. These cells, called thesocytes (= primary archaeocytes, statocytes), are roundish, morphologically similar, and densely packed without any signs of intracellular contacts (Ruthmann, 1965). Each thesocyte contains two touching nucleolated nuclei and abundant reserve substances in two forms: small (0.5–0.8 μm) and large (6–8 μm) granules and lens-shaped, light-refracting vitellin platelets (= yolk platelets, yolk granules, lens-shaped inclusions) (Kauffold, Spannhof, 1963; Ruthmann, 1965; Simpson, Fell, 1974; Höhr, 1977; Simpson, 1984). These nutrient platelets have a sophisticated structure with distinct functional

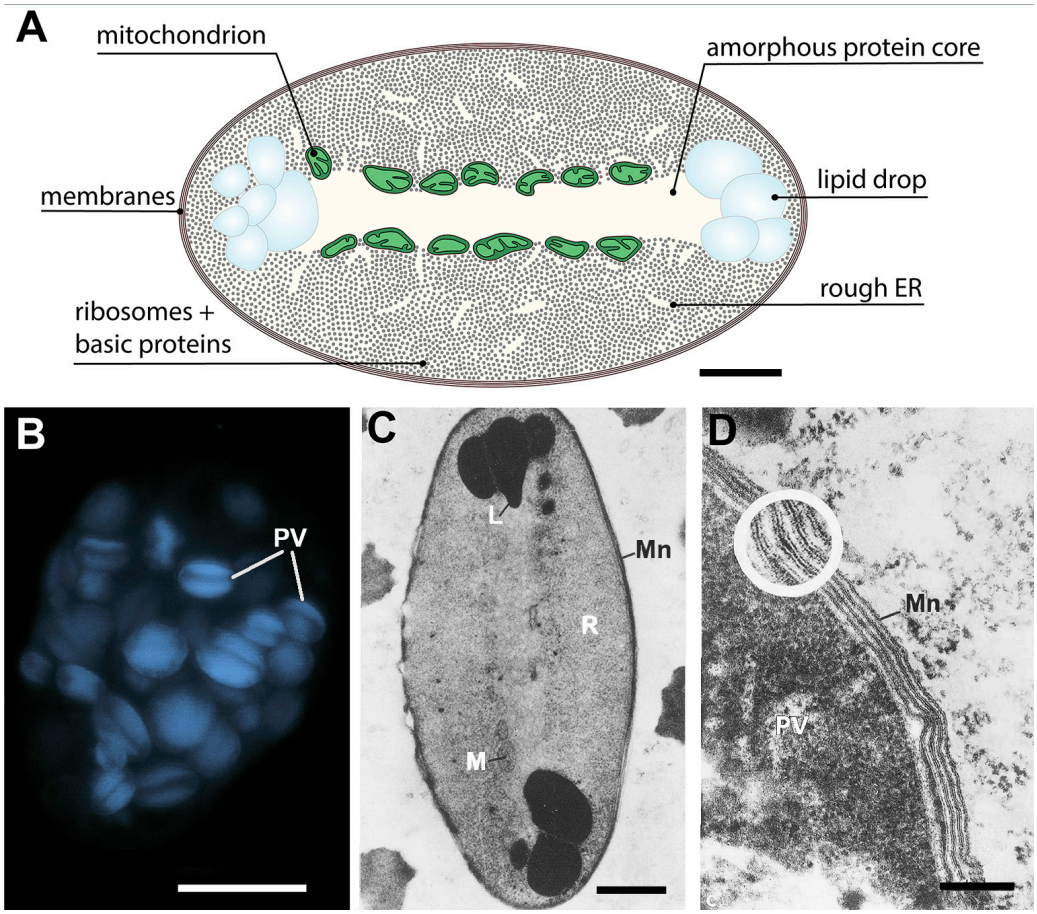


Fig. 2. Vitellin platelets. A — scheme of the platelet structure, redrawn from De Vos, 1971; B — vitellin platelets in thesocytes of *E. fluviatilis* stained with DAPI, non-specific binding. Note the characteristic coffee-bean shape of the platelets with non-fluorescent amorphous core; C — TEM image of *E. fluviatilis* platelet, from De Vos, 1971; D — platelet's membrane, from De Vos, 1971. Permission to reproduce the images C and D was granted by the Societé Français de Microscopie.
Abbreviations: L — lipid drop, M — mitochondrion, Mn — membrane of the platelet, PV — vitellin platelet, R — ribosomes. Scale bars: A — 1 μm, B — C — 0.5 μm, D — 0.2 μm.

zones: a belt of lipid granules, an amorphous acidophilic protein core, and massive lateral valves (= caps) rich in mitochondria, rough endoplasmic reticulum, and ribonucleoproteins (Simons, Muller, 1966; Simpson, 1984) (Fig. 2). Moreover, the platelets contain amino acids, glycoproteins, and polysaccharides (Kauffold, Spannhof, 1936; Simons, Muller, 1966; Tessenow, 1969; De Vos, 1971; Harrison, Cowden, 1975). Finally, each platelet is surrounded by a multilayered membrane system resembling the myelin sheath (Simpson, 1984). This complex and well-organized combination of substances makes the platelet an ideal reserve structure for rapid development after hatching. Its proteins and RNA provide the material for cell proliferation, whereas lipids serve as an energy source (Tessenow, 1969). The stored RNA in these cells likely serves as a template for rapid protein synthesis upon germination, enabling the sponge to develop quickly before other nutritional mechanisms are established (Ruthmann, 1965).

In freshwater sponges that harbor symbiotic zoochlorellae, thesocytes may also house these unicellular algae (Williamson, 1979; Masuda, 1990; Ereskovsky *et al*., 2022; Hustus *et al*.,

2023). Before hatching, the algae can be phagocytosed, and the young sponge develops in an aposymbiotic state (Rasmont, 1970), showing no signs of algal proliferation (Simpson, 1984; Williamson, 1979).

Once activated, thesocytes undergo significant changes to prepare for the next stages of development. Although the biochemical pathways underlying germination are not yet fully understood, several key aspects of this process have been proposed.

## Dormancy-sustaining substances

The endogenous mechanisms that keep gemmules in diapause are largely unknown. Simpson and Rodan (1976) demonstrated that germination is mediated by changes in intracellular cAMP concentration governed by at least two enzymes: phosphodiesterase (PDE) and adenylate cyclase (AC). PDE is known to lower the level of cAMP while AC has the opposite effect. PDE activity increased when gemmules were heated to +20 °C, whereas adenylate cyclase activity decreased (Simpson, Rodan, 1976). The balance between these two enzymes likely plays a significant role in regulation of gemmule dormancy and germination. While low cAMP level is required for cell growth and division, high level promotes the resting stage (Simpson, Rodan, 1976).

Another proposed regulator of the gemmule dormancy is gemmulostasin. This substance is still not characterized, and its nature remains obscure. However, it was extracted from gemmules of non-diapausing species by Rozenfeld (1970, 1974) and was shown to disrupt gemmule development, but only during the first 36 hours. After this period, the percentage of gemmules resistant to its action increased rapidly (Rozenfeld, 1970). Its action was completely reversible (Rozenfeld, 1970). The influence of gemmulostasin on hatching was also confirmed through radionuclide experiments. Gemmulostasin interfered with labeled thymidine incorporation, indicating inhibited DNA synthesis (Rozenfeld, 1974). These experiments showed that gemmulostasin blocks some early stage of gemmule metabolism, likely affecting karyokinesis.

Gemmulostasin could potentially act as an inhibitor of PDE or another participant of the biochemical cascade mediated by cAMP (Simpson, Rodan, 1976). Alternatively, Simpson *et al*. (1973), along with Simpson and Fell (1974), proposed that gemmulostasin might, in fact, represent an increase in osmotic pressure. They discovered that the effects of sodium chloride on germination generally mimic those of gemmulostasin. However, in 1984, Simpson stated that 'there are no compelling data to support the involvement of gemmulostasin in the development of high intragemmular osmotic pressure'. Therefore, the nature of this substance remains questionable.

Regardless of whether it is associated with gemmulostasin, high osmotic pressure is crucial for gemmule development. Loomis *et al*. (2009) clearly demonstrated that germination, metabolic rate, and cell division of resting gemmules are refrained by increased osmotic pressure in the cells. This pressure arises from polyols derived from glycogen during gemmule formation.

## Hatching triggers

Resting gemmules represent a typical example of developmentally programmed dormancy — a state in which metabolic processes and normal life activities are significantly slowed down or completely stopped (Wilsterman *et al*., 2020). This process usually consists of two main phases: diapause, an endogenously controlled metabolic depression, and quiescence, a phase in which dormant stage becomes sensitive to environmental cues but remains inactive due to unfavorable conditions (Hand, Podrabsky, 2000). Diapausing animals must undergo a period of adverse external conditions to terminate diapause and enter quiescence. Once in quiescence, they remain dormant until environmental conditions improve, allowing them to resume metabolism, hatch, and develop into juvenile organism. Thus, both initial adverse conditions and subsequent favorable conditions are essential prerequisites for successful development.

In freshwater sponges, diapause is obligatory for some species, while other can skip this stage. For example, *Ephydatia muelleri*, *Eunapius fragilis* (Leidy, 1851), and *Racekiela ryderi* (Potts, 1882) were described to have an obligatory diapause, while *Eph. fluviatilis* undergoes only quiescence (Rasmont, 1955; Rozenfeld, 1970; Fell, 1990). *Spongilla lacustris* was reported to be a diapausing species with variable depth of the diapause (Fell, 1995; Rasmont, 1954, 1962).

We suggest that dormancy might differ within the same species depending on geographical location.

For diapausing species from boreal and temporal climates, low winter temperatures (~3–5 °C) play a crucial role as diapause breaking factors. Fell (1995) demonstrated that some sponges enter a state he termed *deep diapause*, during which at least one month of cold exposure is required for successful hatching in warmer water. In contrast, *shallow diapause* requires only a few days of exposure to low temperature. When the gemmule transitions to the next stage, quiescence, cold water inhibits hatching to prevent premature emergence during unfavorable conditions in winter waterbodies. However, this inhibition is not absolute — some gemmules can hatch in winter or with the first signs of spring, even when water temperature remains very low (Zeuthen, 1939; Simpson, Gilbert, 1973; Ostrom, Simpson, 1978; personal observations). Remarkably, gemmules may even activate during storage in a fridge (Fell, 1990; Barbeau *et al*., 1989; Fell, 1994; personal data). Therefore, temperature increase alone is not the sole trigger for dormancy termination.

Desiccation is known to contribute to diapause breaking not only in species from arid climates or dry/wet seasons alternation, but also in boreal species. Melão, Rocha (1996) observed increased hatching rates after gemmule drying in the South American species *Metania spinata* (Carter, 1881). Similarly, Fell (1987a) found that gemmules of the cosmopolitan species *Eunapius fragilis*, which failed to hatch after cold treatment, could be activated after short brief drying. He also reported that short desiccation following brief cold exposure significantly enhanced further hatching in warm water (Fell, 1987b, 1990). Moreover, in some populations, desiccation accelerated diapause termination by up to two months compared to cold exposure alone (Fell, 1990).

However, some species, such as *Spongilla lacustris*, cannot survive even short periods of gemmule desiccation (Poirrier, 1969; Fell, 1990; De Santo, Fell, 1996). In addition, desiccation was shown to impair hatching in certain sponges (Ilan *et al*., 1992; Calheira *et al*., 2020). This variability suggests that species-specific adaptations play a remarkable role in diapause termination. Further research is necessary to uncover the molecular and physiological mechanisms underlying these differences.

Experimental investigations suggested that photosynthetic activity of the symbiotic zoochlorellae within thesocytes promotes gemmule hatching. Okuda *et al*. (2022) demonstrated that *Radiospongilla cerebellata* (Bowerbank, 1863) — a species that does not experience harsh winters (Sokolova *et al.*, 2024) — harbors endosymbiotic chlorellae in its resting gemmules and highly depends on light for successful hatching. Oxygen was also found to be crucial for *R. cerebellata*'s hatching, while excessive carbon dioxide level suppressed the process. This indicates that photosynthesis by symbiotic algae may regulate hatching by changing oxygen and carbon dioxide concentrations within the gemmule. Kanayama and Kamishima (1990) showed that a photosynthetic inhibitor suppressed hatching in *R. cerebellata*. Thus, the photosynthetic activity of symbiotic algae is likely essential for the hatching of sponge species whose gemmules contain these algae. Interestingly, *Spongilla lacustris* is known to produce a special type of gemmules filled with symbiotic zoochlorellae and covered with a simple single-layered coat (Fell, Levasseur, 1991). These gemmules appear to be non-diapausing and can germinate immediately when placed in favorable conditions, although exposure to low temperatures was shown to enhance subsequent hatching in warm water (Rasmont, 1962; Fell, Levasseur, 1991).

Gemmule germination is highly dependent on the presence of divalent ions. For instance, $Zn^{2+}$, $Mn^{2+}$, $Ba^{2+}$, and $Sn^{2+}$ were shown to inhibit hatching (Ostrom, Simpson, 1978), although this inhibition can be counteracted by $Ca^{2+}$ and, to a lesser extent, by $Mg^{2+}$. Strekal and McDiffett (1974) discovered that sponges cannot develop normally without $Ca^{2+}$ and $Mg^{2+}$ ions. Calcium, in particular, is crucial for maintaining cellular membrane potential and initiating germination and further growth (Ostrom, Simpson, 1978). Loomis (2010) suggested that the role of ions in germination might be especially important for sponges that produce gemmules specifically to withstand desiccation.

Once cells receive the signal for activation, the survival function of gemmules is fulfilled, and new cycle of growth and development begins. In this review, we define the developmental stages based on Höhr (1977), with minor adjustments to

align with other researchers. It is important to note that the exact timing is highly influenced by water characteristics and gemmule storage conditions.

## Germination: early changes (0–17 hours)

In natural conditions, freshwater sponge diapause typically lasts for 4–6 months in temperate climatic zone. Prior to this period, sponges increase osmotic pressure within gemmules (Zeuthen, 1939; Schmidt, 1970). This helps prevent osmotic rupture of cells and ensures their structural integrity until the sponge is ready to germinate in spring. When the gemmule is ready for germination, the osmotic and hydrostatic pressures significantly decrease (Zeuthen, 1939; Schmidt, 1970; Simpson, Fell, 1974; Loomis *et al*., 2009). This process is driven by the conversion of polyols back into glycogen, which appears to trigger the cascade of events resulting in gemmule activation (Loomis *et al*., 2010).

Another molecular process known to be involved in germination is the reduction of heat shock proteins. Schill *et al*. (2006) reported that within hours of temperature increase, Hsp70 levels in gemmules gradually decline. Heat shock proteins likely help gemmules stabilize cellular components during dormancy if water temperature fluctuates throughout autumn and winter (Schill *et al.*, 2006).

Ruthmann (1965) concluded that thesocyte structure remains largely unchanged after activation, aside from the consumption of reserve substances. Essential organelles such as ribosomes, endoplasmic reticulum, mitochondria, Golgi apparatus, and nucleoli containing RNA are already present in resting thesocytes. However, once gemmules start germinating, local structural changes occur, including increased cytoplasmic density, vesicle formation near the Golgi apparatus, appearance of annulate lamellae, and emergence of a large centriole adjacent to the nuclear membrane.

The next and first morphologically distinct event is the separation of the two nuclei in thesocytes (Berthold, 1969; Rozenfeld, 1970), which occurs several hours after transferring gemmules from cold to room temperature. This is followed by a relatively long pause (~17 hours), during which cell morphology remains unchanged (Berthold, 1969). Eventually, mitoses begin.

## First divisions (20–30 h)

According to Berthold (1969), who conducted the most detailed investigation of cell lineages, the first cells derived from a thesocyte are four mononucleated archaeocytes (Fig. 3). This cytokinesis is preceded by a transient tetranucleated stage (not shown in the figure) which immediately divides into four mononucleated archaeocytes. These archaeocytes subsequently give rise to prohistoblasts — cells with fewer nutrient platelets, smaller nucleoli and a slightly irregular shape.

Prohistoblasts further differentiate into smaller, mononucleated histoblasts, which are essential for the process of hatching. Histoblasts have a spindle-like or irregular shape, lack nucleoli and platelets, and move actively using filopodia. Berthold (1969) observed that mitoses are more frequent during the early stage of differentiation and become significantly less common in prohistoblasts, and especially in histoblasts.

During development, cells undergo significant transformations. With each successive division, cells become smaller, and their shape gradually changes — from rounded thesocytes to oval mononucleated archaeocytes, and finally to spindle-shaped histoblasts (Berthold, 1969). As differentiation continues, vitellin platelets are gradually digested, starting with the lateral valves (Tessenow, 1969; De Vos, 1974). The membranes surrounding the platelets become wavy and form concentric lamellar bodies, which are eventually expelled from the cells. In contrast, small simple granules remain in the cells for a longer period (Höhr, 1977). The nucleolus diminishes in size with each division and eventually disappears in histoblasts (although it was still observed in some *S. lacustris* histoblasts by Wintermann (1951)). Chromatin in histoblasts becomes locally denser, and this, along with the absence of the nucleolus, is considered an indicator of metabolic inactivity (Caspersson, 1950).

The exact location of histoblasts origin remains uncertain. Brien (1932) proposed that histoblast differentiation begins in the cone-shaped region beneath the micropyle. This observation was later confirmed by other researchers (Wierzhejski, 1912; Höhr, 1977), who, however, noted that histoblasts also appear at the gemmule periphery (Fig. 3C). In contrast, Berthold (1969) observed that initially histoblasts and their precursors, prohistoblasts, are scattered throughout

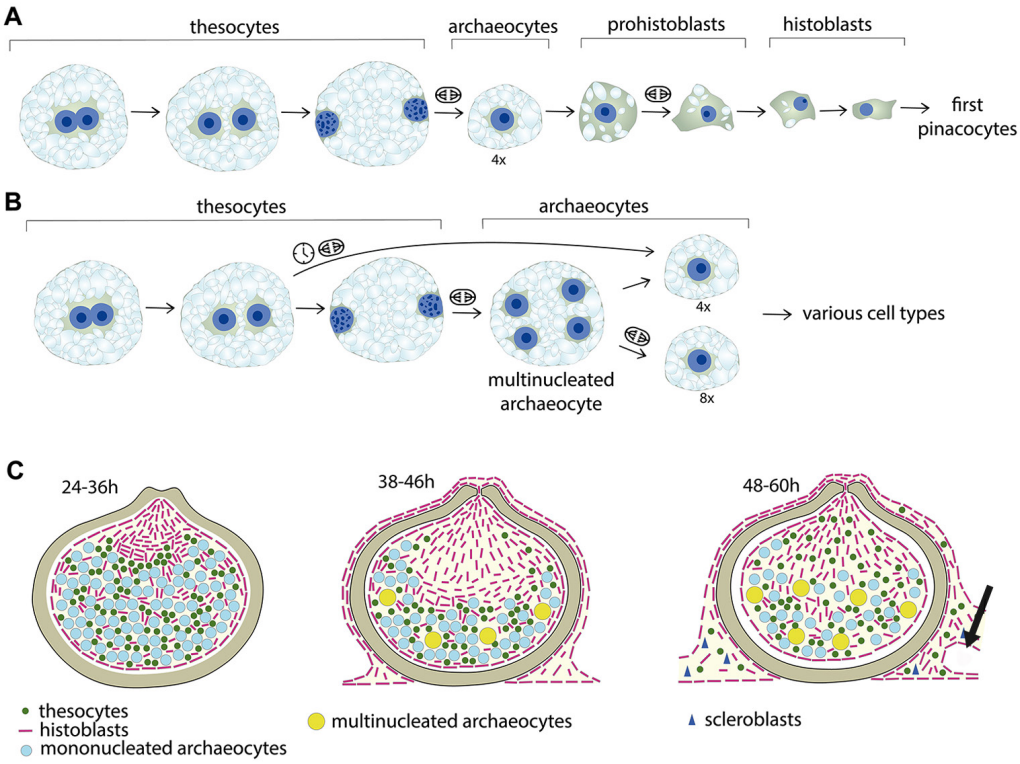


Fig. 3. Morphological events during freshwater sponge development from gemmule. A — thesocyte differentiation pathways leading to formation of the first histoblasts; B — thesocyte differentiation pathways leading to formation of the rest of the cells (redrawn from Berthold, 1969). Sign above some arrows marks mitosis. Note that at the beginning of development, all mononuclear archaeocytes differentiate into histoblasts with no remarkable multinucleated stage. Later, thesocytes give rise to stable multinucleated cells (by fusion or karyokinesis, yet to be determined), which then fell apart to form mononucleated archaeocytes (4x in the figure). Nuclei of multinucleated archaeocytes can also divide prior to fragmentation, but such cells are unstable and immediately disintegrate into mononucleated archaeocytes (8x in the figure). Some thesocytes remain in binucleated stage for a long time and enter the mitosis later, skipping the multinucleated stage; C — key stages of gemmule hatching (redrawn from Höhr, 1977). Left: histoblasts are accumulating under micropyle and at the periphery of gemmule forming a thin epithelium-like layer enclosing other cells; micropyle is closed. Middle: gemmule has hatched and histoblasts are migrating outside; multinucleated archaeocytes appear. Right: cells are migrating in the space between exopinacoderm and basopinacoderm and form the mesohyl with scleroblasts first to differentiate. Arrow shows a lacune of the future exhalant canal.

the gemmule without a clear pattern. She proposed that by the time of germination, histoblasts simply accumulate near the micropyle, as she observed them located beneath the micropyle without prohistoblasts around. This observation challenges the concept of a developmental gradient suggested by others (Wierzhejski, 1915; Brien, 1932; Höhr, 1977). Berthold hypothesized that if such a gradient existed, it would likely consist of a layer of mononuclear archaeocytes beneath the histoblasts, followed by a zone of binucleate cells undergoing mitosis. Therefore, as highly mobile cells, histoblasts might migrate toward the micropyle in response to a chemical stimulus. However, Simpson (1984) suggested that *in situ* differentiation is more likely, given the tight packing of the cells.

Peripheral histoblasts flatten and form a thin epithelium-like layer that encloses the remaining contents of the gemmule (Fig. 3C). This cell layer is regarded as the primary pinacoderm (Höhr, 1977).

Some cell types described at this stage seem questionable. Berthold (1969) reported the presence of rare binucleated histoblasts, but we did not find supporting evidence for this in other sources. Höhr (1977) identified binucleated archaeocytes — the first descendants of thesocytes — as a separate generation; however, this claim is not corroborated by other studies. It is likely that he considered activated thesocytes as a distinct stage compared to their resting predecessors. In any case, the overall outcome of thesocyte division at this stage is the production of two types of mononucleated cells: mononucleated archaeocytes and histoblasts.

## Hatching (36–48 h)

As the cells prepare to exit, the primary pinacoderm becomes more prominent, forming a sac-like structure that encloses other histoblasts and archaeocytes (De Vos, 1974; Höhr, 1977). The cone-shaped area near the micropyle enlarges driven by the continuous generation of histoblasts. According to Berthold (1969), the onset of hatching depends solely on the presence of sufficient number of histoblasts in the cone-shaped zone, which begin to digest the membrane blocking the micropyle (Kilian, 1964; Rozenfeld, 1971). Once the micropyle opens, a milky mass of cells, consisting mostly of histoblasts and pinacoblasts, flows out (Brien, 1932; De Vos, 1974). The histoblasts exhibit strong stereotropism, enabling them to attach to any solid surface. They spread out and flatten, forming a thin layer over the outer surface of the gemmule. Eventually, the cells reach the substrate and anchor to it, creating a sort of epithelial cavity into which mesohylar cells will later migrate (Fig. 3C). Firmly attached epithelial-like cells, which are now called pinacocytes, lose their elongated shape. They expand greatly in all directions, often forming a polygonal pattern (Wintermann, 1951).

Tanaka and Wanabe (1994) noted that histoblasts ‘immediately differentiate into exopinacocytes’. However, it remains unclear whether these cells proliferate to form the exopinacoderm or merely undergo morphological changes after attaching to the substrate. Leaving the micropyle, pinacocytes synthesize collagenous fibers (De Vos, 1974) and facilitate body integrity.

The exact timing and mechanisms of ‘epithelia’ development in freshwater sponges remain unclear (Nakayama *et al*., 2015), but this process give rise to three types of pinacocytes: exopinacocytes, which form the outer surface of the young sponge; basopinacocytes, which create the basal layer; and endopinacocytes, which line the canals and cavities of the aquiferous system (see below). Wintermann (1951) reported that after attaching to the substrate, pinacocytes acquire large vacuoles around the nucleus. She suggested that these vacuoles might be characteristic exclusively of the basopinacoderm. This feature is likely related to the intense spongin synthesis in basopinacocytes, which ensures their adhesion to the substrate and provides a stable foundation for the sponge body (Wintermann, 1951).

At this stage, cellular diversity of the developing sponge increases as a new cell type emerges from thesocytes: large multinucleated archaeocytes (Wierzhejski, 1915; Brien, 1932; Höhr, 1977). According to Berthold (1969), these cells give rise to a new generation of mononucleated archaeocytes which do not specifically participate in histoblast formation but rather contribute to the differentiation of various cell types. The number of nuclei in multinucleated archaeocytes varies, typically ranging from four to six (Wierzhejsky, 1915; Brien, 1932). However, Berthold (1969) found only tetranucleated archaeocytes and reported the absence of five- and six-nucleated cells in her samples. Nevertheless, she put forward an idea that multinucleated state might serve as a mechanism to preserve nutritional structures as long as possible. She suggested that pluripotent cells required for future development originate from those that remain in a multinucleated state for a long time.

The cytoplasm of multinucleated archaeocytes is densely packed with vitellin platelets, sometimes to the extent that the nuclei and cell boundaries become obscured. Wierzejski (1915) proposed that these cells might result from the partial fusion of several thesocytes, since their nuclei are as large as or even larger than those of the initial thesocytes. In addition, he observed that the presumed daughter cells were larger than their predecessors and are heavily laden with vitellin platelets. However, whether archaeocytes actually fuse or undergo karyokinesis with delayed cytokinesis to reach the multinucleated stage remains uncertain.

During hatching, multinucleated archaeocytes go through fragmentation (Figs. 3B; 4),

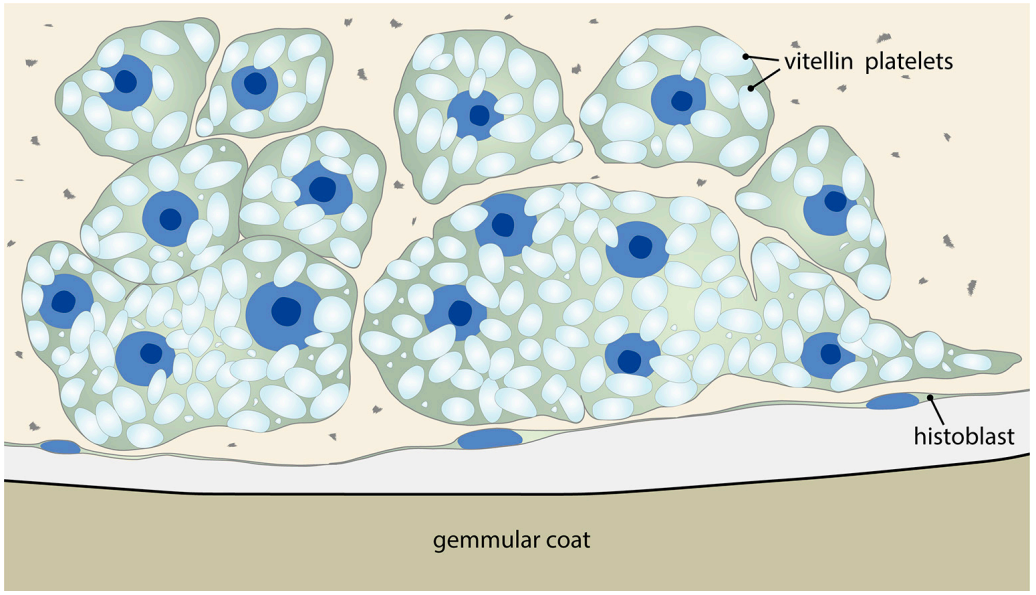


Fig. 4. Multinucleated archaeocytes during fragmentation. Redrawn from Brien, 1932 (figs VII and VIII).

followed by division into smaller cells (Brien, 1932). According to Berthold (1969), the nuclei of multinucleated archaeocytes can also divide prior to fragmentation, but such cells immediately break apart into mononucleated cells (Fig. 3B). For the tetranucleated cells she dealt with, she described the transient formation of unstable eight-nucleated cells, which rapidly break apart into eight mononucleated archaeocytes.

While mononucleated archaeocytes leave the gemmule and differentiate into various cell types, multinucleated cells predominately remain inside, especially the largest ones (Brien, 1932). Berthold (1969) noticed that as all the cells move toward the micropyle, the chemical stimulus she proposed likely affects all of them, but those densely packed with vitellin platelets are unable to move. Only after expending these heavy inclusions during differentiation are they able to crawl out of the gemmule. Some thesocytes remain in binucleated stage for a long time, skipping the multinucleated stage (Fig. 3B). Berthold (1969) suggested that cells resulting from this delayed mitosis, along with the 'old' multinucleated archaeocytes, contribute to the pool of pluripotent cells in the young sponge.

As the cell mass gradually becomes transparent, the central area containing archaeocytes remains whitish (Brien, 1932). These archaeocytes, still filled with inclusions, move in an amoeboid manner along the pathways formed by histoblasts, continuing their development, and differentiating into new histoblasts and other cell types.

## Mesohyl establishment (48–60 h)

Berthold (1969) suggested that cells differentiate into histoblasts mainly during early stages of the development. Later — probably after the formation of epithelium-like sheet that separates the cell mass from the environment — they focus on mononuclear archaeocytes production. Archaeocytes at different stages of differentiation (multinucleated, binucleated, and especially mononucleated) move out in large numbers (Brien, 1932).

The crawling cells form a protrusion, at the top of which the gemmular shell rests (Brien, 1932). The mesohyl of the developing sponge takes shape as cells migrate from the gemmule into the space enclosed by the pinacoderm (Fig. 3C, right). The first mesohyl cells to differentiate from the mononuclear archaeocytes are sclerobLasts — spicule-producing cells (Höhr, 1977).

Thus, histogenetic differentiation begins from the moment of hatching. Layers of pinacoderm form a cavity where future mesohyl cells will

differentiate. It is important to emphasize that the earlier stages of cell differentiation had already established a 'primordium' within the gemmule (Fig. 3C, left), which now move as a complete unit propelled by the advancing cell front of the epithelial-like cells.

## Final Development (72–120 h)

The young sponge takes shape as the remaining cells within the gemmule actively differentiate, with structural support becoming necessary as the body develops. The first spicules are lifted one by one from an almost flat position and positioned by specialized transport cells of unknown nature (Nakayama *et al*., 2015). Needle-shaped spicules are synthesized within sclerocytes. Each spicule is built by silica deposition around the actin (silactin) core (Ehrlich *et al*., 2022, 2024).

Endopinacocytes, derived from histoblasts within the mesohyl, establish the initial canal system of the young sponge. Some of these cells, known as prosendopinacocytes, give rise to the first inhalant canals, while others, termed apoendopinacocytes, form exhalant canals (De Vos, 1974). Lacunae, the precursors of the canals, lack a specialized lining and are surrounded by archaeocytes that still contain vitellin platelets (Tanaka, Watanabe, 1994). They develop and multiply both near the periphery and central region of the sponge (Brien, 1932). Over time, lacunae interconnect and acquire an endopinacocyte lining, transforming into functional canals. Some lacunae and canals dilate and fuse to form a subdermal cavity beneath the outer surface of the developing sponge. This cavity is supported by vertically arranged spicules.

Basopinacocytes build a collagenous base for each spicule, anchoring one end securely, while the other end is oriented vertically by transport cells and pierces through the exopinacoderm (Nakayama *et al*., 2015). Now, the young sponge resembles a tent stretched over spicules much like fabric over the tent poles. However, the walls and roof of such a 'tent' are riddle with holes – these dermal pores allow water to flow into sponge body. During pore development, an exopinacocyte stretches so that its central region becomes thinner, forming an opening, while the nucleus shifts toward the cell periphery (Brien, 1932).

As the structural complexity of the developing sponge increases, additional cell types contribute to its organization. One such type is the collencyte, a collagen-secreting cell. Wintermann (1951) observed these cells in close association with the inhalant canals. Collencytes possess star-shaped, branched pseudopodia and establish connections between opposite sides of the canal. Archaeocytes, in contrast, tend to accumulate near the lining of the exhalant canals.

Choanocytes are among the last cells to differentiate (Tanaka, Watanabe, 1994; Funayama *et al*., 2005). Their appearance was investigated by Tanaka and Watanabe (1994) by imitating the normal process of choanocytes development. The authors inhibited differentiation of the aquiferous system by treating developing gemmules with hydroxyurea and subsequently observed choanocyte emergence *de novo* after its removal. They reported that a single archaeocyte forms a choanoblast cluster through three successive mitotic divisions. The choanoblasts that proceed to a fourth mitosis transform into choanocytes. These newly formed choanocytes undergo an additional mitotic division while retaining their collar and flagellum.

The common origin of choanocytes within a chamber was confirmed by modern techniques (Funayama *et al*., 2005). It was demonstrated that archaeocytes committed to the choanocyte lineage begin expressing the *Ef annexin* gene, a marker characteristic of choanocytes. The authors also observed that choanoblast clusters tend to form in the thin peripheral region of the sponge body, within the narrow space between exopinacocytes and basopinacocytes. Archaeocytes migrate into this narrow space purposefully, suggesting the presence of some spatial or temporal cues that guide choanocyte differentiation.

Mature choanocyte chambers connect the adjacent lacunae through specialized openings: the apopyle and the prosopyle. The prosopyle links the chamber to the incurrent aquiferous system allowing water to flow into the chamber. It is a small opening formed by a single endopinacocyte, in a manner resembling formation of exopinacoderm pores: the opening develops intracellularly, with the nucleus and cytoplasm being pushed to the cell margin (Brien, 1932; Weissenfels, 1981). The apopyle functions in reverse, expelling water from the choanocyte chamber into the excurrent canal of the aquiferous system. Its formation is more complex than that

of the prosopyle. When the chamber approaches the excurrent lacuna, 2–3 choanocytes at the contact point transform into so-called cone cells, which lose their collars but not the flagella. In response, the endopinacoderm develops a single pore cell distal to the cone cell ring (Weissenfels, 1980, 1981; Langenbruch, Weissenfels, 1987). Once connected to the water flow, choanocyte chambers start filtering, and the young sponge no longer relies on vitellin reserves.

The last remaining cells, if any, migrate out of the gemmule, completing the formation of sponge body. The skeleton is reinforced as additional spicules align with the primary spicules, forming bundles that protrude from the sponge's surface, with the exopinacoderm stretched over them (Funayama *et al*., 2005).

The lacunar system takes its final configuration, and the last structure expected to complete development is the osculum. Brien (1932) proposed that the oscular tube forms when the wall of the central lacuna protrudes due to internal water pressure, eventually breaking open. This process results in the formation of a two-layered tube, with the inner layer consisting of endopinacoderm and the outer layer made of exopinacoderm. Endopinacocytes of the inner layer develop specialized sensitive cilia (Ludeman *et al*., 2014), though the details of this process are unknown.

At this stage, the leuconoid organization of the young sponge is fully established, and a Porifera-typical phylotypic stage (spongotype) — the mono-oscular juvenile — is formed (Ereskovsky, 2019). As the sponge expands across the substrate, the empty gemmule capsule remains beneath its body, still visible through the semi-transparent tissues. On the surface, enormous pinacoderm cells with large nuclei can be discerned, while deeper in the body the lacework of choanocyte chambers becomes clearly distinguishable. The osculum rises above the surface, expelling filtered water from the collector canal.

For species whose gemmules lack symbiotic algae (Castro-Rodriguez, 1930; Ilan *et al*., 1996; Hall *et al*., 2021) it is time now to capture them from water column. The young sponge becomes fully functional, no longer dependent on its nutrient reserves and capable of meeting its own needs.

## Genetic basis for development from gemmules

Sponges possess homeobox genes known to guide the developmental processes, determining spatial organization, cell fate, and differentiation in multicellular organisms. These genes include members of POU and Pax classes, Antennapedia superclass, *Msx*, *Tlx/Hox 11*, and *NK2* (check Nikko *et al*., 2021 for references). A new class of homeobox genes called *Sycox* has also been discovered for calcareous sponges (Manuel, Le Parco, 2000).

Seimiya *et al*. (1997) identified two POU-class homeobox genes, *spou-1* and *spou-2*, in the freshwater sponge *Ephydatia fluviatilis*. These genes, which are highly conserved across metazoans, bind to DNA and play an important role in transcriptional regulation (Gold *et al*., 2014). It was found that both genes are expressed in a stage-specific manner during sponge development from gemmules, particularly during the formation of choanocytes and canals (Gold *et al*., 2014). Therefore, *spou-1* and *spou-2* are thought to be involved in the establishment of the aquiferous system.

Another homeobox gene identified in *Ephydatia muelleri, EmH-3*, was shown to be specifically active in pluripotent cells. Using cell fractionation and RT-PCR analysis, Richelle-Maurer *et al*. (1999) demonstrated that *EmH-3* is predominantly expressed in archaeocytes. Both archaeocytes of the fully developed juvenile sponge and early-staged archaeocytes filled with vitellin platelets exhibited high expression levels. In contrast, differentiated cells such as choanocytes and pinacocytes showed almost absent expression, similar to resting thesocytes. Transcript levels increase from undetectable levels in resting gemmules to significantly higher levels during hatching and persists throughout the sponge's life. This indicates a key role for *EmH-3* in the differentiation of pluripotent cells both during gemmule development and in mature sponge, although fully developed adult sponges were not included in the study.

Nikko *et al*. (2021) examined the influence of retinoic acid (RA) — a key regulatory molecule in animal development — on sponge development and *EmH-3* expression. Their study showed that at low RA concentrations, sponge development proceeded normally, with a slightly increase in

*EmH-3* gene expression, indicating a positive developmental effect. However, at higher concentrations, development was severely disrupted: the aquiferous system failed to form, and *EmH-3* expression significantly decreased. Once RA was removed, normal development resumed, and *EmH-3* expression returned to typical levels.

Other genes known to maintain pluripotent state of cells were studied by Funayama *et al*. (2010). These were genes of Piwi proteins, which play a key role in stem cell regulation. Piwi proteins are highly conserved RNA-binding molecules that are crucial for stem and germ cell differentiation in multicellular organisms. In their study, Funayama *et al*. (2010) explored the role of Piwi proteins in the stem cell system of *Ephydatia fluviatilis*. Using *in situ* hybridization, they identified two *Piwi* homologs, *EfPiwiA* and *EfPiwiB*, expressed in archaeocytes and choanocytes of the developing sponge. Their findings highlighted two modes of differentiation: archaeocytes lose *Piwi* expression as they differentiate into specialized cells such as sclerocytes, whereas choanocytes retain *Piwi* expression even in their mature state, suggesting their stem properties. *EfPiwiA* and *EfPiwiB* are supposed to participate in archaeocytes differentiation, maintaining a balance between stem cells self-renewal and differentiation into specialized cell types. In choanocytes, these genes likely enable their transformation into gametes or reversion to archaeocytes.

Archaeocytes of juvenile *Ephydatia muelleri* were shown to express genes of the Wnt signaling pathway: *EmuwntA*, *EmuwntB*, *EmuwntC*, and *b-catenin* (Winsdor, 2014). Treatment of sponges with lithium chloride and alsterpaullon (inhibitors of GSK3, a key component of the Wnt pathway) caused the formation of multiple oscula and disrupted canal structure. This suggests that the Wnt pathway plays a critical role in the correct arrangement and development of the aquiferous system.

One more gene network involved in sponge development from gemmules is the PSEDN (Pax/Six/Eya/Dac Network). This highly conserved group of interconnected homeobox and non-homeobox genes regulates essential developmental processes in animals, including the formation of visual organs, muscles, and endocrine glands (Kozmik *et al.*, 2007). Rivera *et al*. (2013) identified orthologs of Pax and Six family genes in *Ephydatia muelleri*, which were shown to be expressed in pinacocytes and choanocytes using *in situ* hybridization. RNA interference-mediated knockdown of *EmPaxB* and *EmSix1/2* resulted in severe defects in the aquiferous system development.

In addition to gene networks, the expression level of microRNA significantly increases during the transition from undifferentiated gemmule cells to differentiated juvenile sponge cells, indicating their involvement in the process of cellular maturation and organ formation (Robinson, 2015).

Thus, sponge development from gemmules relies on complex molecular mechanisms which are still in the early stages of investigation. Highly conserved genetic pathways, activated by environmental cues, regulate these processes to ensure the proper formation of structures and the transition from undifferentiated cells to a fully developed sponge.

## Perspectives for future researches

Despite significant progress in understanding of gemmule hatching and early sponge development, many fundamental aspects of these processes remain unclear. The complex relationships between environmental cues, morphogenetic events, and molecular mechanisms within gemmules are yet to be fully deciphered. Here, we gather the most obvious unresolved questions that suggest promising directions for future research in this field.

What other substances, besides cAMP and polyols, maintain dormancy in gemmules during diapause? What is the nature of gemmulostasin? Is it a direct regulator of osmotic pressure, as salt ions are, or a component of an enzymatic cascade? Is the mechanism of diapause termination universal, given the varying intensity of the diapause across different species? Does diapause termination differ in species exposed to freezing in cold climate and desiccation in arid climate? Sponges with prominent adaptations to drought, such as a reinforced gemmular case that firmly attaches gemmules to the substrate, are of a special interest. Are there structural differences in thesocytes between these species and sponges that experience low temperatures?

Further investigation is also needed to answer the questions concerning spatial organization and

differentiation processes. For example, does the spatial positioning of thesocytes within the gemmule influence their differentiation and fate? Is a chemical signal involved in guiding histoblast migration toward the micropyle, or does *in situ* differentiation drive the process? How is cellular coordination achieved at the leading edge of the moving cell front during hatching? What mechanisms regulate mesohyl structure once cells emerge from the gemmule? What triggers the transition from archaeocytes into specialized cell types, such as scleroblasts or collencytes, and what factors govern this differentiation? Is the concept of a cellular niche applicable to sponges? How do elements of genetic networks essential for early animal development interact?

Addressing these questions will not only deepen our knowledge of gemmule hatching but will also open up new possibilities for using freshwater sponges as a model in developmental and evolutionary research.

**Compliance with ethical standards**

**Conflicts of interest:** The authors declare that they have no conflicts of interest.

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