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Review

Beauties and beasts: A portrait of sea slugs aquaculture

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ABSTRACT

Research on sea slugs production has steadily increased in the last decades as a result of their use as model organisms for biomedical studies, bioprospecting for new marine drugs and their growing demand for academic research and the marine aquarium trade. However, standardized methods for culturing sea slugs are still limited to a reduced number of species. The main bottlenecks impairing sea slugs aquaculture are the lack of knowledge on suitable larval diets and settlement cues that can induce metamorphosis in competent larvae. Additionally, the stenophagous feeding regime displayed by several species requires the collection and/or culture of their prey, which commonly impairs large-scale production. Nevertheless, significant breakthroughs have been achieved in recent years through the development of innovative culture techniques. The present review summarizes the major issues impairing the culture of sea slugs and presents relevant biological and ecological data that can assist on the development of suitable culture protocols. Information on the most suitable husbandry, larviculture and grow-out techniques are critically discussed, with emphasis to their application on some of the most relevant groups of sea slugs from an academic and commercial point of view: sea hares (*Aplysia* spp.), nudibranchs (e.g., the marine ornamental species *Aeolidiella stephanieae*) and the "solar powered" sacoglossan (e.g., *Elysia* spp.).

Contents

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1. Introduction

Sea slugs are delicate, colored and "sludgy" gastropod mollusks commonly referred to as the underwater version of the butterfly and the caterpillar combined (Debelius and Kuiter, 2007). Outwardly defenseless and often displaying a bizarre silhouette, these gastropods have always been fascinating subjects for marine biologists. Several scientific works already investigated and discussed their morphology (Gosliner, 1994; Mikkelsen, 2002), life cycle (Avila et al., 1997; Clark, 1975; Harris, 1975), ecology (Angeloni and Bradbury, 1999; Carefoot, 1987), feeding habits (Aboul-Ela, 1959; Hoover et al., 2012; Ritson-Williams et al., 2003) and systematics (Bouchet et al., 2005; Jörger et al., 2010; Schrödl et al., 2011).

Sea slugs are currently considered members of the Heterobranchia (according to the most recent classification proposed by Jörger et al., 2010) (Fig. 1), a highly diversified and successful group of marine gastropods presenting a global distribution and occupying a wide range of ecological niches. Sea hares, *Aplysia* spp. (Anaspidea), (Fig. 1) are probably one of the most well studied groups of sea slugs because of their key role in medical research (Sattelle and Buckingham, 2006). Their popular use as model organisms, particularly in neurobiological sciences, prompted researchers to develop suitable culture protocols to allow their mass production under controlled conditions (Capo et al., 2009). Nudibranchs (Nudipleura) (Fig. 1) have been of interest to researchers in biotechnology due to their potential for the bioprospecting of new marine natural products (e.g., *Felimida* spp., formerly known as *Chromodoris*) (Leal et al., 2012a). Additionally, nudibranchs are also widely used as biological tools for scientific research (e.g., *Aeolidiella stephanieae* and *Spurilla neapolitana*), particularly to study their chemical ecology and photosymbiotic associations (Carroll and Kempf, 1990; Cimino and Ghiselin, 2009; Greenwood, 2009). The most dazzling colored nudibranchs are also highly popular among marine aquarium hobbyists, with a number of these ornamental species (e.g., *Felimida* spp.) already being produced in captivity and reaching high retail values in the marine aquarium trade (Olivotto et al., 2011). Sacoglossan sea slugs (Sacoglossa) (Fig. 1) are also popular in the marine ornamental trade (e.g., *Elysia crispata*) because of their ability to control the growth of nuisance algae (Sprung, 2002). Nonetheless, the culture of sacoglossans (e.g., *Elysia chlorotica*, *Elysia timida* and *Elysia viridis*) has mostly provided biological material to researchers addressing one of the most puzzling features displayed by marine invertebrates — the ability to keep functional algal chloroplasts within their animal cells (Johnson, 2011). This remarkable feature has provided these sacoglossans the nickname of "solar-powered sea slugs" or "photosynthetic animals" (Rumpho et al., 2000, 2011).

The present review provides a comprehensive overview of the most significant breakthroughs on sea slugs aquaculture, as well as

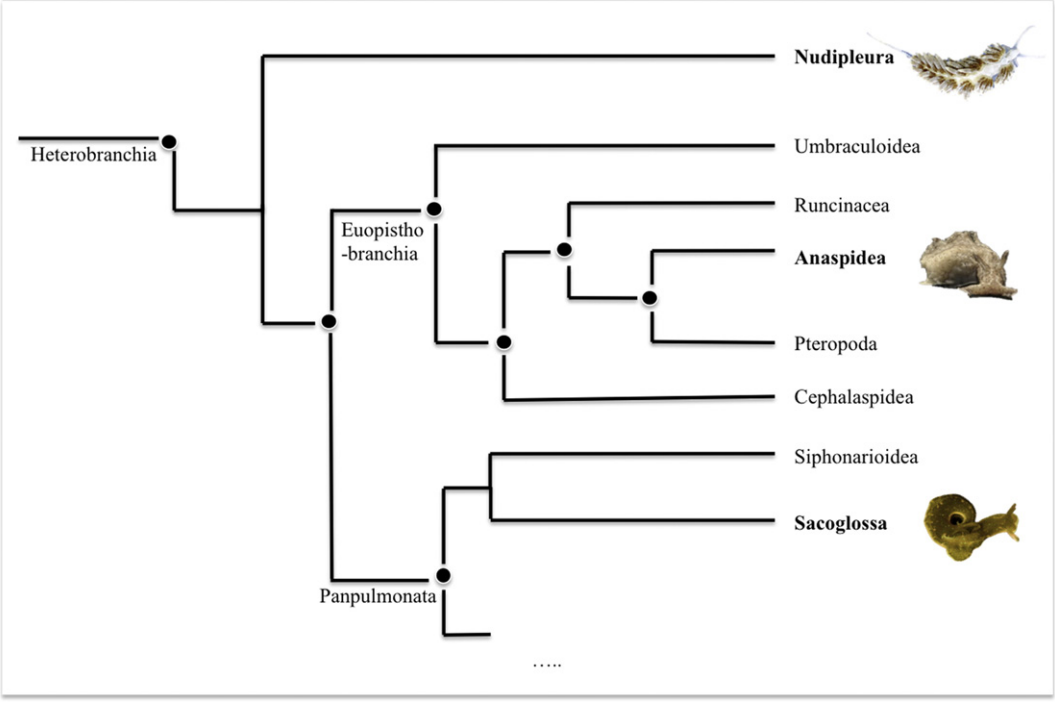

Fig. 1. Simplified classification of the most commonly cultured groups of sea slugs (adapted from Jörger et al., 2010). The differences between branches reflect a measure of divergence time. Black nodes show significant support. Nudipleura are represented by *Aeolidiella stephanieae*, Anaspidae by *Aplysia* sp. and Sacoglossa by *Elysia viridis*. (Note: the group Panpulmunata is not represented in the figure.)

current bottlenecks impairing their large-scale production. Special emphasis is given to species playing an important role for biomedical and academic research, for bioprospecting of new drugs and for the marine aquarium trade. The husbandry requirements of reproductive broodstock are addressed, along with the most relevant aspects of sea slug reproduction from an aquaculture perspective. Larviculture protocols for the most commonly cultured sea slugs are reviewed, with emphasis to culture systems, diets and cues known to trigger metamorphosis. Current methodologies available for the grow-out of juvenile sea slugs are presented, with particular focus on the challenging task of providing suitable diets to stenophagous species.

2. Why culture sea slugs?

2.1. Sea slugs as biological models and tools

Sea slugs are often used as a "biological model" or "biological tool". The term "model" describes non-human biological systems that are used to better understand human disorders. A biological tool is used on research studies that are not related with human disorders (Sive, 2011). Species within genus *Aplysia* are good examples of biological models that have been successfully used on a broad range of experimental studies addressing biomedical topics (see Capo et al., 2009). In contrast, "solar-powered" sea slugs (e.g. *Elysia* spp.) are biological tools used to study endosymbiotic associations between metazoan cells and functional chloroplasts (Pelletreau et al., 2011; Rumpho et al., 2011). Fig. 2 provides a schematic overview of different research topics where sea slugs culture is performed for commercial or academic purposes.

2.1.1. Sea hares, Aplysia spp., as models in scientific research (the beasts)

Aplysia species are acknowledged as one of the most important invertebrate model organisms in biomedical studies (Kandel, 2001). Species within genus *Aplysia*, namely *Aplysia californica*, have relatively simple biological systems. The use of these organisms in scientific research allowed significant breakthroughs on the clarification of molecular mechanisms involved in all phases of implicit memory and cellular basis of behavior (Hawkins et al., 2006; Kandel, 1982). *Aplysia* species are also important models for research on neural control of hormone secretion (Wayne, 1995), aging (Bailey et al., 1983) and Alzheimer's disease (Shemesh and Spira, 2010).

2.1.2. "Solar-powered" sea slugs as tools for research on symbiosis between metazoan cells and functional chloroplasts

The popularity of "solar-powered" sacoglossan sea slugs (e.g., *Elysia* spp.) has grown among researchers since the 1960s. These organisms are able to "steal" functional chloroplasts from their algal prey and keep them functional in animal tissue, somehow continuing to photosynthesize without the support of the whole native algal cell (Pierce and Curtis, 2012; Rumpho et al., 2011). These highly specialized organisms feed by slicing or puncturing siphonaceous algal cells and sucking out their contents. Most cell contents, including the algal nucleus, are digested, whereas chloroplasts are retained as functional organelles within the cells of its new host — the sea slug (Rumpho et al., 2000). This puzzling behavior has been termed chloroplast symbiosis, plastid sequestration or kleptoplasty (Johnson, 2011; Pelletreau et al., 2011). Several levels of kleptoplasty (i.e., different retention abilities of non-functional and functional chloroplasts) have been recorded among genera of sacoglossans (e.g., *Alderia*, *Bosellia*, *Caliphylla*, *Elysia*, *Hermaea*, *Limapontia*, *Mourgona*, *Oxynoe*, *Plakobranchus*, *Tridachia*, and *Thuridilla*), although not all sacoglossan species display the ability to retain functional chloroplasts (e.g., *Placida dendritica*, *E. catulus*, *Acobulla ulla*) (Evertsen et al., 2007; Rumpho et al., 2011). The mechanisms of interaction between the foreign organelle (the stolen chloroplast or kleptoplast) and its host animal cell have just started to be unraveled by the scientific community (Händeler et al., 2009; Pelletreau et al., 2011; Pierce et al., 2012; Rumpho et al., 2008; Vieira et al., 2009; Wägele et al., 2011). Different strategies to retain functional chloroplast within animal cells have been recorded among sacoglossan: species retaining non-functional chloroplasts for some hours, and species retaining functional chloroplasts for days, weeks or even months. Such differences may provide a unique opportunity for researchers to witness an ongoing evolutionary process of endosymbiosis. Most efforts to culture "solar-powered" sea slugs in the laboratory have targeted

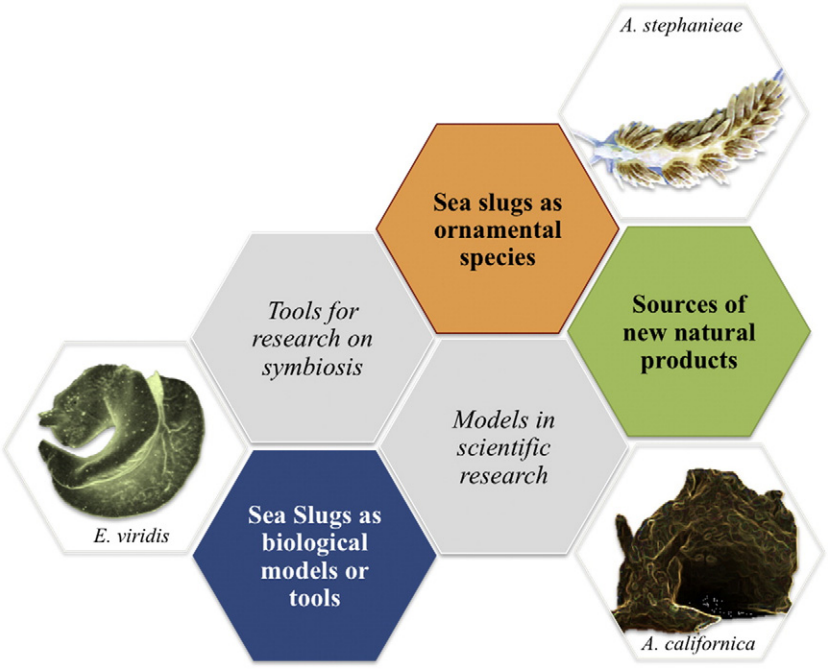

Fig. 2. Schematic overview of different research topics where sea slugs culture is performed for commercial or academic purposes. Sea slugs as biological models and tools (blue hexagon) divided in two major themes: tools for research in symbiosis and models in scientific research (grey hexagons) (e.g., biological model — *Aplysia californica*; biological tool — *Elysia viridis*), as ornamental species (orange hexagon) (e.g., *Aeolidiella stephanieae*), and as sources of new marine natural products (green hexagon) (e.g., *Aplysia* spp.)."

Costasiella lilianae, *E. chlorotica*, *Elysia clarki*, *E. timida* and *E. viridis* due to their rapid embryonic development and the ease of rearing their larvae and juveniles (Curtis et al., 2006; Rumpho et al., 2008; Trowbridge, 2000).

2.2. Sea slugs as sources of new natural products

A large number of natural products with remarkable bioactivities have been discovered during the last few decades from marine invertebrates (Blunt et al., 2012). The Heterobranchia follows the same trend and several new marine natural products have been extracted from this group of organisms (Leal et al., 2012a), particularly amino acids and peptides (Leal et al., 2012b). While not all molecules discovered from sea slugs have interesting biomedical applications, some of them have been the starting point for a number of potential drug candidates (Barsby, 2006; Putz et al., 2010). In some cases, the origin of natural products in sea slugs has been attributed to bio-accumulation or biotransformation of molecules acquired through the ingestion of their prey (Avila, 1995). However, sea slugs may also synthesize their own molecules de novo, which is one of the more striking aspects of the ecology of the Heterobranchia (Cimino and Ghiselin, 2009). While significant breakthroughs have been achieved in the field of organic synthesis in the recent years, several molecules recorded from marine organisms, such as sea slugs, exhibit structural peculiarities that are still difficult to recreate through chemical synthesis (Baran et al., 2007). Another restrictive step in the development of new marine drugs from sea slugs, as for other marine organisms, is the frequent lack of an adequate and consistent supply of raw material for standard screening assays, especially for rare and/or small-sized species. The paucity of cost-effective techniques for molecular isolation, identification and synthesis are additional bottlenecks that impair the development of new marine drugs from sea slugs. Under this scenario, aquaculture may provide a potential solution to some of these constraints, as it can supply the required biomass throughout the drug discovery pipeline (Leal et al., 2012a). Under a controlled environment, the aquaculture of sea slugs in the laboratory may be fine-tuned to maximize the production of particular molecules, namely by shifting biotic and/or abiotic factors that can favor certain metabolic pathways. Aquaculture may also be advantageous for those species that acquire their natural products through their prey, as the long-term husbandry of sea slugs may allow the maximization of the concentration of target molecules. The rationale for this claim is that sea slugs, commonly intensify their chemical weaponry through the ingestion of their prey (Molinski et al., 2009).

From all sea slugs screened so far for new products, members of genus *Aplysia* are unquestionably the ones yielding the highest number of compounds over the last two decades (58 new compounds representing 10.2% of new natural products obtained from marine mollusks) (Leal et al., 2012a). Some of these molecules identified from *Aplysia*, as well as from other sea slugs, have been shown to be feeding-deterrents and to display cytotoxic and ichtyiotoxic properties (Avila et al., 2006). Table 1 summarizes the most relevant molecules yielded so far from sea slugs, as well as their bioactivities.

2.3. Sea slugs as ornamental species (the beauties)

In the last decade, several studies have addressed the aquaculture potential of marine invertebrates commonly traded for marine aquariums (Olivotto et al., 2011). Nevertheless, little information is currently available on the commercial relevance of sea slugs in this trade. Most sea slugs traded as ornamental species are dazzling coloured (e.g., *Felimida* spp.), but commonly have very low chances of surviving in captivity and invariably starve to death. The most common reason for such poor husbandry success is their stenophagous feeding regime. These organisms only accept one type (or at the most a limited range) of prey as food, such as cnidarians, bryozoans, tunicates, sponges or even other nudibranchs (Calfo and Fenner, 2003; Sprung, 2001). The nudibranch *A. stephanieae*, formerly known as *Berghia verrucicornis*, is a good example of this rule, as it preys exclusively upon one of the most feared pests by marine aquarium hobbyists — glass anemones of genus *Aiptasia* (Carroll and Kempf, 1990; Leal et al., 2012c). Commonly employed as a biological weapon to control the outbreaks of these sea anemones in reef aquariums, *A. stephanieae* easily thrives and reproduces in the presence of large populations of *Aiptasia* (Banger, 2011). Due to its popularity in the marine aquarium trade, *A. stephanieae* commonly fetches high market values (up to 25 € per specimen – retail values for 2012) being a highly prized species for hobbyists breeding marine ornamentals. Another group of sea slugs commonly traded for marine aquariums are those that eradicate nuisance algae. The presence of certain sea slugs, such as *E. crispata*, slows down the growth rate of undesired algae in aquarium reefs, such as the green-hair like algae *Bryopsis* (Sprung, 2002). It is important to highlight that many Heterobranchia are relatively short-lived, thriving for only a couple of months to one year under optimal husbandry conditions (Calfo and Fenner, 2003). From a commercial point of view, this may be an advantage for breeders, as species employed to control nuisance organisms (e.g. glass anemones and undesirable algae), may have to be regularly replaced by new specimens, thus ensuring a continuous demand for these organisms.

3. Bibliometric analysis of sea slugs culture

Sea slug's research has experienced a considerable increase over the last decades, as a result of the above mentioned drivers. In the early 20th century, the main research topics on sea slug were their biology and taxonomy. At present, most studies performed with sea slugs have focused on neurobiology. As bibliometric analysis is a powerful tool to evaluate research priorities across entire disciplines (Neff and Corley, 2009), the published scientific literature on sea slugs over the last half-century (1958–2012) was surveyed and all

Table 1
List of molecules and respective bioactivities isolated from marine sea slugs that have been determinant for drug development.

Source organism	Compounds	Bioactivities	References
Aplysia dactylomela (A)	Escapin	Antimicrobial	1
Aplysia kurodai (A)	Pericosine A and B	Anti-tumour	2
Bursatella leachii (A)	bursatellanin-P	Anti-HIV	3
Dolabella auricularia (A)	Dolastatin 10, Dolastin 15 and Synthadotin	Anti-cancer	4
Chromodoris aspersa (N)	Various sesquiterpenes	Antimicrobial, antifungical	5
Dendrodoris carbunculosa (N)	Various dendocarbins	Anti-cancer	6
Doris kerguelenensis (N)	Palmadorin A, Labdane, Austrodorin	Antibacterial, anti-foulant, among others	7
Leminda millecra (N)	Toluquinone	Anti-cancer	8
Elysia rufescens (S)	Kahalalide F and Kahalalide A	Anti-tumour and anti-tuberculosis (respect.)	9–10

A — Anaspidea, N — Nudipleura, S — Sacoglossa; 1) Yang et al. (2005); 2) Numata et al. (1997); 3) Rajaganapathi et al. (2002); 4) Pettit et al. (1987); 5) Gunthorpe and Cameron (1987); 6) Sakio et al. (2001); 7) McClintock et al. (2010); 8) Whibley et al. (2007); 9) Hamann and Scheuer (1993); 10) Hamann et al. (1996).

published papers listed on the online database Web of Knowledge published by Thompson Reuters (available at http://apps.webofknowledge.com, and consulted the 24th of August 2012) were retrieved. The following search factors were used in the field "topic" as search request for referenced publications until August 2012: "Opisthobranchia" (for the general search) and "Opisthobranchia" AND "culture" (for the restricted search on sea slugs culture). While the term "Opisthobranchia" is no longer used (changed to Heterobranchia in 2010; see the Introduction section and Fig. 1), its use allowed a more complete survey of all previous works published on sea slugs. The search performed retrieved 948 works referring to Opisthobranchia from 1958 to August 2012. Overall publication activity was characterized by an increase in the number of published articles per year. Most articles on this topic have been published only in the last 12 years (2000–2012) (418 articles, representing 44% of total publications on this topic) (Fig. 3). Only 30 of the retrieved publications specifically address the culture of sea slugs. Apart from recent efforts targeting culture, given their importance for mainstream scientific areas such as neurobiology, sea slug production at a large scale has been poorly investigated. This trend in publications on sea slug's culture reflects how limited our scientific knowledge on this topic is and the need for further research to help our understanding of this important group of organisms.

4. Broodstock husbandry and reproduction

4.1. Collecting broodstock

Sea slugs usually occur in intertidal and coastal areas, with large sized species (e.g., *Aplysia* spp.) being easily detected even by inexperienced collectors. However, the detection of most sea slugs in the wild requires a careful inspection, mostly due to the remarkable mimetic ability displayed by some species (Debelius and Kuiter, 2007). Sea slugs can either be manually collected after visual detection, or researchers may employ underwater suction devices to collect specimens that were brushed from a surface commonly covered by the prey species of the sea slug (Bleakney, 1969; Clark, 1971; Franz, 1975). When employing SCUBA diving equipment, collecting the most frequent dietary prey of the target sea slug species (e.g. a sponge or a coral) is often a good option to harvest small-sized or highly mimetic animals (the limited air supply of the collector may impair a detailed inspection *in situ*). Harvested samples can later be easily inspected in the laboratory (Franz, 1975), where the use of flexible tweezers is recommended for manipulating sea slugs. Collected sea slugs can be shipped in aerated containers for short distances, or placed in round-bottomed plastic bags with one third of their volume filled with seawater and the two thirds with oxygen for long distances (as described by Wabnitz et al., 2003 for marine ornamental species).

Seasonal variability on the abundance of Heterobranchia is probably most pronounced in temperate regions, such as north-eastern Atlantic coasts, where significant seasonal shifts in water temperature occur (Franz, 1970). Knowledge on the zoogeography and reproductive ecology of target species will certainly be helpful to maximize collection efficiency. Local restrictions and collecting permits should be assessed prior to collecting in order to avoid any illegal actions that may involve fines or other legal sanctions.

4.2. Husbandry

The husbandry of sea slugs broodstock strongly depends on the availability of adequate food to keep breeding pairs under proper nutritional conditions and allow stocked animals to produce large numbers of high quality embryos. The rule of thumb in sea slugs husbandry is that the more intense the animal's color, the healthier it is. Nonetheless, stocking breeding pairs in captivity under optimal conditions is far from an easy task for some species, particularly when researchers ignore their feeding regimes. Additionally, potential environmental or nutritional stressing events that may have affected sea slugs prior to collection can also negatively affect the success of their husbandry, regardless of employing optimal stocking procedures. The life history of field collected-specimens, such as age, parental lineage and reproductive state may also significantly influence their breeding performance in captivity (e.g., *A. californica*, *E. viridis*).

The optimal type of system used to stock and breed sea slugs mostly depends on the target species and the purpose of its production, i.e., research scale vs. semi-commercial or commercial scale. So far, the only sea slug being commercially produced at a semi-industrial scale is *A. californica* (University of Miami/NIH, USA), with an overall production of 30,000 animals per year (Capo et al., 2009). While the ornamental sea slug *A. stephanieae* is currently only commercially cultured at a small scale (Olivotto et al., 2011), the work by Banger (2011) reports that semi-industrial scale production of this species can be achieved by using an innovative breeding system (see the Section Larviculture techniques for a detailed description).

Different broodstock systems have already been successfully employed for sea slugs production, from flow-through systems (Capo et al., 2002), such as the one used by the National Resource of *Aplysia* (University of Miami/NIH), to recirculated systems (Banger, 2011; Peretz and Adkins, 1982), that are often employed for academic research and/or small scale production of ornamental sea slugs. Recirculated systems operating with synthetic seawater have already allowed the culture of large numbers of sea slugs in a small space, with biological, chemical and mechanical filtration assuring high water quality and little system maintenance (Banger, 2011).

While no data is currently available to reliably compare the success of different broodstock systems for sea slugs, the location of the breeding facility (coastal areas vs. inland) and the availability of natural seawater with a suitable quality seems to rule the choice between flow-through and recirculated systems. As sea slugs have

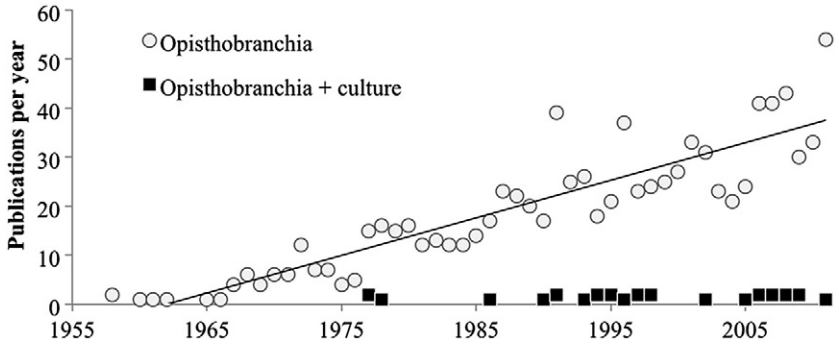

Fig. 3. Number of scientific articles published between 1958 and 2012, according to Web of Knowledge online database (on 24th August, 2012), using the search terms "Opisthobranchia" and "Opisthobranchia AND Culture".

already been successfully cultured employing either synthetic or natural seawater (Banger, 2011; Capo et al., 2002; Carroll and Kempf, 1990), it seems evident that the production of these organisms can be successfully achieved at inland facilities with no access to natural seawater. Sea slugs may be housed in glass, fiberglass, or plastic tanks, with some interesting examples of sea slug broodstock systems being provided by Capo et al. (2002) and Banger (2011) (Fig. 4A and B).

Sea slugs generally require seawater at a salinity of 30–35 and a pH of 8.0–8.2, with water temperature being dependent on the temperate or tropical origin of the sea slug species being cultured. As temperature is an important factor for gonadal maturation, it is possible to induce breeding throughout the year in captivity through the manipulation of water temperature (Kriegstein et al., 1974). Furthermore, it is vital to adjust this parameter to optimal values when stocking species with symbiotic associations, as it is known that abnormal temperatures may disrupt the association between animal hosts and endosymbiotic photosynthetic dinoflagellates (e.g., *Symbiodinium*) (Venn et al., 2008). Light is also an important factor to consider when stocking sea slugs with photosynthetic endosymbionts (see the Section "Solar-powered" sea slugs as tools for research on symbiosis between metazoan cells and functional chloroplasts). Successful husbandry has been achieved with fluorescent lamps (e.g., Vieira et al., 2009), light emitting diodes (LEDs) (e.g., Cruz et al., 2012), or natural sunlight (e.g., Schmitt et al., 2007). Table 2 summarizes some of the useful conditions for the successful husbandry of the most relevant sea slugs for academic research. The use of inadequate physico-chemical water parameters, along with nutritionally unbalanced diets, may result in poor egg quality, abnormal egg loss during incubation, and ultimately on the death of reproductive breeding pairs (Schlesinger et al., 2009).

4.3. Feeding preferences and nutrition

Feeding preferences within the Heterobranchia are known to vary largely, with some species being able to feed on a range of prey, while others display a stenophagous feeding regime (preying on a single species). Nonetheless, all Heterobranchia species seem to be insatiable, displaying a voracious appetite and often requiring a daily supply of food. In order to assure the suitable feeding of stocked specimens, both quantitatively and qualitatively, prey organisms must be easy to collect from the wild or to culture in captivity. It is therefore of paramount importance to know before-hand the dietary preferences of the target sea slug species to be cultured, as well as how easy it will be to collect or culture its prey.

Feeding specificity is usually higher in shelled Sacoglossa (Jensen, 1980), although some species shift their dietary preferences throughout development (Thompson and Jarman, 1989; Trowbridge and Todd, 2001). Nudibranchia can also be stenophagous, preying only on a single genus or species (Carroll and Kempf, 1990). At present, molecular techniques can allow researchers to determine the feeding regime of sea slugs (in the case of species with unknown feeding regimes) by analyzing DNA barcodes of their gut content. The dietary algal prey of sacoglossan sea slugs may also be investigated by analyzing the chloroplast DNA from whole animals (Händeler et al., 2010). Not all sacoglossans feed on algae, as some species feed exclusively on embryos of other mollusks (Jensen, 1997). Table 2 summarizes the dietary items required for successfully stocking breeding pairs of some of the most important sea slug species for research.

Several protocols are already available to culture some of the macroalgae commonly employed to feed juvenile stages of *A. californica* (e.g. *Gracilaria ferox*, *Agardhiella subulata*, *Ulva* spp. *and Laurencia* spp.) (Capo et al., 1999, 2002; Smith et al., 2011). Similarly, sea anemones have also been successfully propagated in captivity to culture the nudibranch *S. neapolitana* and *A. stephanieae* (Leal et al., 2012d; Schlesinger et al., 2009, respectively). For prey organisms with challenging life cycles and/or unsuitable for captive culture, such as certain sponges, ascidians, invertebrate embryos or even other sea slugs, the only option to sustain breeding pairs is to collect and stock their prey. While live prey are commonly employed, frozen sponges have already been successfully used as food items for certain nudibranchs (e.g., genus *Felimare*) (G. Calado, unpublished data).

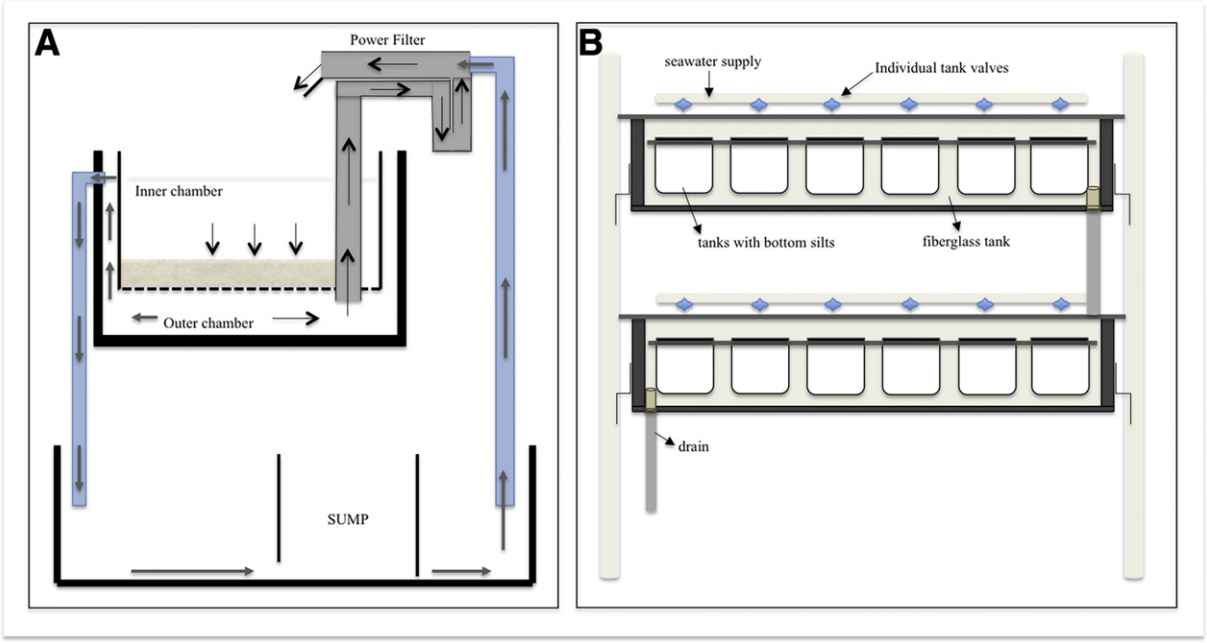

Fig. 4. Illustrations of two different systems employed for commercial sale production of sea slugs: A) Recirculated "breeding chamber" culture system for *Aeolidiella stephanieae* (adapted from Banger, 2011). Filtered seawater enters the inner chamber from the sump, passes through the substrate, and exits the breeding chamber via a drain located on the side of the outer chamber and returns to the sump for filtration; broodstock, larvae and juveniles remain inside the inner chamber. B) Flow-through culture system for *Aplysia californica* (adapted from Capo et al., 2009). Polycarbonate chambers held in a large fiberglass tank are continuously supplied with chilled seawater; each chamber presents an open top to facilitate the unidirectional flow of seawater supplied through individual valves: supplied seawater passes through the slits at the bottom of the chamber to the fiberglass tank and is discharged.

Table 2
Stocking conditions employed for the successful husbandry of marine sea slugs.

Species	Temperature (°C)	Diet	References
Aeolidiella stephanieae (N)	24	*Aiptasia sp.*	1
Aplysia californica (A)	13–18	*Gracilaria ferox*; *Agardhiella* sp.; *Ulva* sp.; *Laurencia* sp.	2–6
Aplysia dactylomela (A)	23–28	*Ulva* sp.; *Spyridia filamentosa*; *Laurencia* sp.	7
Aplysia juliana(A)	23–28	*Ulva* sp.; *Enteromorpha intestinalis*	7
Aplysia oculifera (A)	24	*Ulva* sp.; *Enteromorpha intestinalis*	8
Elysia chlorotica (S)	10	*Vaucheria litorea*	9–10
Elysia timida (S)	20	*Acetabularia acetabulum*	11–12
Elysia viridis (S)	10–18	*Codium fragile*; *C. tomentosum*	13–16

A — Anaspidea, N — Nudipleura, S — Sacoglossa; 1) Carroll and Kempf (1990); 2) Smith and Carefoot (1967); 3) Kriegstein et al. (1974); 4) Capo et al. (2002); 5) Capo et al. (2009); 6) Smith et al. (2011); 7) Switzer-Dunlap and Hadfield (1977); 8) Plaut et al. (1995); 9) West et al. (1984); 10) Green et al. (2000); 11) Marin and Ros (1989); 12) Wägele et al. (2011); 13) Weinmayr (1992); 14) Trowbridge (2000); 15) Trowbridge and Todd (2001); 16) Unpublished data.

A viable alternative to live feeds would be feeding sea slugs with inert microdiets. Significant efforts have been made in last years to design specific artificial diets suitable for commercial aquatic species such as fishes, crustaceans and bivalve molluscs (Luzardo-Alvarez et al., 2010). Successful advances such as diet binders, agglutination chemicals, inclusion of different enzymes and pre-hydrolyzed proteins among others, are good examples for future research in sea slug nutrition.

It is known that lipids and fatty acids (FA) play an important role in developing embryos of mollusks (Joseph, 1982). However, few studies are currently available on this topic for sea slugs (Martínez-Pita et al., 2006). As most marine invertebrates, sea slugs cannot synthetize certain FA de novo, which must be derived from their dietary prey or provided through symbiotic relationships with microalgae or algal chloroplasts (Zhukova, 2007). FA profiles on early developing embryos are primarily regulated by broodstock nutrition and clearly reflect the pool of fatty acids available for parental diets (Leal et al., 2012d; Martinez-Pita et al., 2005).

4.4. Reproduction

Sea slugs display complex reproductive modes and strategies (Ghiselin, 1966). They are hermaphrodites with internal cross-fertilization (Beeman, 1970; Painter et al., 1985). Allosperm resorption has been shown to occur in several species (Rivest, 1984) and can be assumed to be widespread due to the presence of a gametolytic gland in most groups (Schmitt et al., 2007). Sea slugs typically donate and receive sperm reciprocally in the head-to-tail cross-position (Carefoot, 1987). Besides this standard insemination mode, a variety of alternatives exist. Some species form mating chains (Angeloni, 2003; Switzer-Dunlap and Hadfield, 1984; Yusa, 1996), alternate sex roles (Anthes et al., 2006; Michiels et al., 2003), or transfer sperm via externally attached spermatophores (Karlsson and Haase, 2002). Hypodermic insemination, in which sperm is injected through the partners' body surface, is also widespread, particularly among Sacoglossa (Angeloni, 2003; Jensen, 1999; Rivest, 1984; Schmitt et al., 2007). The duration of different mating phases, such as courtship behavior and the timing of the penial gland eversion, are known to be species-specific (Reise, 2007). Species in genus *Aplysia* frequently mate with several partners (Angeloni et al., 2003; Yusa, 1996) and readily mate with a second partner immediately after an initial mating encounter of 30–40 min (Ludwig and Walsh, 2008). As a consequence of this behavior and reproductive anatomy, these organisms display sperm competition, as well as post-copulatory female choice (Michiels, 1998; Yusa, 1994). Furthermore, size-differences between mating partners have been shown to influence mating behavior in sea slugs (Angeloni and Bradbury, 1999; Angeloni et al., 2003; Gianguzza et al., 2004). In this way, a mixture of strategies or gender preferences can be employed by these organisms, depending on the unique set of circumstances associated with each mating encounter (Anthes et al., 2006).

In order to achieve good results on broodstock reproduction the following issues must be carefully addressed: 1) food must never be a limiting factor, nor negatively affect water quality parameters (Capo et al., 2002; Plaut et al., 1995); 2) pairing similar sized animals may increase the number of produced embryos (mated specimens will reproduce both as male and female), as size-differences can influence mating behavior and small sized animals are more prone to mate in female role (Angeloni and Bradbury, 1999; Angeloni et al., 2003; Anthes et al., 2006); 3) stocking groups of breeding organisms is advisable, as long as animal density is maintained between the limits that induce matting strategies and those that would cause suppressed somatic growth (e.g., between 5 and 7 animals per breeding tank for *Aplysia*; see Capo et al., 2002). Animals stocked at high densities may display slower growth rates and contrasting final weights, but often display a synchronous onset of sexual maturity independently of stocking densities (Capo et al., 2002).

4.5. Embryos incubation

The number of embryos produced per spawning in sea slugs is species specific and depends on parental size (Hadfield and Switzer-Dunlap, 1984; Switzer-Dunlap and Hadfield, 1979). Switzer-Dunlap and Hadfield (1979) found that the lifetime egg production of certain anaspideans to be up to 272×10^6, while small sacoglossans have been reported to produce between 1000 and 2250 eggs (Chia, 1971).

The shape of egg masses, as well as the attachment mode, may vary among families and genera, and may even be a useful feature to distinguish between sea slug species (Franz, 1975). Eyster (1986) reviewed the ultrastructure of nudibranch egg capsules, while the histology and ultrastructure of egg masses of several Heterobranchia have been reviewed by several authors (Klussmann-Kolb and Wägele, 2001; Wägele, 1989, 1996).

While the majority of sea slugs readily spawn on any submerged surfaces, some species may display more specific requirements. As an example, *S. neapolitana* will only lay its egg masses if the substratum is clean (Schlesinger et al., 2009). For sacoglossan sea slugs, oviposition is usually facilitated if the host algae is present in the breeding tank (sea slugs will lay their embryos on the surfaces of the algae) (Franz, 1975). For some species, portable egg-laying substrates can be used as they provide shelter for breeding pairs, increase the available area for attaching egg masses and allow a better monitoring of developing embryos. These shelters can be made of PVC tubes, which are set into breeding tanks (Schlesinger et al., 2009). The inspection of these structures can be facilitated if PVC pipes are split in two longitudinally and held together with rubber bands; by removing the rubber bands it is possible to perform a close inspection of the embryos in egg masses attached to the inner walls of the PVC pipe. Another alternative often employed to culture *A. stephanieae* is to place clay pots with holes drilled in their bottoms upside down in broodstock tanks; breeding pairs commonly lay their embryos inside the pot, which can later be easily removed and inspected (Banger, 2011). Some small sea slugs

from the genera *Aeolidiella*, *Cuthona* and *Calma* can also spawn at the air–water interface but egg masses must be submerged artificially to develop and avoid contact with air (G. Calado, unpublished data).

It is a common practice to carefully take/detach the egg masses from the substrate and transfer them to sterile beakers, or Petri dishes filled with filtered seawater, which are latter placed in an incubator (to control for temperature and photoperiod) until the hatching of veliger larvae. Recent advances were made by Banger (2011) on the development of a system that avoids the physical contact with egg masses during incubation. The "Banger breeding chamber" consists of an outer chamber that houses an inner chamber holding breeding sea slugs and a deep "flow through sand bed" (Fig. 4A). The deep "flow through sand bed" provides a natural barrier that prevents newly hatched larvae or juveniles from being drained through the outflow and damaged by filtration systems. It is well known that sea slug's egg masses are commonly sensitive to external factors, namely water quality and circulation. In static egg mass cultures water changes are usually performed daily or every other day to prevent water quality deterioration. Temperature, light, pH and salinity should be identical to that of parental stocking tanks and maintained constant during the incubation period (a feature that is more easily achieved when employing flow-through or recirculated systems than in static incubation tanks). The most suitable option to aerate sea slugs egg masses during incubation appears to be species-specific, as Carroll and Kempf (1990) recommend a gentle air bubbling for *A. stephanieae* and Capo et al. (2009) advocate the use of a vigorous aeration to incubate the embryos of *A. californica*.

5. Larviculture

Larval culture is often a challenge for the production of marine invertebrates, and most sea slugs are no exception to the rule. Researchers studying the early life history of Heterobranchia of interest for biomedical research provided detailed descriptions of their larviculture trials (Kempf and Willows, 1977; Kriegstein et al., 1974; Paige, 1988; Strenth and Blankenship, 1978), particularly those targeting *Aplysia* (Kriegstein, 1977; Switzer-Dunlap and Hadfield, 1977). Larviculture of other ecologically important sea slugs (e.g. *Alderia modesta*, *Hermissenda crassicornis*, *Doridella obscura*, *Adalaria proxima*, *Dendronotus frondosus*, *Dollabella auricularia*) has also been described by several authors (Clark, 1975; Harrigan and Alkon, 1978; Krug and Zimmer, 2000; Perron and Turner, 1977; Sisson, 2005; Switzer-Dunlap and Hadfield, 1977). However, scientific information on larval feeding is still extremely limited (Avila et al., 1997; Hubbard, 1988; Plaut et al., 1995; Switzer-Dunlap and Hadfield, 1977). While a small number of sea slugs can already be mass cultured in captivity (Banger, 2011; Capo et al., 2002; Schlesinger et al., 2009), the small size displayed by the newly hatched larvae of most species, along with their unknown feeding requirements, are still a challenge for researchers. The need to develop suitable systems/techniques for rearing the larval forms of sea slugs is essential for a successful commercial scale production of these organisms. It is therefore very useful to gain further knowledge on sea slugs larviculture system design, larval feeding and nutrition, as well as optimal culture conditions.

5.1. Larval development modes

As for several other marine invertebrates, the initial development of sea slugs occurs in protected egg masses, which is often followed by a free-swimming larva — the veliger (Oyarzun and Strathmann, 2011). Sea slugs may undergo metamorphosis inside the egg capsules and crawl out as fully developed juveniles (direct development), or metamorphose outside the capsules (indirect development) (Carroll and Kempf, 1990). If they hatch from the egg capsule as larvae, they may develop without feeding stages (lecithotrophic development, e.g., *A. proxima*) (Thompson, 1976) or they may need to feed on phytoplankton for days, weeks, or even months (planktotrophic development, e.g., *A. californica*) (Capo et al., 2009). Although the nutritional modes of marine invertebrate larvae are typically ranked in a dichotomy between planktotrophy or lecithotrophy (Strathmann, 1985), sea slugs are known to exhibit poecilogony - the existence of variable larval development modes within the same species (e.g., *Alderia willowi*, *Elysia pusilla*, *Elysia cause*, *E. chlorotica*) (Allen and Pernet, 2007; Vendetti et al., 2012) (see Fig. 5). In other words, the type of larvae produced from the egg masses of certain sea slugs can be modulated through culture conditions; as an example, *Alderia willowi* is known to produce a higher proportion of lecithotrophic larvae under high temperature and salinity, whereas the production of plankthrophic larvae is favoured under lower temperature and salinity (Krug et al., 2012).

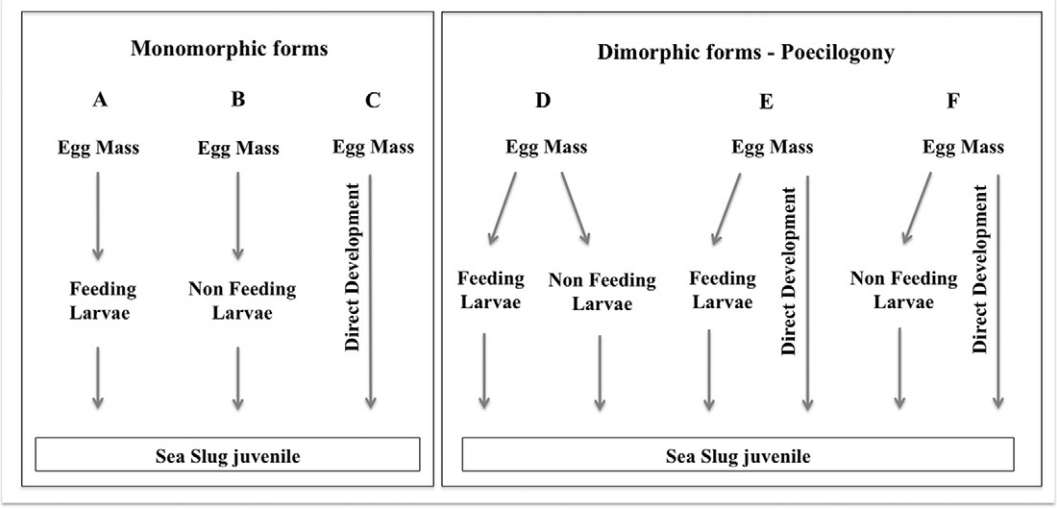

Fig. 5. Overview of monomorphic and dimorphic modes of development in sea slugs. Monomorphic forms: A) feeding larvae (planktotrophic, require a suitable supply of phytoplankton after hatching to reach metamorphosis), B) nonfeeding larvae (lecitotrophic, no exogenous food is required after hatching to reach metamorphosis), and C) direct development (metamorphosis occurs inside the egg capsule and an *imago* of the adult crawls from egg mass): dimorphic forms (different modes of development can occur within the same egg mass - poecilogony): D) feeding and non-feeding larvae can hatch from the same egg mass, E) The release of feeding larvae and *imagos* of the adult (direct development) can be recorded from the same egg mass, and F) the release of non-feeding larvae and *imagos* of the adult (direct development) can be recorded from the same egg mass.

Several studies on the larval development of sea slug species displaying planktotrophic larvae are available, particularly for nudibranchs (Hadfield and Miller, 1987; Kempf and Willows, 1977; Todd et al., 2001), sacoglossans (Clark, 1975; Clark and Goetzfried, 1978; Harrigan and Alkon, 1978; Krug and Zimmer, 2000; Seelemann, 1967; Trowbridge, 1998, 2000; Weinmayr, 1992; West et al., 1984) and aplysiids (Hadfield and Switzer-Dunlap, 1984; Thompson and Jarman, 1989). From an aquaculture point of view, the most desirable sea slug species for culture, in increasing order of preference, will be those displaying a short planktotrophic larval development, a lecithotrophic larval development, or a direct development (the most desirable for culture).

5.2. Larviculture techniques

Early trials of sea slugs larviculture performed during the 1960s and 70s relied on static culture approaches (e.g., Franz, 1975; Kriegstein et al., 1974; Switzer-Dunlap and Hadfield, 1977 and references therein). While most research efforts have targeted the culture of sea slugs displaying planktotrophic larvae (Avila et al., 1997; Harris, 1970; Kempf and Willows, 1977; Nadeau et al., 1989), it is not surprising to verify that the most successful larviculture experiments were achieved when addressing species displaying lecithotrophic larval development (Harris, 1970, 1975; Swennen, 1961; Tardy, 1962, 1970; Thompson, 1958).

In general, researchers transfer newly hatched veligers to beakers (e.g., Trowbridge, 2000) or, less commonly, to sterile dishes (Capo et al., 2009; G. Dionísio, unpublished data) for posterior culture. Filtered seawater is commonly used, with water changes being performed from 1 to 4 times a week with the help of mesh screens to retain cultured larvae (mesh screens of variable sizes can be easily constructed using PVC rings as frames, or replacing the bottom of a beaker with mesh) (e.g., (Avila et al., 1997; Capo et al., 2009; Carroll and Kempf, 1990; Franz, 1975; Trowbridge, 2000). Larvae retained on the mesh screen can be washed to Petri dishes using a pipette and then poured into a new culture beaker with filtered seawater and algal food (Trowbridge, 2000). Young sea slug larvae are positively phototactic and may be trapped at the air–water interface when swimming toward the top of culture vessels illuminated from above. The trapping of veligers in the air–water interface is due to surface tension and can be reduced by keeping culture vessels in the dark (Franz, 1975). Hurst (1967), Harris (1970) and Harrigan and Alkon, 1978 reported that cetyl alcohol can be successfully used to reduce larval mortality due to this phenomenon, namely by sprinkling lightly flakes of this compound on the water surface of the culture vessel. Capo et al. (2009) successfully used an iodine-based surfactant to re-suspend any larvae entrapped at the air–water interface.

Another bottleneck often faced during the larviculture of sea slugs is the susceptibility of cultured larvae to infections by bacteria, fungi and protozoans. Cleanliness is the best solution to prevent this problem. Some authors use antibiotics, such as penicillin and streptomycin sulfate (Franz, 1975; Switzer-Dunlap and Hadfield, 1977), anti septic solutions (e.g. poly-iodine complex and fish-grade Trizma (Capo et al., 2009), Chloramphenicol and EDTA (Avila et al., 1997; Harrigan and Alkon, 1978; Sisson, 2005) or keeping cultures in the dark to suppress the growth of unwanted microorganisms. Several studies have already addressed the effect of initial larval density on growth, metamorphic competency and survival of sea slugs larvae (Avila et al., 1997; Capo et al., 2009; Schlesinger et al., 2009; Switzer-Dunlap and Hadfield, 1977). The general trend of recording decreasing survival rates at increasing larval densities has been demonstrated for several species (Avila et al., 1997; Hubbard, 1988). This trend may be attributed to collisions among developing larvae, which may promote a range of deleterious effects, namely feeding inhibition and physical injuries, bacteria and other micro-organisms growth due to an increased of larval metabolic wastes. A higher rate of disease transmission under high larval densities was also suggested by Capo et al. (2009) in order to explain lower survival rates under such culture conditions.

5.3. Larval feeding

As previously mentioned, larvae can hatch either as feeding or non-feeding forms. Feeding veligers rely on the ingestion of phytoplankton for a variable period of time (weeks to months) until reaching metamorphosis. The duration of the larval phase is known to be species-specific and mainly regulated by culture conditions (e.g., water quality, temperature). The biggest constraint for the large-scale culture of sea slugs is the provision of an adequate larval diet, especially for larvae that possess undeveloped or rudimentary feeding structures (Franz, 1975). Feeding larvae will only grow at maximum rates if the prey provided fulfills all of their nutritional requirements (Pechenik and Heyman, 1987). “Non-feeding" veligers rely on yolk reserves provided by parental organisms to fuel their energetic demands until metamorphosis; while non-feeding larvae commonly develop in a relatively short period of time to metamorphosis, in some cases such larvae have the potential to feed secondarily and persist in the plankton for long periods (Kempf and Hadfield, 1985). Species that display direct development commonly produce larger sized juveniles than those resulting from the metamorphosis of planktotrophic or lecithotrophic veligers.

Feeding protocols currently available for sea slugs provide a supply of phytoplankton species (e.g., *Isochrysis*, *Tetraselmis*, *Rhodomonas*) to their developing larvae, either as pure or mixed diets (Avila et al., 1997; Harrigan et al., 1978; Harris, 1975; Kempf and Willows, 1977; Perron and Turner, 1977; Schlesinger et al., 2009; Trowbridge, 2000) (see Table 3). Previous studies have already demonstrated that species composition of microalgal diets (either monospecific or mixed), as well as their concentration, play a key role in the success of sea slug larviculture trials (Capo et al., 2009; Hubbard, 1988; Schlesinger et al., 2009; Switzer-Dunlap and Hadfield, 1977). Algal uptake in sea slugs veligers is regulated by the size and density of the microalgae provided (Chia and Koss, 1978). As shown for other organisms, larger microalgae may decrease total ingestion as a consequence of particle interference at the velar edge, longer handling time inhibiting the simultaneous ingestion of smaller particles, or post-ingestive rejection (Strathmann, 1987). Most studies report algal concentrations ranging from 10×10^3 (Chia and Koss, 1978; Plaut et al., 1995; Trowbridge, 2000) to 10×10^4 cell.ml^{-1} (Avila et al., 1997; Kempf, 1981; Kriegstein et al., 1974; Paige, 1986; Switzer-Dunlap and Hadfield, 1977) as suitable to culture sea slug veligers (Table 3). Nonetheless, under higher algal concentrations, it is common to record a faster growth, a higher survival and a shorter period required for larvae to reach metamorphic competence (Capo et al., 2009; Hubbard, 1988).

Concerning the larviculture of *Aplysia*, static rearing conditions proved to be unsuccessful (Capo et al., 1987; Kriegstein et al., 1974; Nadeau et al., 1989), as water movement is needed to keep food in suspension so that it remains available for larvae under culture (accelerated particles are more likely to encounter the vela cirri and be ingested more easily). The use of roller bottles that maintain algal preys in constant suspension while providing a homogenous non-turbulent environment for rapid larval growth were a significant breakthrough for the successful larviculture of *Aplysia* (Capo et al., 2009).

5.4. Metamorphosis and settlement cues

Planktonic larvae preferentially metamorphose in response to specific cues (Avila, 1998; Krug and Zimmer, 2000; Pawlik, 1992; Trowbridge and Todd, 2001) (see Table 4). However, there are several sacoglossan sea slugs (e.g., *Tenellia fuscata*, *Toranatina canaliculata*) (Franz, 1975), and nudibranchs (e.g., *A. stephanieae*, *Melibe leonine*) (Bickell and Kempf, 1983; Carroll and Kempf, 1990) that do not require any specific stimulus to trigger metamorphosis.

Table 3
Larviculture conditions employed to raise sea slugs, with emphasis to larval diet, density (larvae ml^{-1}) and days required to reach metamorphis.

Species	Temp. (°C)	Diet	Density (larvae/ml)	Metamorphosis (DAH)	References
Aeolidiella stephanieae (N) [a]	21–26	No exogenous food required	–	0 or 13–15	1–2
Alderia modesta (S) [b]	25	Mixed: *Rhodomonas* sp., *Isochrysis galbana*, *Pavlova lutheri* (10^4 cell ml^{-1})	–	3–6	3
Aplysia californica (A) [c]	22	Mixed: *Isochrysis sp.* and *Chaetoceros muelleri* (1:1) (250×10^3 cells ml^{-1})	0.5–1	35	4–5
Aplysia dactylomela (A) [d]	24–26	*P. lutheri*, *I. galbana*, *Dunaliella tertiolecta*, *Nannochloris sp.*	0.8–1	30	6
Aplysia juliana (A) [e]	23–28	*Ulva* sp.	0.8–1	28	6
Aplysia oculifera (A) [e]	24	*I. galbana* (10^4 cell ml^{-1})	<1	28	7–8
Bursatella leachii plei (A) [f]	25	*I. galbana* (10^4 cell ml^{-1})	1–5	20	9
Elysia chlorotica (S) [g]	10	*I. galbana* (10^4 cell ml^{-1})	–	25	10–11
Elysia timida (S) [d]	20	No exogenous food required	–	0	12
Elysia viridis (S) [d]	15	*Rhodomonas baltica* (10^4 cell ml^{-1})	–	28–30	13
Hermissenda crassicornis (N) [h]	12	Mixed: *Isochrysis sp.* and *Rhodomonas salina* (1:1) ($10–25 \times 10^3$ cell ml^{-1})	1–4	42	14
Melibe leonine (N) [e]	12–14	*P. lutheri* (10^4 cell ml^{-1})	2–3	30–48	15
Phestilla sibogae (N) [e]	24–27	No exogenous food required	–	24–29	16
Spurilla neapolitana (N) [i]	24	Mixed: *I. galbana* (10^5 cell ml^{-1}) + *Tetraselmis tetrathele* (10^3 cell ml^{-1})	4	25	17
Tochuina tetraquetra (N)[d, e]	20–22	Mixed: *Isochrysis sp. and Monochris lutheri* (1:1) (10^4 cell ml^{-1})	2	34	18

A — Anaspidea, N — Nudipleura, S — Sacoglossa; DAH — days after hatching; a) cultured in Petri dishes and in recirculated systems (described by Banger, 2011) using Millipore-filtered aged natural seawater and artificial seawater, respectively; b) cultured in beakers and requiring 20 mM K^+ in the form of KCl to induce metamorphosis (Yool et al., 1986); c) cultured in roller bottles with aeration and filtered sea water containing chloramphenicol and Na2EDTA; d) cultured in beakers using filtered sea water; e) cultured in beakers with filtered sea water containing antibiotics (e.g., Penicillin G and Streptomycin sulfate); f) cultured in beakers with artificial seawater; g) cultured in beakers with artificial seawater containing antibiotics; h) cultured in roller bottles with filtered sea water containing chloramphenicol and EDTA; i) cultured using the double beaker method (as described by Strathmann, 1987) with filtered sea water containing antibiotics; 1) Carroll and Kempf (1990); 2) Banger (2011); 3) Krug (1998); 4) Capo et al. (2002); 5) Capo et al. (2009); 6) Switzer-Dunlap and Hadfield (1977); 7) Plaut et al. (1995); 8) Kempf (1981); 9) Paige (1988); 10) West et al. (1984); 11) Rumpho et al. (2011); 12) Marin and Ros (1989); 13) Trowbridge (2000); 14) Avila et al. (1997); 15) Bickell and Kempf (1983); 16) Kempf and Hadfield (1985); 17) Schlesinger et al. (2009); 18) Kempf and Willows (1977).

In the laboratory, sea slugs larvae may settle and metamorphose in response to a variety of artificial or natural cues (e.g., aqueous extracts from dietary prey). An increase in seawater concentration of potassium can trigger metamorphosis in some sea slug species (Todd et al., 1991; Yool et al., 1986). Nevertheless, this stimulus is not a universal substitute for natural cues promoting metamorphosis (Pechenik and Rice, 2001; Pechenik et al., 1995). Sea slug larvae will also metamorphose when exposed to neuroactive agents, such as choline (Todd et al., 1991) or organic compounds (e.g., acetone, ethanol, methanol) (Avila, 1998; Pechenik et al., 1995). Water agitation may also be very effective to increase metamorphosis when natural inducers are present (Pechenik et al., 1995). A step increase in water temperature can induce an increase in the percentage larvae metamorphosing, but may also negatively affect later life stages (Avila, 1998).

Table 4
Sea slugs already cultured in captivity and respective settlement cue(s) employed to trigger metamorphosis (all species listed under settlement cues are algae, unless indicated otherwise).

Species	Settlement cue(s)	References
Aplysia californica (A)	*Gracilaria ferox*; *Agardhiella* sp.; *Ulva* sp.; *Laurencia* sp.	1–2
Aplysia juliana (A)	*Ulva* sp.	3
Bursatella leachii plei (A)	*Microcoleus lyngbyaceous*, *Schyzothrix calcicola*, *Porphyrosyphon notarisii*	4
Corambe obscura (N)	*Electra crustulenta* (bryozoan)	5
Dendronotus frondosus (N)	*Obelia geniculate* (hydroid)	6
Elysia chlorotica (S)	*Vaucheria litorea*	7
Elysia viridis (S)	*Codium fragile*, *C. tomentosum*, *Cladofora rupestris*, conspecifics	8
Elysia timida (S)	*Acetabularia acetabulum*	9
Hermissenda crassicornis (N)	*Tubularia crocea and Pennaria* sp. (hydroid); *Metridium senile* and *Haliplanella luciae* (anemone)	10
Phestilla sibogae (N)	*Porites* sp. (coral)	11–13
Phestilla melanobranchia (N)	*Tubastrea aurea* (coral)	14

A — Anaspidea, N — Nudipleura, S — Sacoglossa; 1) Pawlik (1989); 2) Nadeau et al. (1989); 3) Switzer-Dunlap and Hadfield (1977); 4) Paige (1988); 5) Perron and Turner (1977); 6) Paige (1988); 7) Rumpho et al. (2011); 8) Trowbridge and Todd (2001); 9) Marin and Ros (1993); 10) Avila (1998); 11) Switzer-Dunlap and Hadfield (1977); 12) Ritson-Williams et al. (2003); 13) Ritson-Williams et al. (2009); 14) Ritson-Williams et al. (2007).

Most Heterobranchia are known to be feeding specialists, with adult preys commonly acting as the metamorphic trigger for developing larvae (Avila, 1998). Metamorphosis of several sea slugs is known to be stimulated by chemical cues released from their invertebrate (Hadfield and Koehl, 2004; Ritson-Williams et al., 2003 and references therein; Krug, 2009) or algal prey (Kriegstein et al., 1974; Nadeau et al., 1989), as these cues probably indicate that newly metamorphosed juveniles will be provided a suitable environment for their grow-out. It is also important to highlight that, at least for some herbivorous sea slug species, both prey and non-prey algae may induce competent larvae to metamorphose. (Trowbridge, 2000; Weinmayr, 1992).The introduction of invertebrate or algal species known to trigger metamorphosis must be carefully timed, as only competent larvae (larvae which already have enough energetic reserves to undergo metamorphosis) will be receptive to any potential cues and metamorphose (Avila et al., 1997; Franz, 1975; Kempf and Willows, 1977). Between and within-culture variations in the timing of larval competence are well known and inevitable, being largely inherent to larval variability (Kempf, 1981; Plaut et al., 1995). The source of such variability has been attributed, among other aspects, to genetic differences, dietary or water quality deficiencies, the use of antibiotics and bacterial loads (Avila, 1998).

6. Juvenile grow-out

6.1. Feeding and nutrition

Sacoglossans are specialized suctorial feeders, with most species being stenophagous herbivores (Jensen, 1994) that may switch their food preference during grow-out (Thompson and Jarman, 1989; Trowbridge, 2004). The same level of specialization is valid for the majority of nudibranchs, with their diets being commonly limited to a single species (Thompson and Jarman, 1989). An electronic register of the worldwide food habits of nudibranchs was created by Gary R. McDonald and James W. Nybakken and can be consulted on the www.theveliger.org webpage. In general, aplysiids are more

generalist feeders and can feed upon more than one species of algae (Carefoot, 1987). Increasing evidences suggests that feeding preferences for certain sea slugs are related to the secondary metabolites they will acquire by feeding upon a certain prey (Barile et al., 2004).

Artificial diets have already been successfully used to raise juvenile sea slugs. As an example, a diet made up of chemicals and set in agar with a small amount of water extract of the green alga *Ulva fasciata* promoted acceptable growth levels and even induced spawning in the sea hare *A. dactylomela* (Carefoot, 1980).

Studies specifically addressing the nutritional requirements of juvenile sea slugs during grow-out seem to be non-existent in the scientific literature. The information retrieved from ecological based works is also scarce, being limited to one study addressing lipid classes and fatty acids (FA) in two nudibranch genera (*Felimida* and *Phyllidia*) (Zhukova, 2007). In this study, phospholipids were shown to be the dominant lipid class, followed by sterols. A wide diversity of fatty acids was also recorded, with the typical marine $n-3$ polyunsaturated fatty acids (PUFA) comprising only a small proportion of the total pool of FAs (0.6 to 1.3%). On the others side, $n-6$ PUFA represented up to 25% of the total pool of FAs. It would therefore be useful to promote further research on the nutrition of juvenile sea slugs and determine if formulated diets can be a reliable alternative to the use of live/frozen prey during grow-out.

6.2. Culture systems

Controlling animal quality and information on their age, parental background and reproductive state, are the main drivers stimulating research on the grow-out of sea slugs. Successfully controlling water temperature, light, stocking density and food quantity and quality are the key-issues for culturing juvenile sea slugs at a commercial scale (Capo et al., 2009). Several factors have already been identified as potential growth inhibitors: 1) the release of animal pheromones that trigger mass spawning and/or suppress somatic growth (Audersirk, 1979; Levy et al., 1997); 2) high ammonia levels promoted by animal waste that are detrimental to the health of stocked organisms (Handy and Poxton, 1993); 3) toxic compounds secreted from uningested prey in response to grazing stimulus (Handy and Poxton, 1993; Toth and Pavia, 2001); and 4) high prey biomass that may deplete important nutrients from the sea water that are required for the somatic growth of juvenile sea slugs (Capo et al., 2009). It has been suggested that crowding *per se* does not affect the timing for sexual maturity as long as food is available, but food limitation *per se* may negatively affect the onset of sexual maturity (Capo et al., 2009; Plaut et al., 1995). Grow out systems used for juvenile sea slugs are usually the same used for broodstock maintenance. The most successful systems employed so far for growing sea slugs are those by Banger (2011) for the mass grow-out of the ornamental nudibranch *A. stephanieae* (Fig. 4A) and by Capo et al. (2009) for the commercial scale culture of the sea hare employed in biomedical research, *A. californica* (Fig. 4B).

6.3. Live shipping

As there are no specific live shipping protocols for sea slugs, we recommend the use of identical procedures to those described by Wabnitz et al. (2003), which are commonly employed to ship marine ornamental invertebrates. At their origin, sea slugs should be quarantined and starved for at least 48 h prior to shipment; this procedure will ensure that they do not excrete any undesirable compounds into the water of the shipping container. As is the case for most invertebrates, sea slugs should be packed in polyethylene bags with rounded bottoms, filled with one third seawater and two thirds oxygen, sealed and placed in a polystyrene box (for insulation and shock resistance). Another method employed for shipping small sized sea slugs is the use of plastic bottles or vials. To avoid excessive risks, a maximum travel time of 48 h is recommended for shipments of live sea slugs. At the destination, newly arrived specimens should be slowly acclimated to the water chemistry of their new stocking system, with the dripping acclimation method commonly being a suitable solution. It is also important to highlight that shipping water should always be discarded and never added to the new husbandry system that will house the sea slugs.

7. Concluding remarks

While new advances have been achieved in the lasts decades in the culture of sea slugs, large scale production is still restricted to a reduced number of species. Commercial scale production has only been implemented for *A. californica*, although recent data using small-scale recirculated culture systems (e.g. for culturing *A. stephanieae*) open good perspectives for the production of several other species. Future research efforts should target the standardization of larviculture systems and feeding protocols, as well as the clarification of the mechanisms involved in triggering metamorphosis in the most desirable species for captive culture. Another topic that should be investigated is the potential to replace live, freshly harvested or frozen prey by formulated diets that can be customized to meet species-specific nutritional requirements of adult and juvenile sea slugs. The successful establishment of reliable culture systems and protocols would certainly provide the necessary know-how to prompt the large scale production in those sea slugs most commonly desired for academic research or commercial purposes.

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