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REVIEW ARTICLE

Sink and swim: a status review of thecosome pteropod culture techniques

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The widespread distribution of pteropods, their role in ocean food webs and their sensitivity to ocean acidification and warming has renewed scientific interest in this group of zooplankton. Unfortunately, their fragile shell, sensitivity to handling, unknowns surrounding buoyancy regulation and poorly described feeding mechanisms make thecosome pteropods notoriously difficult to maintain in the laboratory. The resultant high mortality rates and unnatural behaviours may confound experimental findings. The high mortality rate also discourages the use of periods of acclimation to experimental conditions and precludes vital long-term studies. Here we summarize the current status of culture methodology to provide a comprehensive basis for future experimental work and culture system development.

INTRODUCTION

Pteropods are a cosmopolitan group of holoplanktonic molluscs, which consists of thecosome (shelled) and gymnosome (shell-less) species, both of which play an important role in trophic and biogeochemical fluxes (Lalli and Gilmer, 1989). For decades, pteropods have been understudied, but recently thecosome species have become the focus of renewed scientific interest within the rapidly expanding field of ocean acidification research, and as such are the primary focus of this review. Since the industrial revolution the oceans have absorbed approximately one-third of anthropogenic CO_2 (Sabine *et al.*, 2004), altering seawater carbonate chemistry. Ocean pH has dropped by 0.1 pH units since the industrial revolution and is projected to decrease by up to 0.3–0.4 units by 2100 (Orr, 2011).

Pteropods are predicted to be particularly vulnerable to the effects of lowered carbonate saturation states resulting from ocean acidification as their thin shells are made of aragonite, a form of calcium carbonate more soluble than calcite in sea water (Mucci, 1983). Studies have confirmed that pteropod larvae and adults exhibit negative calcification responses to projected future conditions with decreased calcification (Comeau *et al.*, 2010a,b; Lischka *et al.*, 2011) and shell dissolution (Lischka *et al.*, 2011). Studies of metabolic rate have also revealed that CO_2 acts in concert with other environmental stressors, such as temperature and salinity, influencing swimming speed and oxygen consumption in some pteropod species (Comeau *et al.*, 2010b; Maas *et al.*, 2011, 2012a, 2012; Bednaršek *et al.*, 2012a). These studies have been primarily of short-term duration due to constraints imposed by the difficulties of keeping pteropods in culture.

In their natural environment, pteropods have periods of active swimming, sinking and neutral buoyancy (Lalli and Gilmer, 1989). The weight of the shell causes the animals to sink relatively quickly, and culture vessels do not provide a sufficiently deep water column and/or adequate current conditions for the animals to attain neutral buoyancy. This often results in the animals repeatedly hitting the bottom of the culture vessel, causing shell breakage and the production of stress-induced mucus. The mucus can stick their wings together, impairing swimming ability and can adhere them to the bottom of the tank.

Lack of buoyancy, in turn, impedes feeding behaviour. Thecosome pteropods are suspension feeders, producing mucous webs from their wings (Fig. 1), several times their body size, to entrap phytoplankton and small motile prey and other particles (Gilmer, 1972). Production and retraction of the web can be relatively fast as little as 5 s for deployment and <20 s for retraction, although this is highly species specific (Gilmer and Harbison, 1986). Whilst the web is deployed the animal hangs motionless with the web between the parapodia; eventually the animal consumes the entire mucous structure with its attached particles. In culture, there is insufficient time to extend the wings and deploy the feeding web. Food deprivation has been shown to function as a synergistic stressor with CO_2 (Seibel *et al.*, 2012), and thus also acts as a confounding variable in long-term studies of captive animals.

In the course of experimental work with pteropods, various methods have been used to culture animals in the laboratory, with varying degrees of success. At the time of writing, only one laboratory has successfully reared a single species of pteropod, *Limacina retroversa*, through a full life cycle. Here we report, for the first time, the methods used. A common problem in developing culture systems is the patchy and seasonal abundance of pteropods, which limits opportunities for method testing and development. As negative results are often not published, these rare chances are often wasted by unknowingly repeating the unsuccessful tests of others, for this reason we also provide a summary of the varied and often-unpublished techniques tested in pteropod culture work. The aim of the present assessment is to summarize the different approaches used and discuss their efficacy as a starting point for the much-needed further development of pteropod culturing systems.

METHOD

Collection

Pteropods are often classified as gelatinous animals in zooplankton collections and are often in a poor state when retrieved using classical net tows (Hamner *et al.*, 1975; Robison, 2004). The shells of thecosome pteropods are fragile and many of the more highly evolved groups, including the subfamily *Cavolinidae* and the pseudothecosomes, have extremely delicate gelatinous pseudoconches and extensive external mantle structures. Damage by nets becomes particularly problematic when animals are collected for experimental or culturing purposes. Thus, collection of pteropods using specialized capture methods can greatly increase the proportion of undamaged animals. Although primarily open ocean animals, in some regions the topography and hydrography allow the collection of pteropods from the shore or using small boats. In these locations, researchers have used "jelly dippers" (beakers strapped to long handles) to allow the collection of these delicate animals (Seibel *et al.*, 2007; Maas *et al.*, 2011).
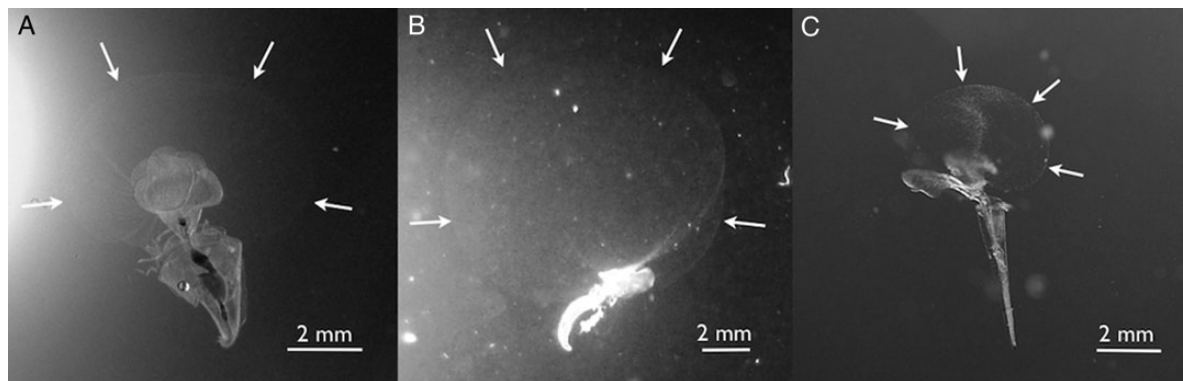
Fig. 1. Mucous web productions observed in pteropods maintained in temperature-gradient kreisel (Gorsky *et al.*, 1986). (**A**) The mucous web being retracted and (**B**) the mucous web at its maximum extension in Mediterranean *C. inflexa*. (**C**) The mucus production of the Mediterranean pteropod *C. virgula*. Photos: S. Comeau.

Hand collection can also be performed by sampling pteropods using SCUBA diving techniques (Gilmer, 1974; Fabry, 1990; Maas *et al.*, 2012a) to collect larger organisms with minimal stress. Successful collection using diving is limited by animal density and is much more time and effort intensive than net sampling (Haddock and Heine, 2005). It has, however, proven particularly useful in tropical regions (where cavolinids and pseudothecosomes are more abundant) and at night (when diel migratory individuals congregate in surface waters). Finally, a few species are exclusively meso- or bathy-pelagic, and gentle collection of these animals is particularly difficult. Such species have been sampled using remotely operated vehicles (Seibel *et al.*, 2007) and using a net with a thermally isolated cod end that closes at depth (i.e. Childress *et al.*, 1978), maintaining the animals in water from the depth of collection until retrieval.

Plankton net tows remain the most widely used for collection since they are relatively easy to operate and offer a variety of mesh sizes and designs (e.g. Bongo, WP-2, MOCNESS). Many pteropod species can be captured using >330-μm mesh nets, although small species and juveniles may be better sampled using 153-μm mesh or less (Lischka *et al.*, 2011; Bednaršek *et al.*, 2012b). Plankton tows have the advantage that they enable the user to easily collect large numbers of pteropods, including small individuals (<500 μm) and/or individuals that occur at great depths (>50 m). The disadvantage of damage by nets to both the soft tissues and the shell for work with live animals can be minimized if ship speeds of <1 knot are employed in conjunction with short duration, relatively vertical tows (Bednaršek *et al.*, 2012a). The use of a specialized cod end such as an extra-large cod end (Ikeda and Mitchell, 1982; Bouquet *et al.*, 2009) or thermally protected cod end (Childress *et al.*, 1978) can further limit physical and thermal stress.

After collection, pteropods should be inspected to ensure that the shell and soft tissue have not been damaged during collection. Damaged organisms must be discarded in order to avoid subsequent bacterial infection and the contamination/death of the whole population. Following inspection, it is recommended to immediately transfer the organisms into a large volume of filtered sea water at *in situ* temperature from the site of collection.

Water treatment

Pteropods produce mucous coverings, mucous webs and pseudofaeces, and rejected particles, expelled in mucous strings (Gilmer and Harbison, 1986), during feeding and buoyancy maintenance. These structures can attract bacterial populations that, in turn, can impact experiments and contaminate laboratory cultures. To deal with these issues researchers have used a combination of filtration, antibiotics and ultraviolet (UV) treatment of sea water (Comeau *et al.*, 2009, 2010b; Lischka *et al.*, 2011; Maas *et al.*, 2012a). Additionally, 0.5-mg L^{-1} disodium ethylenediaminetetraacetate (EDTA) can be used to chelate toxic metals from the sea water, even in areas where local water sources are relatively clean. Egg clutches/strings and juveniles are particularly susceptible to bacterial infections, and successful rearing through to adult stages is only possible with rigorously clean techniques using antibiotics and careful handling procedures (see below). For adult cultures, water filtration through <5-μm membrane filters is recommended as this reduces fouling and entanglement in aggregations of mucus and detritus; juvenile cultures require filtration using 1-μm filters.

To test directly whether antibiotics influenced the metabolism of pteropods, single adult *Limacina helicina* was held in glass syringe respiration chambers either treated or untreated with streptomycin and ampicillin (each

25 mg L^{-1}). During each experiment, a control syringe, without an animal, was simultaneously run as a control. After $\sim 10-15$ h, their oxygen (O_2) consumption (μmol g^{-1} h^{-1}) was measured using the methods detailed by Maas *et al.* (Maas *et al.*, 2012a). There was a no significant effect of exposure to antibiotics for a period of ~ 12 h (ANCOVA $F_{1,15} = 0.098$, $P = 0.760$) on the metabolic rate of *L. helicina* (Fig. 2). These results indicate that antibiotics may be an effective way to control bacterial activity during short duration respiration runs without physiological side effects. This is also particularly useful when oxygen consumption is being measured at warmer temperatures ($>10°C$) and longer duration experiments (>8 h) where bacterial respiration can be significant.

UV and microwave sterilization may also be used to sterilize culture water against fungi and viruses as well as bacteria. UV treatment is also more appropriate for use with large volumes than with antibiotics that need to be regularly replenished. A major drawback of UV sterilization, however, is its inefficiency in closed, recirculating systems (Spotte and Adams, 1981). In the Ny Ålesund Marine Laboratory, sea water is UV treated and 20-μm filtered, thus UV sterilization has been used for a number of pteropod perturbation experiments (Comeau *et al.*, 2009, 2010b; Lischka *et al.*, 2011). To our knowledge microwave sterilization has never been used in conjunction with pteropod culturing.

Aeration and gas bubbling

Gas bubbling is often used for aeration or to control the carbonate chemistry in ocean acidification perturbation experiments; however, it can also cause shell damage; particularly in juvenile pteropods. Thus, in closed vessels the bubbling should be performed before addition of pteropods, while in open vessels, bubbling should be made in such a way that the animals are not in direct contact with the stream of bubbles. This can be achieved
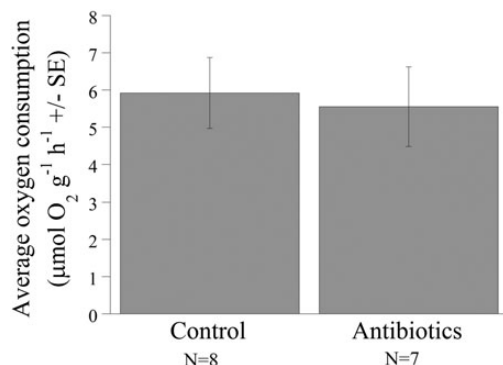
by bubbling within a vertical tube placed in the vessel, thereby offering excellent control of the carbonate chemistry while also gently stirring the media. Veliger shells are hydrophobic (Gallager, personal observation), which can cause them to be trapped at the surface during bubbling or for gas bubbles to attach to the shell, causing buoyancy issues, in this instance the surface of the air−water interface can be covered with a few granules of polyvinyl alcohol, which forms a non-toxic surface coating eliminating the hydrophobic/hydrophilic interface. If bubbling occurs from a compressor, a series of filters decreasing in porosity to 0.45 μm should be used to avoid introducing airborne contaminates to the cultures. In humid conditions, air should be dehydrated using $CaCl_2$ or silica gel (Utting and Spencer, 1991).

Diet

To date, very little experimental work has been undertaken to establish the appropriate dietary regime for cultures. Previous studies utilizing pteropod cultures have used enrichment of phytoplankton monocultures (Comeau *et al.*, 2010a). Alternatively, cultures have not been enriched with food (Lischka *et al.*, 2011) or *in situ*, unfiltered sea water is used. The use of motile species ensures that the entire water column remains evenly saturated with food with minimal agitation of culture vessels.

Successful cultures of *L. retroversa* have been maintained from juvenile stages through to adulthood with a mixed algal diet. Based on feeding studies with artificial microspheres (unpublished data), adults and juveniles were capable of feeding on particles 0.5 −30 μm in diameter. Accordingly, the flagellate *Isochrysis galbana* (clone T-ISO) and a cryptophyte *Chroomonas salina* (clone 3C) were used for larval and metamorphosing juvenile culture (Table I). Concentrations of 1×10^4 cells mL^{-1} are suitable for culture densities of ~ 1 veliger mL^{-1}. As parapodia developed, the dinoflagellate *Ceratium longipes* was added in addition to the two other species of microalgae. This dinoflagellate was used because of the frequent association of *C. longipes* and *Limacina* in the field (Gallager *et al.*, 1996). *Ceratium longipes*, *C. salina* and the flagellate *I. galbana* at a final concentration of 5×10^4 cells mL^{-1} are an optimal combination of microalgae for juveniles and adults.

Food density was also found to be important during feeding trials using *L. helicina* juveniles to assess mixed algal diets at varying concentrations (Table I). Two cultures were maintained with high food concentrations (5×10^4 cells mL^{-1}) and one with low food concentration (1×10^4 cells mL^{-1}). Animals incubated in low food concentrations survived for 21 days, whereas animals in the high food concentrations survived for 37 days and

Fig. 2. The effect of antibiotics (25 mg each of streptomycin and ampicillin L^{-1}) on the respiration rate of *L. helicina*.

Table I: Summary of dietary regimes used in thecosome pteropod culturing

Diet	Algal species	Conc.	Pteropod species	Survival (days) and mortality (%)	Culture system	Reference
Unfed	–	–	*L. helicina* (j)	29 ~25% ~57% ~68% mortality	440 mL Jars, 3°C 5°C 8°C	Lischka *et al.* (2011)
Unfiltered seawater	–	–	*L. helicina* (j)	8 6% 8% 9% mortality	125 mL bottles, 1.6°C pH 8.05 7.90 7.75	Comeau *et al.* (2012)
Single algal species	*Isochrisis galbana*	–	*C. inflexa* (j)	13 70% mortality	1 L bottle, plankton wheel, 13°C	Comeau *et al.* (2010a)
Mixed algal diet	*Tetraselmis* spp., *Skelotonema* spp., *Gymnodinium simplex*, *Amphidinium carterii*, *Rhodomonas salina* *Pavlova*	1000 cells mL^{-1}	*L. helicina* (j) 100–2800 μm	5, 100% mortality	Stirred batch 2 μm filtered seawater, ~7°C	Doubleday, (personal observation)
Mixed algal diet	*Tetraselmis* spp., *Skelotonema* spp., *Gymnodinium simplex*, *Amphidinium carterii*, *Rhodomonas salina* *Pavlova*	>50 000 cells mL^{-1}	*L. helicina* (j) ~500 μm	40, 50% mortality	Stirred batch 2 μm filtered seawater, ~7°C	Doubleday, (personal Observation)
Mixed algal diet high conc.	*I. galbana, Chaetoceros calcitran Pavlova*	>50 000 cells mL^{-1}	*L. helicina* (j) 100–200 μm	37, 100% mortality	Stirred batch 2 μm filtered seawater, ~7°C	Doubleday, (personal observation)
Mixed algal diet low conc.	*I. galbana, Chaetoceros calcitran Pavlova* *I. galbana, C. salina* *I. galbana, C. salina, C. longipes*	1000 cells mL^{-1} 1000 cells mL^{-1} 50 000 cells mL^{-1}	*L. helicina* (j) 100–200 μm Larvae *L. retroversa* *L. retroversa*, adult, juvenile	21 100% mortality to maturity	Stirred batch 2 μm filtered seawater, ~7°C 2 L flasks, 10°C 1000 L tubes, 10°C	Doubleday, (personal observation) Gallager, (personal observation) Gallager, (personal observation)

For pteropod life stages: (j) juveniles, with shell diameters where available. It should also be considered that many of the methods listed were employed as part of perturbation experiments, therefore other stressors outside of diet regime may have contributed to mortality.

ciliary feeding was observed. These results suggest similar optimal algal concentrations for the two *Limacina* species.

Culture methods

A wide range of approaches have been used to maintain pteropods for experimental purposes. They can be grouped into three broad categories: batch cultures (with various modifications; agitation, tethering) (Table II), flow systems (circulating and flow through) (Table III) and mesocosm systems.

Closed systems

Small individuals (<1.5 mm) such as *Limacina inflata*, and juveniles of *L. helicina* (>5 mm) or *L. retroversa* can survive reasonably well in closed batch cultures. These small individuals can be maintained in smaller closed flasks with no water changes for up to 29 days at 3, 5.5 and 8°C (*L. helicina*, for percentage mortality see Table II) (Lischka *et al.*, 2011), and 7 days at 14°C (*L. inflata*) (Howes, personal observation). Adults and large species, however, appear to require much larger volumes for successful rearing. An appropriate culture vessel size is difficult to define, although the greater the water column depth the better the chance of mucous web production.

Often if the culture vessels are not gently swirled or shaken periodically, food particles, mucus and/or pseudofaeces collected at the bottom of the vessel then become entangled around pteropods incubated in the batch cultures, contributing considerably to mortality (Bednaršek *et al.*, 2012a). As with most culturing systems, particulate waste should be gently siphoned out routinely, along with periodic water changes. To maintain pteropods in suspension, tests have explored the effects of gently agitating cultures, either using external methods to move the whole vessel or by placing stirrers directly into the culture (Fig. 3 and Table II). While agitation methods work well for rearing egg clutches and juveniles (Table II) (Comeau *et al.*, 2010a) due to their light weight and slow sinking speed which allows them to be maintained in constant suspension, these techniques have been less successful for maintaining cultures of adults or juveniles >3 mm in size. Several of the agitation methods tested did appear to improve adult buoyancy, however, this was partially due to the pteropods swimming continually against the water currents precluding successful feeding behaviour. Alternatively, stirring can tumble pteropods either across the bottom of the culture vessels or into each other, and also cause an accumulation of mucus and detritus at the bottom of the culture vessel that can entangle

individuals. Vibrations produced by some of the systems also appeared to disturb the animals.

To avoid some of the pitfalls of agitation, individual pteropods have been attached to mounted needles, pre-fixed in culture vessels, using gel-based superglue (Table II, Fig. 4). This method allows the use of smaller volumes of water and less space requirement when culturing adults. Great care and dexterity are needed to avoid damaging the fragile pteropod shell, the process is time consuming and likely very stressful. The success of the method depends greatly on the shell shape: those species with simple, straight or conical shells responded better than those with more complex shell forms. This can probably be attributed to the animals being fixed at slightly the wrong orientation. Tethered animals were never observed feeding. To date, the most successful culture of pteropods has been accomplished using a closed system with regular water changes, developed over eight separate attempts at culturing. In overview, egg strands of *L. retroversa* were collected from adults already in culture for several weeks. Larval development at 10°C in the egg capsules took ∼10 days, followed by ∼40 days for the veligers to reach metamorphic competency and begin to develop parapoda typical of the juvenile and adult. Metamorphosis took ∼10 days to make the complete transition and loss of the velum. Development from metamorphosis to reproductive adult took ∼40–50 days (Fig. 5). Egg strand production then began 90 days after hatching and continued until the F1 generation began to die out. After 110 days high mortality occurred and by 117 days all of the adults had died. Complete generation time from reproductive adult to reproductive adult was around 90–110 days at 10°C. Several F2 generations were again raised to reproduction.

To begin, the overall process adults were maintained for ∼4 weeks in the laboratory in either 1000 L tubes or large tanks. Egg masses were separated by screening adult culture water through a 200-μm Nitex screen, rinsed and re-suspended in 2000 mL Erlenmeyer flasks in 1-μm filtered sea water. Water in the ova cultures was aerated at ∼100 mL min^{-1} with 1-μm filtered air and not changed during the ova incubation period. Before hatching, as the ova strands darkened, eggs were treated to reduce bacterial and fungal contamination for 30 min with 2-mg L^{-1} polyvinylpyrrolidone iodine complex (PVP-I, Sigma Chemical) sea water solution whose pH has been adjusted to ∼8.3 with Tris−HCL buffer (Trizma, pH 8.3). The egg masses were then treated with 0.1-M sodium thiosulphate to reduce the iodine to iodide prior to re-suspension in culture flasks prepped with antibiotics, EDTA and algal mix. Gentle agitation of flasks might also be useful in keeping egg masses off the bottom, minimizing the risk of bacterial infection

Table II: Summary of variations in batch techniques attempted

Technique description	Species	Survival (days) and mortality (%)	Reference	Comments
Batch: 440 mL jars, 10 individuals. Unfed. 3, 5, 8°C	*L. helicina* (j)	29 ~ 25%, 57%, 68%	Lischka *et al.* (2011)	–
Batch: 1l L, opaque, polyethylene jars. 1–6 individuals	*C. pyramidata*	≤1, 0%; short	Fabry (1989)	Feeding not observed
	L. helicina	incubation	Fabry (1990)	
Batch: 1 litre Nalgene bottles, ≤15 individuals, −2°C	*L. helicina*	13, 0%	Maas *et al.* (2012a)	Feeding not observed
Batch: 2 L Schott bottles (opaque)	*L. helicina* ant.	14	Bednaršek *et al.*	*L. inflata was* not observed feeding, but guts
2 L Schott bottles 14°C	*L. inflata*	3, 5%	(2012a)	were full when inspected with a binocular
			Moya *et al.* (in prep)	microscope
20 L, 0°C, 4°C	*L. helicina*	2, 0%; short incubation	Comeau *et al.* (2010b)	Feeding not observed
1000 L tubes, 10°C	*L. retroversa*	To reproduction	Gallager (personal observation)	Webs not observed, but guts full
50 000 L mesocosm (KOSMOS units)	*L. helicina* (a)	7, 95%	Riebesell *et al.*	Floating animals were observed
		28, 99%	(2013)	
77 000 L mesocosm (KOSMOS units) (Fig. 6)	*L. retroversa* (a,j,l)	40, 50%	Büdenbender *et al.*, in prep	All life stages present until the end of the experiment
Larvacean system:	*L. helicina*	5, 0%	Comeau *et al.* (2009)	Technique effective for small individuals, egg
Plastic stirrers rotate (<10 rpm) in round culture vessels (e.g. 20 L beakers).	*C. inflexa* (e, j)	40, 50%	Doubleday (personal	clutches and larvae but failed to maintain
5°C		10, 100%	observation	pteropods >2 mm; should be used
7°C			Howes (personal	preferentially with individuals of reduced
15°C			observation)	sizes
Rollers 6 rpm: (Fig. 5A)	*L. inflata*	7, 60%	Howes (personal	Slow speeds were more suitable for
Incubation 14°C in 2 μm filtered seawater in 2 L glass bottles enriched with *I. galbana*. Bottles sealed with parafilm, ensuring no air bubbles. Every second day water was changed by gently pouring through a 100-μm sieve. Inspection and removal of dead individuals			observation)	maintaining cultures
Rotatory shakers (varying speeds) (Fig. 5B): Preparation as described for rollers	*L. inflata*	7d, 60%	Howes (personal observation)	Slow speeds were suitable for maintaining cultures.
Plankton wheel (Fig. 5C)	*C. inflexa* (e, j)	13, 70%	Comeau *et al.*	Successful for individual <3 mm
1 L glass bottles prefilled with 0.2 μm-filtered seawater. Partial water change every second day, 13°C			(2010a)	
Tethering: Rapid setting gel-based glue used to attach pteropods to needle. Needles	*C. acicula*	7, 100%	Howes (personal	Stressful and time-consuming attachment
and pteropods introduced to seawater (14°C) immediately after attachment	*C. inflexa*	2, 100%	observation)	procedure
(Fig. 8). Disposable pipettes used to keep pteropods wet at all stages of	*C. pyramidata*	1, 100%		Response species specific
procedure. Fed with *I. galbana*	*S. subula*	5, 100%		Feeding not observed

For pteropod life stages employed: (a) adults (j) juveniles, (l) veliger larvae, (e) eggs. Note that the survival time represents the point at which the experiments were concluded and does not represent the time at which all individuals died. It should also be considered that many of the methods listed were employed as part of perturbation experiments, therefore other stressors outside of the culture environment may have contributed to mortality.

Table III: *Summary of variations in flow systems*

Technique description	Species	Survival (days) and mortality (%)	Reference	Comments
Temperature-gradient plankton Kreisel (Fig. 3)	*C. inflexa* *C. virgula*	7	Gorsky *et al.* (1986)	Successful feeding observed at high algal concentrations
Up flowing (Fig. 6A) Main body cylindrical water inflow at the base, positioned under 100 μm mesh, to disperse the flow evenly. Mesh at the top of the water column prevents animals being washed out	*C. inflexa*	3, 100%	Howes (personal observation)	Animals sunk beneath the lowest current and struggled to swim back up through water flow. Use of meshes traps biofilm and pteropods. Requires exact flow rate control
Circular water current (Fig. 6B): Circular current provided by water inflow at top of kreisel	*C. acicula*	2, 100%	Purcell *et al.* (2013)	System did not provide enough flow to maintain buoyancy and water column too small to allow production of mucous web
Circulating upward flow (Fig. 6C) Main body is cylindrical. Three equally spaced inflows running vertically down one side. Each inflow fed by its own tube allowing the speed of the flows to be differentially controlled	*C. acicula*	3, 70%	Graeve (unpubl.), Howes (personal observation thesis)	System was more successful for the smaller individuals, <1 cm, and one was observed with mucous feeding web. Once settled, they struggled to swim up the water column through water flows
Bi-directional elliptical flow (Fig. 6D) Rectangular design. Two elliptical flows: one from the top of the tank flowing vertically and a second from one end, flowing horizontally	*C. acicula*	3, 80%	Purcell *et al.* (2013)	The flows did not maintain the animals in suspension and forced them to swim continually or sink to the bottom
110 L aquaria: 110 L V-shaped aquaria (40 × 40 × 70 cm) ca. 80 individuals	*L. helicina*	14, 90%	Böer *et al.* (2006)	Feeding not observed Water column too short, sinking time ~15 s
600 L system: (Fig. 7) Horizontal circular current, constant flow (10 L min^{-1}) 20 μm filtered water directly pumped from Kongsfjord 100 individuals per tank	*L. helicina*	14, 50%	Büdenbender (personal observation)	Feeding not observed Animals were observed floating in water column Large volumes of water unsuited to perturbation experiments

For pteropod life stages: (j) juveniles, (e) eggs. Note that the survival time represents the point at which the experiments were concluded and does not document the time at which all individuals died.
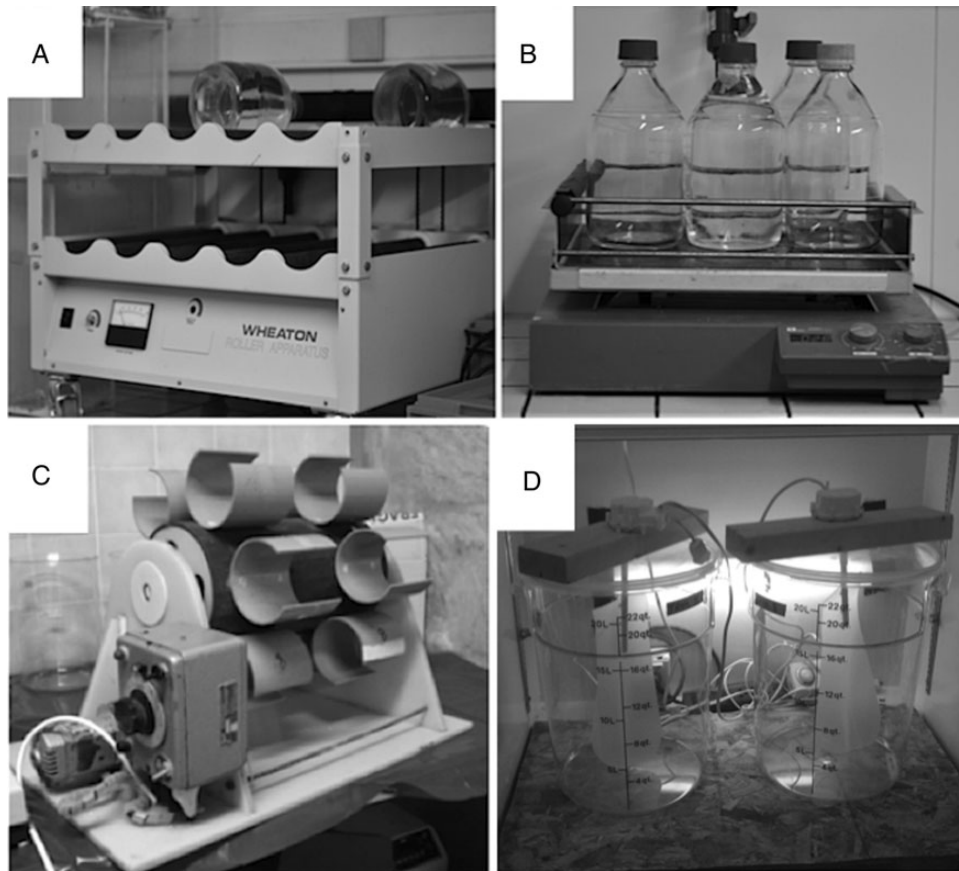
Fig. 3. Agitation methods: (**A**) rollers with sealed 2 L bottles containing *L. inflata* specimens. (**B**) Gyratory shaker with 2 L bottles containing *L. inflata* cultures. (**C**) Plankton wheel used for the culturing of *C. inflexa* egg clutches. (**D**) The larvacean system, used for appendicularian culturing. In this system, the organisms are maintained in suspension by creating a gentle current using plastic paddles that rotate slowly (<10 rpm) (Comeau *et al.*, 2009).
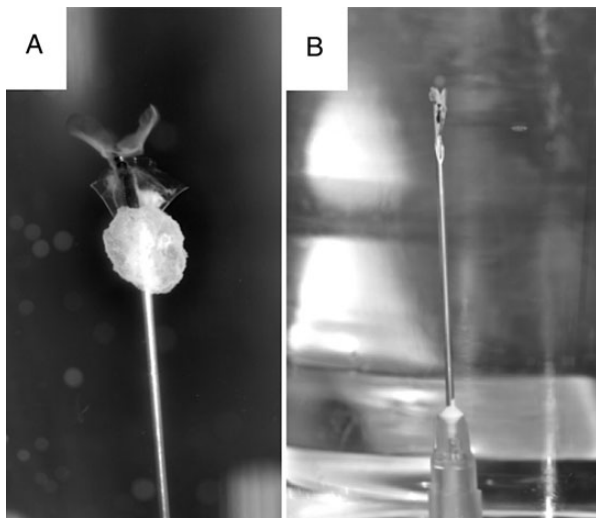

Fig. 4. Tethering method using needles and superglue. (**A**) *C. pyramidata*. and (**B**) *S. styliola*.

(Comeau *et al.*, 2010a). To avoid entrapment at the surface, the flask was filled to near overflowing and covered with Parafilm to eliminate air bubbles.

Initially, ∼1 veliger mL^{-1} of culture water was considered optimal. Algae were added to the cultures to achieve a final concentration of 1×10^4 cells mL^{-1}. Flasks were inverted several times a day to mix algae and larvae. Cultures were screened using a 100-μm mesh and changed weekly at a minimum, and optimally every 3 days. At each culture water change, larvae were inspected and dead individuals removed using two drops of a 1:24 dilution of surgical scrub surfactant (Purdue Frederick, Norwalk, CT, USA) to re-suspend and facilitate handing the hydrophobic shells. Larvae were subsampled for measuring as necessary (typically 10–30 larvae were preserved per flask in 1% buffered formalin), and then concentrated into the centre of the dish by swirling. The larvae were then pipetted directly into a freshly prepared culture flask containing filtered sea water, algae and antibiotics. This
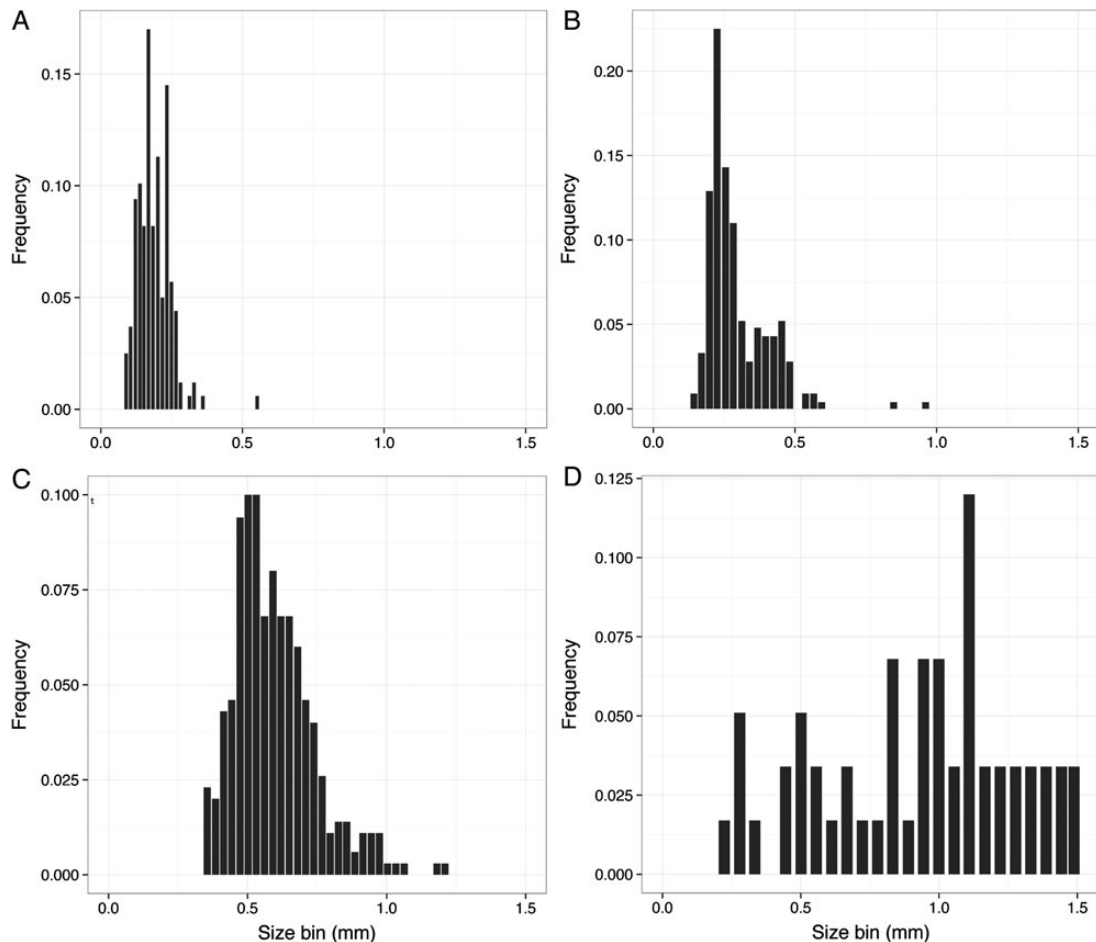
Fig. 5. Size distributions of juvenile and adult *L. retroversa* cultured at 10°C. (**A**) Metamorphosis, 50 days post-hatch, mean shell length 0.262 mm. (**B**) Sixty-two days post-hatch, mean shell length 0.459 mm. (**C**) Eighty days post-hatch, mean shell length 0.693 mm. (**D**) Ninety-eight days post-hatch, mean shell length 1.370 mm.

process minimized carryover of the surfactant from the glass dish. The flask is then sealed as before with parafilm or polyvinyl alcohol. As larvae developed and approached metamorphic competency, densities were reduced to 1 individual 10 mL^{-1}, and once metamorphosed, each flask of juveniles was transferred to 50-L culture vessels.

Recirculating systems

Variants on the original design of the plankton kreisel (Greve, 1968) have been used successfully to culture a variety of gelatinous zooplankton species (Dawson, 2000; Purcell *et al.*, 2013). A range of kreisels with different flow patterns have been tested for pteropod culture including circular, up-flow and multidirectional flow patterns (Fig. 6) (Table III). Notably, one of the only observed instances of pteropods producing mucuos webs under laboratory conditions (Fig. 1) was obtained using a variant of the "planktonkreisel" (Fig. 6E) (Gorsky *et al.*, 1986).

Kreisel systems typically use large volumes of water, and the movement produced by the currents gives buoyancy aid. The flow rate appears to be critical, and very fine control is required to keep the animals in suspension. If the flow is too strong, the pteropods will swim continuously, preventing feeding and resulting in rapid depletion of energy reserves. If too weak, it does not counteract the negative buoyancy of the shell and the animals come in contact with the sides and bottom of the vessel, causing shell damage and excess mucus production.

Flow-through systems

Flow through, or single-pass systems, utilize water currents and flows in the same way as circulating systems; however, in these aquaria, when the overflow water leaves the aquaria it is discarded. Gymnosome pteropods have been successfully maintained in large numbers (ca. 80 individuals) for 365 days using a 110-L, V-shaped
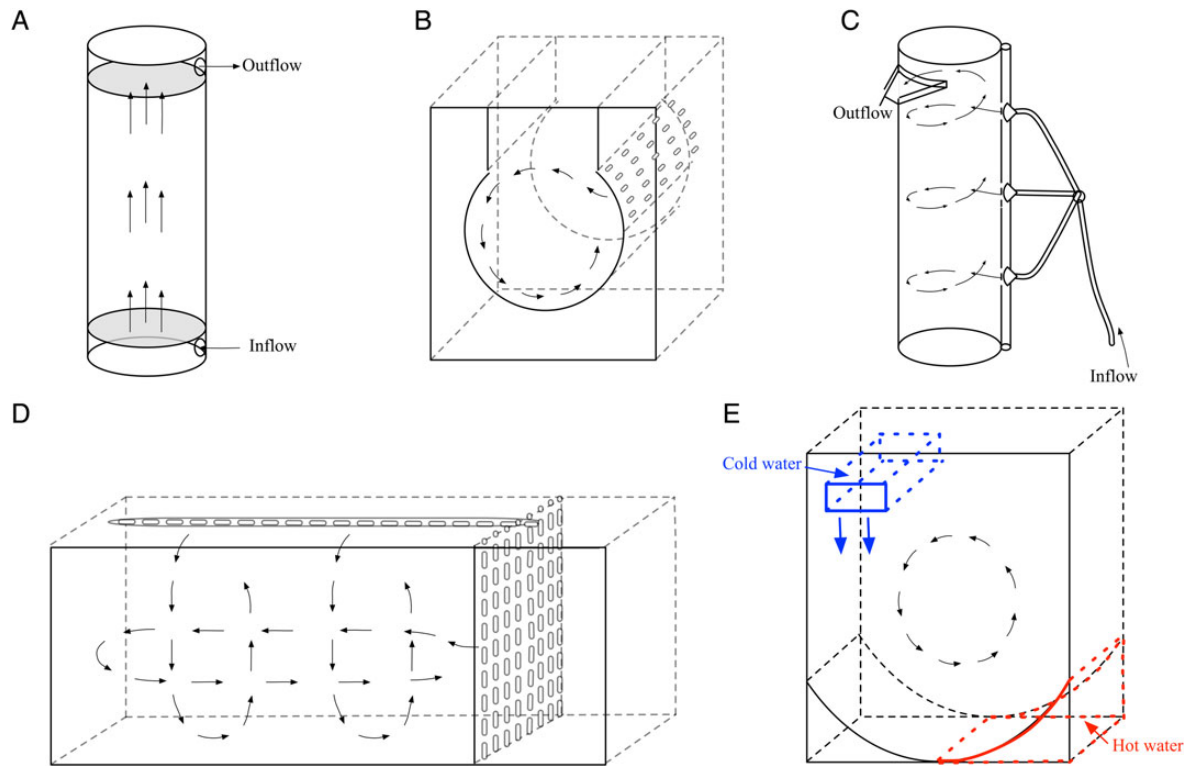
Fig. 6. Representations of the water flow kreisel systems, direction of water current represented by arrows. (**A**) Upflow system designed in Villefranche-sur-Mer (Mahacek, Moya and Howes). (**B**) Circular flow system used for ephyrae culture at Institut de Ciences del Mar, CSIC, Barcelona (Purcell *et al.*, 2013). (**C**) Circular upflow kreisel designed at Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research Dr Martin Graeve. (**D**) Multidirectional jellyfish kreisel, Institut de Ciences del Mar, CSIC, Barcelona (Purcell *et al.*, 2013). (**E**) The temperature-gradient plankton kriesel (Gorsky *et al.*, 1986). The water current (black arrows) is generated by the difference in water density between low density water on one side of the tank on the bottom (created by warming up the water) and high density water on the top of the tank on the opposite side (created by cooling down the water).

aquaria (40 × 40 × 70 cm) (Lange and Kaiser, 1995) with constant circulation, to keep the animals swimming (Böer *et al.*, 2007). The same system was tested with *L. helicina*: the sinking from surface to the bottom of the aquaria was ∼15 s and after 14 days only 0–5 of 100 individuals survived.

Larger flow-through systems have been more successful. In the Marine Laboratory in Ny-Ålesund adult *L. helicina* were reared in 600-L flow-through system (10 L min^{-1}) (Table III) successfully for 14 days at 100 individuals per tank; mortality was <50%. A horizontal circular current transported the pteropods extending the time from surface to bottom by up to 1 min during which no swimming activity or interface collision occurred. Mucous feeding structures were not observed.

Mesocosm systems

The presence of a large water column, which can allow time for animals to deploy their mucous webs, makes mesocosm system candidates with high potential for successful pteropod culturing. To our knowledge, there have only been a few but promising attempts in this direction (Table II).

In 2010, nine KOSMOS mesocosms were deployed for 35 days in a high-arctic Fjord. The mesocosms contained ∼50 m^3 of natural fjord water, the enclosed water column was 15-m deep (Riebesell *et al.*, 2013). Controlled quantities of pteropods collected using jelly dippers were added to each mesocosm (4 individuals per m^3). Most died during the first 2 weeks due to a design shortcoming that trapped pteropods in the dead volume below the built-in sediment trap or in the sediment trap itself (Czerny *et al.*, 2012; Riebesell *et al.*, 2013). Only single individuals survived for the full period of the mesocosm deployment (30 days).

During the 2011 Bergen mesocosm campaign, nine KOSMOS units (Fig. 7) of ∼77 m^3 volume and the total bag length of 25 m were deployed in the Raunefjord close to the Espegrend Biological Station, Bergen, Norway. *Limacina retroversa* individuals of all life stages were part of the natural plankton community and were enclosed in the mesocosms during the filling process making the stressful collection process unnecessary.
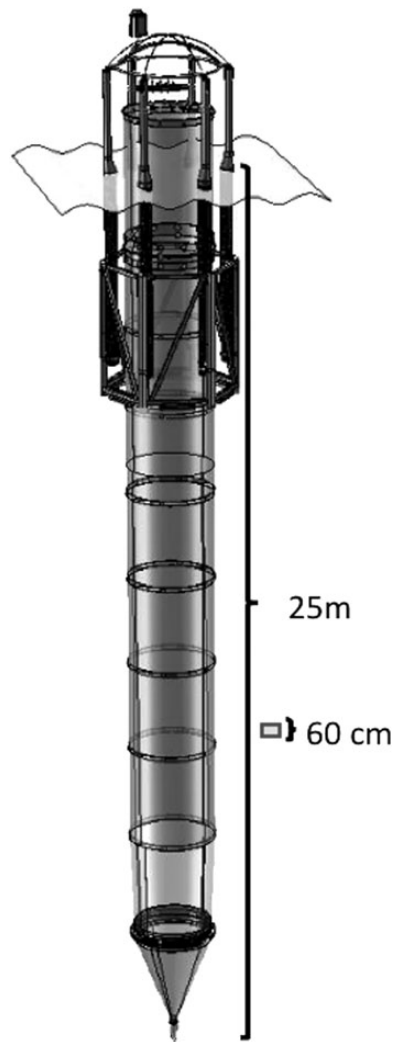
Fig. 7. KOSMOS system deployed for 40 days in Raunefjord Bergen Norway 2011, enclosing a natural plankton community including *L. retroversa*. Mesocosm drawing by D. Hoffmann.

Limacina retroversa as well as potential predators (e.g. *Clione limacina* and fish larvae) remained present for the full experiment (40 days) (Büdenbender *et al.*, in prep.). Mortality rates of veliger larvae under control conditions were ~50%.

DISCUSSION

Species selection

In general, when culturing thecosomes it seems that the size of the animals and the size of the culture vessels are both important. Although small species/juveniles (<1.5 mm) can be cultured in smaller vessels, larger species/adults require larger water volumes. Species of *Limacina* seem to be more robust than other body forms,

with *L. retroversa* reared through larval stages to F3 generation under laboratory conditions. Healthy cultures of *L. inflata* have been maintained for at least 7 days in low densities (fewer than 10 individuals L^{-1}) in simple closed containers. Also, the slower metabolic rate at colder temperatures for polar Limacinidae allows longer survival when individuals are unable to feed (Hopcroft, personal observation).

Cavoliniidae are more problematic, possibly due to the increased shell and mantle complexity making them more prone to damage. Tethering methods showed some early promise when applied to Cavoliniidae species with simple conical shells, while some success has been reported with *Creseis virgula* and *Cavolinia inflexa* in the temperature-gradient plankton kreisel. Future attempts to improve culture systems should take into account the fact that the different forms respond differently to the same culture vessels; one size does not fit all. With the goal of performing successful perturbation experiments, effort should focus on developing techniques for a several key and widespread cosmopolitan species.

Larval rearing

The use of eggs laid in culture has been the only method that has successfully reared reproductive adults and refining this technique for polar species, e.g. *L. helicina*, and adapting it for Cavoliniidae should be a priority for future work. Whilst *L. retroversa* is relatively robust, there may also be advantages of working with F2 generations. The egg masses are not subjected to the rigours of sampling from the wild and can be kept in the most sterile conditions possible, limiting the risk of bacterial infestations. Experimentalists are able to work with many individuals of the same life stage that are already acclimatized to culture conditions. Successful work has already been undertaken using the egg clutches of *C. inflexa* (Comeau *et al.*, 2010a), with clutches raised through veliger stages to metamorphosis with low mortality. However, once individuals reach sizes of >1 mm, culture methods must be adapted, as mortality rapidly decreases populations by up to 50% in 24 h. Large adults can be induced to mass spawning in the laboratory when held in crowded conditions or exposed to mild heat or light stress (Howes, personal observation) making them good candidates for further work with careful application and adaptation of the methods presented here for *L. retroversa*.

Vessels

Batch cultures are easy, non-labour intensive and cheap for pteropod cultivation, but are much more suitable in small volumes for juveniles and small species than for

large adults as demonstrated for *L. retroversa*. With refinement, batch culturing should allow for easy experimental replication and could be implemented under field conditions. Nonetheless, fully closed batch culturing has its drawbacks: changes in sea water chemistry generated by biological activities (i.e. changes in oxygen availability, carbonate chemistry and the accumulation of potentially lethal nitrogenous wastes), along with the accumulation of faecal wastes and bacteria. Small closed volumes are also handicapped by increased contact rates with the sides or the base of the culture vessel. General husbandry of culture vessels and water changes appear critical to rearing success. In this regard, recirculating systems such as plankton kreisels or flow-through systems may offer some intermediate level of self-sufficiency albeit with higher cost and effort. With some specializations, manipulations of temperature and water chemistry are possible with kreisels and flow-through systems, e.g. as implemented in the indoor mesocosm facility at GEOMAR, Kiel.

Mesocosms such as those employed in the 2011 Bergen campaign show potential for new insights into pteropod ecology, particularly when tested with smaller *Limacinid* species. Mesocosms can provide conditions in which populations/species of pteropods are able to reach a state of neutral buoyancy and are able to feed with a mucous web on their natural food sources in a close to *in situ* environment. There is a need for new instruments, which allow *in situ* observation and controlled capture of single individuals within these systems due to their large body size/volume to water volume ratio (1:5.5 billion) (e.g. volume based camera systems and remotely operated sampling devices like small ROV's). Mesocosms like the KOSMOS system, due to their size, provide the most realistic and favourable conditions for pteropod culturing. As demonstrated in the 2010 experiments, the size and design of mesocosms must be taken into careful consideration in relation to the size and sinking rates of the pteropod species. Mesocosms with built-in sediment traps should be avoided as they act as a one-way gate, trapping animals at the bottom of the mesocosm (Büdenbender, personal observation). The labour, cost and logistics of setting up mesocosms are major drawbacks for experimental work where several treatments and replicates are required; the costs can, however, be greatly reduced by using pre-existing facilities such as the Espegrend biological station or by co-operation with existing projects. Further development of this work should focus on testing with non-polar or Cavoliniid species as it is not yet known how these less robust forms will respond to mesocosm conditions.

The common problem for all thecosome species is buoyancy and, as a direct result, feeding behaviour. As described above, many flow systems destabilize or trap animals and even the use of much larger culture vessels does not allow adequate sinking space. In a range of culture vessels used by Gallager with *L. retroversa*, healthy adults and juveniles of various species tended to congregate at the surface. Surface aggregation may have been a response to gravity, illumination, tank currents or food, with individuals observed vigorously swimming upwards and across the surface, followed by drifting downwards at various rates, usually quite slowly.

In the wild, pteropods undergo diurnal vertical migration (DVM), for some species of >300 m, which has been suggested to be linked to control of metabolic rate (Wormuth, 1981; Lalli and Gilmer, 1989; Maas *et al.*, 2012b). The migratory behaviour might account for sinking and could exacerbate feeding difficulties experienced by culture specimens. The development of culture vessels, dark incubations, opaque tanks or graduated opaque tanks with darker bases and transparent tops, might exploit the natural upward migrations during dark hours and reduce sinking periods in laboratory animals. Variegated mesocosms have been used to culture jellyfish found in meromictic lakes (Dawson, 2000) and such an introduction of a false halo, thermo or chemocline might counteract the downward migrations of the pteropods or satisfy metabolic needs related to DVM.

Diet and feeding

As a result of abnormal feeding behaviour in many culture vessels, it is unclear whether inadequate diet may be a further stressor for laboratory animals; several taxa of gelatinous zooplankton require mixed diets when maintained in artificial environments (Raskoff *et al.*, 2003). Sub-optimal food might account for the relatively slow growth rates encountered during culture of *L. retroversa*. Many other commonly cultured zooplankton genera can only achieve optimal fitness when provided with specific diets (Demott and Müller-Navarra, 1997; Sommer, 1998; Knuckey *et al.*, 2005). Gut content analysis of wild-caught, adult *L. helicina* indicates an omnivorous and, often, cannibalistic diet with juvenile life stages apparent in the gut contents (Gilmer and Harbison, 1991). Small disturbances to the mucous web provoke rapid ingestion and it has been hypothesized that adults may, in fact, be mucous trappers, using their webs as a spider would (Gilmer and Harbison, 1991). The guts of juveniles and smaller (<2.6 mm shell diameter) individuals contained only phytoplankton (Hopkins, 1987), suggesting a possible dietary shift in adult life stages. These *in situ* observations suggest that the mechanical feeding difficulties experienced by thecosomes in laboratory cultures of adults may be compounded by sub-optimal diets. The species selected for algal diets should
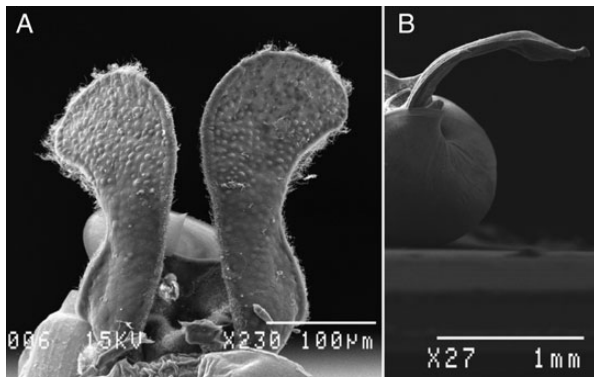
Fig. 8. (**A**) Long cilia fringing the periphery of the parapodia of *L. retroversa* (arrow). (**B**) Food groove travelling around the circumference of the parapodia of *L. retroversa* (arrow).

be tailored to the size and developmental stage of the culture individuals. Algal species that occur concurrently with the culture species of pteropods should be used where possible.

Observations of cultured *L. retroversa* have shown periods of negligible and sometimes slightly buoyant drift usually associated with web production. However, at no point during culture to the F3 generation were feeding webs observed, although phytoplankton was present in the guts of all life stages. The same phenomenon was also observed in healthy cultures of *L. inflata* (Howes, personal observation) and *L. helicina* (Doubleday, personal observation). Close examination of the parapodia of tethered *L. retroversa* indicated that the edges of the parapodia were fringed with several sets of cilia (Fig. 8A). Long cilia ($\sim$30 um) beat at a rate of $\sim$15 Hz and together produced sufficient lift to keep the organisms floating with the parapodia extended but not moving. The longer cilia also provided flow to carry food particles past the parapodia edge where they were captured by the shorter cilia. These shorter cilia ($\sim$5 μm) surrounded the parapodia and formed a food groove (Fig. 8B) that transported particles to the mouth. Particles were observed moving at $\sim$20 μm s^{-1} in the food groove towards the mouth where some form of particle selection occurred. Feeding appeared to occur when the parapodia were extended and not moving as the organism slowly drifted about as if it were neutrally buoyant. Yonge (Yonge, 1926) proposed ciliary feeding in pteropods and it was later described, in *L. retroversa* by Morton (Morton, 1954). Since the scuba observations of Gilmer and Harbison (Gilmer and Harbison, 1986), the mucous web has become the accepted feeding mechanism in all pteropods, but these recent studies suggest that a mucous web is not essential for feeding under at least some conditions. If the mucous trapper theory is correct, production of a mucous net

might only be triggered when larger, motile prey is detected: future studies should explore if mixed cultures of pteropods with smaller zooplankton prey induce web production. Alternatively, the high food concentrations in culture means may only require ciliary feeding mechanisms and that webs, which are energetically expensive to produce, are only used to maximize capture when food is sparse.

Healthy *L. helicina* can survive starvation for 2–4 weeks (depending on condition at capture and body size) at 0–5°C by living off lipid and body reserves (Lischka *et al.*, 2011; Lischka and Riebesell, 2012), as can *L. helicina antarctica* at −2°C (Maas *et al.*, 2011) and also at higher temperatures of up to 4°C (Bednaršek *et al.*, 2012a). Cultures of veliger stage *L. helicina* have been observed to survive starvation for up to 7 days near 0°C (Hopcroft, personal observation). Ideally, successful culture methodology should confirm that feeding is taking place either by measuring metabolic rate (which decreases with food deprivation), or by direct observation of feeding structures or full guts. Designs utilizing camera systems may be required for feeding observations as production, and retraction of feeding webs can be very rapid (Lalli and Gilmer, 1989).

Established protocols

Culture of other taxa of gelatinous zooplankton has been markedly more successful than that of pteropods, although many of the challenges faced are very similar. The production of mucous feeding structures was a major issue for appendicularian culturing just as it is for thecosome pteropods (Paffenhöfer, 1973). Delicate gelatinous bodies are prone to damage from collection and handling and must be kept in suspension, protected from impacts with the walls and base of culture vessels. The traditional plankton kreisel (Greve, 1968) is suitable for many species and, in other cases, has provided an excellent starting point for more complex designs (Sommer, 1992, 1993). Pteropod culture tests have been relatively successful using flow through and temperature-gradient kreisels, and these prototypes should now be expanded upon. Many attempts at culture development are side projects or a means to an end for a wider research project, such that the inevitable lack of time prevents the further development of promising systems. It is our belief that progress could be vastly improved with dedicated projects focused on improving systems and increased co-operation with aquaculturists. In this manner, it would be possible to capitalize on the potential economic value to fisheries as well as usefulness to the scientific community.

The successful culture techniques used during rearing *L. retroversa* were developed in collaboration with mariculturists raising the opisthobranch *Aplysia californica* (Capo *et al.*, 1997).

Current pteropod culture techniques are insufficient to address many questions about their future under changing climate conditions. There is a pressing need to develop improved cultivating methods, as thecosome pteropods are major exporters of carbon to the deep oceans in some regions (Berner and Honjo, 1981) and important prey items for a number of animals, including commercially important species (Armstrong *et al.*, 2008). Focus should first be on establishing successful culture methods before attempting to adapt these methods for suitability in undertaking perturbation experiments.

Recommendations

(i) Dedicated projects to develop systems and increased co-operation with aquaculturists. In this manner it would be possible to capitalize on the potential economic value to fisheries as well as usefulness to the scientific community. This would also allow the further development of reasonably successful methods reported here, several promising methods have been attempted once or twice and then abandoned due to lack of time or other priorities.

(ii) At present, perturbation experiments should focus on smaller (<2 mm) species, larval or lipid rich life stages to ensure healthy cultures and minimize the effects of experimental stress on observed variables until solutions can be found for larger species and adults.

(iii) In the case that perturbation experiments with adults and larger species are attempted, large-scale facilities (>500 L) should be considered (e.g. Bergen mesocosm facilities, Kiel KOSMOS system).

(iv) Tailoring of mixed algal diets depending on species size, life stage and algal species that are associated with pteropod blooms. Introduction of small zooplankton prey items for large species should be explored. Since web production has been observed with the temperature-gradient kreisel, this apparatus should be used for investigation of potential links between food concentration and web production and

(v) Improved communication between researchers: whilst compiling this paper it has become clear that the same, largely unsuccessful, methods have been attempted and re-attempted by several research groups. Thecosome pteropod abundance is patchy and difficulties in developing useful techniques are often compounded by limited access to specimens.

As such, it is vital that opportunities are not lost repeating unsuccessful methodology.

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