**Title**: Prudent female allocation by modular hermaphrodites: female investment is promoted by the opportunity to outcross in cyclostome bryozoans.

**Authors**: Helen L. Jenkins[1-3], John D.D. Bishop[1] and Roger N. Hughes[2]

[1]Marine Biological Association of the U.K., Plymouth, Devon, UK.

[2]School of Biological Sciences, Bangor University, Bangor, Gwynedd, UK.

[3]Department of Life Sciences, Natural History Museum, London, UK.

**Running title**: Female investment promoted by outcrossing

Author for correspondence: Helen L. Jenkins, The Marine Biological Association of the U.K., The Laboratory, Citadel Hill, Plymouth, Devon, PL1 2PB, UK.
Tel: +44 (0) 1752 633207; Fax: +44 (0) 1752 633102
e-mail: helnki@mba.ac.uk

**Abstract**

Many sessile, suspension-feeding marine invertebrates mate by spermcasting: aquatic sperm are spawned and gathered by conspecific individuals to fertilise eggs that are retained during development. In two phylogenetically distant examples, a cheilostome bryozoan and an aplousobranch ascidian, the receipt of allosperm has previously been shown to alter sex allocation by triggering female investment in eggs and brooding. Here we report experiments demonstrating that two species of cyclostome bryozoan also show restrained female investment in the absence of mating opportunity. In *Tubulipora plumosa*, the production of female zooids and progeny is much reduced in reproductive isolation. In *Filicrisia geniculata*, development of distinctive female zooids (gonozooids) begins but halts in the absence of mating opportunity, and no completed gonozooids or progeny result. Reduced female investment in the absence of a mate thus occurs in at least two orders of Bryozoa, but significant differences in detail exist and the evolutionary history within the phylum of the mechanism(s) by which female investment is initiated might be complex. The broadening taxonomic spectrum of examples where female investment appears restrained until allosperm becomes available may signify a general adaptive strategy among outcrossing modular animals, analogous to similarly adaptive sex allocation typical of many flowering plants.

**Key words**

Bryozoa — Cyclostomata (Cyclostomatida) — *Filicrisia geniculata* — reproductive assurance — resource allocation — simultaneous hermaphroditism — spermcasting — *Tubulipora plumosa*.

## Introduction

Allocations to male and female function in hermaphrodites need not be equal, and hermaphrodites can direct resources between sex functions to maximise the return on reproductive investment and thus enhance fitness (Charnov, Maynard Smith & Bull, 1976; Charnov, 1979, 1982). Flexibility in sex allocation is thus considered a major advantage afforded to simultaneous hermaphrodites over gonochorists (Michiels, 1998). Observed sex allocation may reflect an individual's adjustment in investment within its lifetime (phenotypic plasticity) or optimisation over an evolutionary timescale (Schärer, 2009; Avise, 2011).

Modular organisms should be capable of exceptionally variable sex allocation throughout their lifetime (for flowering plants see Lloyd & Bawa, 1984, for colonial invertebrates see Hughes & Cancino, 1985). The modular architecture of simultaneously hermaphroditic colonial marine invertebrates has the potential to facilitate flexibility in resource allocation, enabling colonies to shunt resources between growth (addition of modules) and reproduction, and between gender roles. However, the fixed costs of producing different sexes in simultaneous hermaphrodites are expected to constrain sex allocation (Heath, 1977). Mating systems of many colonial invertebrates, including all bryozoans (Ryland & Bishop, 1993), involve internal fertilisation of a retained egg by waterborne sperm (spermcast mating) (Bishop & Pemberton, 2006). Resultant embryos are often retained during development and in many cases receive ongoing maternal nourishment (matrotrophy; see Ostrovsky, 2013a-b; Ostrovsky *et al*., 2009, 2015). Whilst this strategy has several benefits (e.g. enhancement of the survival of embryos), it incurs the fixed costs of formation of female zooids and/or incubation chambers and the cost of extensive resource provisioning of the embryos in matrotrophic or macrolecithal species. Accordingly, mechanisms to control female investment may be in place. For example, the receipt of conspecific allosperm triggers the development of female zooids in the gymnolaemate bryozoan *Celleporella*

*hyalina* (Bishop, Manriquez & Hughes, 2000; Hughes, Manriquez & Bishop, 2002). A similar triggering of female investment by allosperm is also observed in the colonial ascidian *Diplosoma listerianum*, although this occurs in the absence of zooidal polymorphism (Bishop *et al.*, 2000). In species committed to outcrossing, restrained female investment may thus be advantageous as it postpones fixed costs until mating is assured, allowing redirection of investment. Self-fertilisation may be possible in simultaneous hermaphrodites (Darwin, 1876; Stebbins, 1950; Jarne & Auld, 2006) but it is generally avoided in colonial marine invertebrates (Ryland & Bishop, 1990, 1993; Knowlton & Jackson, 1993; Bishop, Jones & Noble, 1996; Hoare, Hughes & Goldson, 1999; Hoare & Hughes, 2001; but see Hughes & Wright, 2014). Such delayed or restrained female investment until male genetic input is assured in colonial invertebrates such as bryozoans and ascidians is a trait shared with flowering plants and is just one of several life history analogies identified between modular animals and angiosperms (see Richards, 1997; Bishop *et al.*, 2000; Bernasconi *et al.*, 2004; Hughes, 2005).

Patterns of sex allocation in bryozoans are predominately understood from studies of female investment in *Celleporella hyalina* (Order Cheilostomata). The present study again focuses on allocation to female function but extends investigations to a different major clade of Bryozoa, the exclusively marine Order Cyclostomata (Class Stenolaemata), in which colonies are formed from autozooids of cylindrical morphology (Borg, 1926; Hayward & Ryland, 1985). Cyclostomes are understood to exhibit colonial hermaphroditism, but individual zooids within colonies are gonochoristic (zooidal gonochorism) (Reed, 1991; Nielsen, 2012), as spermatogonia are found only in zooids without oogonia (Harmer, 1929). These calcified colonies are often small and lack the degree of zooidal polymorphism found in other bryozoans such as the previously studied *Celleporella hyalina.* However, some degree of polymorphism is present with the enlargement of female zooids to form voluminous chambers, termed gonozooids, for the intracoelomic incubation of multiple embryos (Borg, 1926). Viviparous reproduction in the Cyclostomata is characterised by the enigmatic

phenomenon of polyembryony — the splitting of a primary embryo into multiple genetically identical clone-mates (Craig, Slobodkin & Wray, 1995) — which is believed to occur within the gonozooids of all or almost all living species (Harmer, 1893, 1896, 1898; Calvet, 1900; Robertson, 1903; Borg, 1926; Jenkins, 2013). Fertilisation is internal, with a retained egg fertilised by water-borne sperm (i.e. spermcast mating), the uptake of which may involve the transitory lophophore in the developing gonozooid (Borg, 1926; Silén, 1972). Cyclostome larvae receive extraembryonic nutrition within gonozooids from the specialised nutritive tissue that surrounds them (Borg, 1926). Nourishment of embryos relies upon the transfer of energy from surrounding autozooids, as gonozooids are non-feeding at this stage. Furthermore, the production of genetically identical larvae from a single brood chamber may be prolonged (Jenkins, 2013). Thus, incubation is likely to incur significant costs, although as yet of unknown scale, and to create competition for resources within the colony, with the potential for conflict between the maternal colony and developing offspring. Inter-brood competition for resources may also occur where multiple broods of differing genotype are present within the same colony. Other costs of sex are expected to be minimal as in *C. hyalina* (e.g. no risks associated with sexual display or mating, uptake of sperm integrated with feeding process, costs only of sperm production) – though this is an assumption in both cases (Hughes *et al.*, 2002).

Using gonozooid development as a proxy, the prediction of restrained female investment pending an opportunity to mate was investigated in two species of bryozoan from the Order Cyclostomata. *Tubulipora plumosa* Thompson in Harmer, 1898 (Suborder Tubuliporina) and *Filicrisia geniculata* (Milne Edwards, 1838) (Suborder Articulata) are cyclostomes of contrasting colony form and gonozooid morphology. They represent different cyclostome suborders, broadening the phylogenetic perspective of the investigation. *T. plumosa* is an encrusting bryozoan, forming colonies of a single, broad lobe or multiple, narrower lobes (Fig. 1A-B). Autozooids are arranged in radiating, linear, comb-like rows within the lobes. Gonozooids are of irregular shape, often extensive, extending between rows of autozooids

and entirely or partially occupying lobes. Colonies of *T. plumosa* are common in shallow-water rocky habitats, where they are found encrusting a variety of substrata, including various algal species (Hayward & Ryland, 1985). *F. geniculata* is an erect bryozoan with a rather straggly or weedy colony form (Fig. 1D). Branches are formed from a single series of long and slender zooids, successive zooids being separated by a non-calcified joint. Gonozooids are inflated and club-shaped (clavate) (Fig. 1F). Colonies of *F. geniculata* are often found entangled with other species of Crisiidae among the sessile sward communities of the low shore, located below large boulders and overhangs (Hayward & Ryland, 1985). In this present investigation, colonies of *T. plumosa* and *F. geniculata* in laboratory culture were exposed to a source of conspecific allosperm and its effect on brood chamber development, and ultimately female investment, is reported.

## Material and Methods

### Material collection and founding of clones

Colonies of *Tubulipora plumosa* and *Filicrisia geniculata* were founded from larvae originating from wild parental colonies collected from the Hoe foreshore, Plymouth, Devon (*T. plumosa*), and from Wembury, Devon and Hannafore Point, Looe, Cornwall (*F. geniculata*). Parental *T. plumosa* colonies were collected on fronds of the non-native brown alga *Sargassum muticum* ((Yendo) Fenshott, 1955). Isolated wild colonies with single gonozooids, each from different *S. muticum* plants, were placed in separate crystallising dishes filled with aged, 0.2µm-filtered, UV-sterilised natural seawater (FSW) and lined with seawater-preconditioned acetate sheet. Larvae released overnight subsequently settled and metamorphosed onto the acetate. Individual metamorphs (at the ancestrula stage) were isolated on trimmed acetate, mounted onto a larger piece of acetate fixed to a microscope slide and clipped into separate stirred tanks. Colonies were grown from the ancestrulae, and each was propagated by artificially dividing and re-culturing the sections to form a clone of

169 independent, equal-sized ramets ('subcolonies'; n = 16). One clone per parental (wild) colony
170 (n = 5) was selected for experimentation. Hence, a 'clone' refers here to a genetically distinct
171 genet represented by a set of ramets. For *F. geniculata*, individual small colony fragments,
172 each with a single gonozooid, were mounted onto a piece of acetate sheet on a microscope
173 slide, held in place by a loop of very fine fishing line, and clipped into separate stirred tanks
174 (one fragment per tank). Tanks were filled with aged FSW and lined with seawater-
175 preconditioned acetate sheet. Colony fragments were maintained in culture and the acetate
176 sheet was monitored daily for ancestrulae. After ~10 days, individual ancestrulae were
177 isolated into separate stirred tanks as described for *T. plumosa* and maintained in culture
178 conditions until attaining a suitable colony size for experimentation. One cultured colony
179 derived from each parental (wild) colony (n = 4), was divided into a clone of equal-sized
180 ramets (n = 12), each with ~8-12 branch tips with feeding autozooids. Two colony types were
181 identified whilst rearing colonies in isolation prior to experimentation: 'Type 1' colonies,
182 composed solely of regular autozooids, and 'Type 2' colonies, which also developed
183 incomplete gonozooids (Fig. 2). The following experiment involved two clones of each colony
184 type: Clones A & D were Type 1 colonies and Type 2 colonies were represented by Clones B
185 & C.
186
187 **Culturing conditions**
188
189 *Tubulipora plumosa* and *Filicrisia geniculata* ramets were maintained in stirred tanks (for *T.*
190 *plumosa*, two ramets per tank) filled with ~850ml FSW at 16$^{o}$C±1$^{o}$C with 15:9 hour light:dark
191 regime, and fed twice daily with a mixture of *Rhinomonas reticulata* (Novarino, 1991) and
192 *Isochrysis galbana* (Parke, 1949) (Bishop *et al*., 2001). Feeding with *R. reticulata* alone
193 supports growth of *Celleporella hyalina* (Cheilostomata) to maturity (e.g. Hunter & Hughes,
194 1993, as *Rhodomonas baltica*; Manríquez, 1999; Manríquez, Hughes & Bishop, 2001), while
195 the mixture of two species used here enables indefinite growth and reproduction of the
196 compound ascidian *Diplosoma listerianum* (e.g. Bishop *et al*., 2001). Water was replaced

weekly and ramets were observed and regularly cleaned with a soft artist's brush (~one week intervals). Precautions were taken against any unwanted transfer of sperm between tanks as detailed in Ryland & Bishop (1990).

**Experimental procedure**

All clones of each species were exposed to two experimental treatments: 'single-clone' and 'mixed-clone' treatments. In the 'single-clone' treatment, two ramets from the same clone were placed within a tank. In the 'mixed-clone' treatment a single ramet from each of two different clones was placed within a tank. Consequently, each tank contained two ramets, so that the degree of crowding was equal in both treatments. Furthermore, for each species, the number of tanks in each treatment was equal, as were the number of ramets per clone in each treatment (these varied between the species due to number of available clones). Thus for *Tubulipora plumosa*, the single-clone treatment comprised four tanks per clone (total no. ramets per clone = eight) and the mixed-clone treatment comprised 20 tanks, two tanks per cross (total no. ramets per clone = eight). For *Filicrisia geniculata*, the single-clone treatment comprised three tanks per clone (total no. ramets per clone = six) and the mixed-clone treatment comprised 12 tanks, two tanks per cross (total no. ramets per clone = six). Experiments were conducted under culturing conditions identical to those used previously to maintain ramets. Tank order on shelves was randomised to reduce any potential effects of shelf position. All tanks were subject to an equal number of water changes, so opportunities for the potential loss of sperm and larvae were equal.

**Supplementary study – exposure of *Filicrisia geniculata* to a single dose of allosperm**

Six virgin (unmated) fragments of Type 2 *Filicrisia geniculata* Clone C, all with developing gonozooids (Fig. 2A), were mounted onto separate slides as described above. Two experimental treatments were conducted: 'exposure' and 'control'. In the 'exposure'

treatment, three fragments were placed into a tank containing allosperm in suspension from Type 1 colonies (but not containing Type 1 colonies themselves). The presence of allosperm in suspension was confirmed using techniques described by Bishop (1998). In the 'control' treatment, the three remaining fragments were placed in a tank containing clean FSW only. Tanks were maintained under standard culture conditions and the fragments were monitored for completion of gonozooids.

**Data collection and analysis**

All experimental ramets were monitored for the appearance of gonozooids and progeny. Counts were made of the number of completed gonozooids per ramet and the number of progeny produced per tank. Only settled progeny could be recorded, as swimming larvae were not readily visible. Any bias in the effect of larval loss was minimised by undertaking an equal number of water changes for all tanks.

Statistical analysis of count data was conducted where possible to assess the effect of conspecific allosperm on gonozooid development and progeny production. With *Tubulipora plumosa*, a replicated G-test was conducted in Excel to assess the overall effect of the treatments on gonozooid production and on the overall response of clones (McDonald, 2009). Progeny production between treatments was assessed using a Mann-Whitney U test in Minitab.

**Results**

***Tubulipora plumosa***

Gonozooids were first observed developing by Week 4 in some single- and some mixed-clone ramets but no progeny were observed at this time. At Week 8, counts of gonozooids

and settled progeny were made. Progeny at this time were evidently recently settled, either at primary disc/ancestrula stage (Fig. 1C) or three to four autozooids in size. Adult ramets were transferred onto new slides in new tanks at this time to reduce the risk of cross-fertilisation with developing progeny and to enable counting of further progeny. At Week 12, final counts of gonozooids per ramet and progeny per tank were made. Ten mixed-clone ramets (two per clone) were kept beyond Week 12 and the release of larvae was observed (but not counted) for a further four weeks.

Gonozooids were produced by all clones in the mixed-clone treatment (present in 30 ramets out of 40) and by four clones in the single-clone treatment (present in 16 ramets out of 40). The presence of gonozooids in the single-clone treatment provides evidence of gonozooid development in the absence of allosperm. One clone (Clone 4) developed only a single gonozooid in the mixed-clone treatment and none in the single-clone treatment; however, in the mixed-clone treatment, gonozooids and progeny were produced by the companion clone.

Gonozooid production depended on treatment (replicated G-test, pooled $G_1$ = 211.8, $P$<0.0001). Thus, the number of gonozooids produced differed between treatments, with more in the mixed-clone treatment (mean = 6.63, SD = 6.02, n = 40) than in the single-clone treatment (mean = 0.78, SD = 1.23, n = 40) (Table 2).

Clones did not differ statistically in their response to the two treatments (replicated G-test, heterogeneity $G_4$ = 7.693, $P$ = 0.1035) — in all clones gonozooid production was greater in ramets in the mixed-clone treatment. Figure 3 shows this relatively homogenous response across clones and that clones rank the same in both treatments.

Progeny counts per tank give an estimate of progeny production per gonozooid in each treatment. Greater estimated progeny production was shown by gonozooids in mixed-clone tanks (mean= 69.21, SD = 56.06, n = 20) compared with those in single-clone tanks (mean =

15.56, SD = 20.62, n = 9) (Mann-Whitney U, W = 3620, $P$ = 0.0037) (Table S1). An absolute figure of progeny production per gonozooid was not possible as: (a) progeny cannot be assigned to a particular ramet or gonozooid within a tank (due to multiple gonozooids present within each tank); (b) progeny cannot be counted directly *in situ* within a gonozooid (as prevented by the opaque, calcified outer skeleton); and (c) some larvae may be lost through water changing (an effect balanced between treatments by the equal number of water changes undergone by all tanks). Counts of metamorphosed (i.e. settled) larvae per tank were used to estimate mean larval production per gonozooid. There was wide variation in progeny per gonozooid between tanks in both treatments (Table S1). Progeny production in single-clone tanks provides *prima facie* evidence for self-fertilisation in Clones 1, 2, and 5; although gonozooids were produced, no progeny were recorded from single-clone tanks of Clone 3. The overall frequency of tanks with progeny depended on treatment (Chi-squared: $X^2_1$= 15, $P$<0.001), with 18 out of 20 mixed-clone tanks and 6 out of 20 single-clone tanks having progeny.

***Filicrisia geniculata***

A transitory lophophore was observed in early (approximately funnel-shaped) female zooids in Clones B and C in both treatments (Fig. 2B).

After 20 weeks, no completed gonozooids developed in any ramet (of any clone) in the single-clone treatment. In the mixed-clone treatment, only Clones B and C produced completed gonozooids but only when crossed with ramets of Clones A and D; in single-clone tanks clones B and C only produced incomplete gonozooids. Clones A and D developed only autozooids in all ramets in both treatments.

Gonozooid production therefore differed markedly between clones, with completed gonozooids only produced in Clones B and C (Chi-squared: $X^2_3$= 161.738, $P$<0.0001) (Table

1). A very clear-cut pattern of two distinct colony types was thus observed. Type 1 colonies (Clones A & D) were composed solely of autozooids; Type 2 colonies (Clones B & C) formed incomplete gonozooids, in addition to autozooids, in reproductive isolation and when reared with one another. Completed gonozooids were only produced in Type 2 colonies in the presence of Type 1 colonies.

Progeny were recorded from a total of three tanks over the duration of the experiment, all from the mixed-clone treatments (Table 2). Larvae were released within tanks between Weeks 13 and 20. Despite efforts to thoroughly examine colonies, metamorphs could potentially settle onto branches of ramets and would then be difficult to count or could be obscured altogether (even in tanks where gonozooids were present but no progeny were scored). Therefore, alongside the reasons outlined for *Tubulipora plumosa*, progeny counts should be considered as estimates.

**Supplementary study – exposure of *Filicrisia geniculata* to a single dose of allosperm**

Following exposure to allosperm, completed gonozooids were found only in those fragments of Type 2 Clone C reared in the 'exposure' tank (i.e. receiving a single dose of Type 1 allosperm): a single completed gonozooid was present in two of the three fragments, with incomplete gonozooids being budded subsequently. No completed gonozooids were produced in any of the three control fragments.

## Discussion

Female investment was restricted in the absence of conspecific allosperm in both *Tubulipora plumosa* and *Filicrisia geniculata*. In *T. plumosa,* reproductive isolation (i.e. the single-clone treatment) reduced gonozooid production 8.5-fold, and progeny were released in a smaller number overall and in a smaller proportion of replicate tanks. Colonies of *T. plumosa* are

337 therefore apparently able to defer a proportion of their potential resource provisioning to
338 female function until receipt of conspecific allosperm is assured. In *F. geniculata*, our
339 observations indicate that some degree of female investment, in the form of incomplete
340 gonozooids, is made prior to the receipt of allosperm in Type 2 colonies. In this case, part of
341 the cost of gonozooid production and the subsequent investment in brooding offspring is
342 delayed until fertilisation is assured and this is apparently controlled by allosperm availability.
343 The result of the supplementary study strongly suggests that it is allosperm that triggers the
344 completion of developing gonozooids, rather than the presence a conspecific colony of Type
345 1 as such, although additional experiments would be required to rule out the role of an
346 additional substance released along with sperm (cf. Bishop *et al.*, 2000). Furthermore, the
347 resumption of production of incomplete gonozooids after the transient formation of completed
348 one(s) in response to a single exposure to allosperm (as seen in the supplementary study)
349 suggests a zooid-by-zooid basis to gonozooid development. This also implies the absence of
350 a sperm storage mechanism, known in some spermcasting colonial invertebrates (Bishop &
351 Ryland, 1991; Hughes *et al.*, 2002), which might counteract fluctuations in sperm supply.
352
353 In the absence of allosperm, *T. plumosa* colonies do reproduce (presumably by intraclonal
354 self-fertilisation), but this involves significantly fewer gonozooids and progeny than when
355 paired with another genotype. Under the same conditions of reproductive isolation,
356 gonozooid development in *F. geniculata* begins but is aborted, and no progeny result. Thus,
357 in *T. plumosa*, the general degree of investment depends on allosperm availability whereas
358 in *F. geniculata*, the completion of gonozooids followed by incubation depends on allosperm
359 availability. This suggests that the exact mechanisms controlling female investment differ
360 between the two species. In both cases, restrained female investment presumably allows
361 reallocation of resources to other colony functions, potentially with compensatory allocation
362 to female function when allosperm become available.
363

Aborted gonozooids in *F. geniculata* remained as wide, approximately funnel-shaped, structures (Fig. 2B) either with the opening sealed over, or with a short autozooid-like opening extending from within it. Borg (1926) described that the developing gonozooid has a modified polypide with shortened tentacles and rudimentary gut in the Crisiidae (not including *F. geniculata*). If not becoming gonozooids, such zooids resorb their ovaries, regenerate a functional polypide and become feeding zooids with a cystid morphology intermediate between autozooid and gonozooid. The ability of aborted gonozooids to feed and the resorption of the ovaries require confirmation in *F. geniculata*.

Evidence here for restrained female investment in two cyclostomes pending receipt of conspecific allosperm is generally in accordance with that from the gymnolaemate bryozoan *Celleporella hyalina* (Bishop *et al*., 2000; Hughes *et al.*, 2002). All share the ability to defer costs of female investment until cross-fertilisation is assured, but differences are apparent. In *C. hyalina* production of mature female zooids is triggered by the translocation of sperm captured by feeding zooids and stored within the colony (Hughes *et al.*, 2002), whereas in cyclostomes sperm uptake is understood to be via the feeding or non-feeding lophophore of the zooid that developed an ovary and will become female (Borg, 1926; Silén, 1972). Such differences in detail at an ordinal level suggest that the evolutionary history of the mechanisms involved in the initiation of female investment may be complex. It should be noted that *C. hyalina* may itself represent an unusual case amongst gymnolaemates because the possession of separate male, female and feeding modules is uncommon, but gives the potential for three-way resource trade-off between functions at the level of modules.

Restrained female allocation in the absence of allosperm should be expected only if selfing is absent or provides a lower return on reproductive investment, in fitness terms, than outcrossing. Obligate outcrossing is typical of the *Celleporella hyalina* clade, but the congeners *C. angusta* and *C. osiani*, which belong to a separate phylogenetic clade, freely self-fertilise without incurring detectable inbreeding depression and show undiminished

391 female investment in the absence of allosperm (Hughes *et al.*, 2009; Hughes & Wright,
392 2014). In contrast, Johnson (2010) documented substantial inbreeding depression following
393 selfing in another cheilostome bryozoan, *Bugula stolonifera*. *Tubulipora plumosa* might be an
394 example of restrained investment in selfing when outcrossing is not possible, in a species
395 susceptible to inbreeding depression.

396 Borg (1926) noted the presence of a transitory lophophore (having shortened tentacles and
397 rudimentary digestive tract) in young crisiid zooids destined to become female. Silén (1972)
398 reported that lophophore tentacles can be seen extending from the developing gonozooid, as
399 confirmed here in *Filicrisia geniculata*, and proposed that sperm uptake was via this organ.
400 Evidence here from exposing Type 2 colony fragments to a single dose of allosperm
401 suggests that incipient gonozooids may need to be at a particular developmental stage to be
402 receptive to sperm uptake. Despite experimental fragments possessing many developing
403 gonozooids, only one completed gonozooid was produced in two out of three recipient
404 fragments after exposure, suggesting the capture of allosperm by individual zooids must be
405 during a relatively brief critical interval between polypide development and degeneration.
406 However, before any firm inferences can be drawn, the fertilisation mechanisms of *F.*
407 *geniculata* require further investigation, including confirmation of the transitory lophophore as
408 the actual site of sperm uptake.
409
410 Our observations reveal two distinct patterns of female allocation amongst cyclostome
411 bryozoans. Phenotypic plasticity in allocation is suggested in *T. plumosa* by greater female
412 investment in response to allosperm availability. This apparent colony-level within-lifetime
413 adjustment of female allocation is likely achieved via the shifting of resources between sex
414 functions – flexibility that is enhanced by modularity and has been demonstrated in other
415 bryozoan studies (*C. hyalina*: Hughes & Hughes, 1986; Hughes *et al*., 2002, 2003). This
416 supports the view of simultaneous hermaphroditism as a mechanism to avoid gamete
417 wastage, allowing the re-direction of resources between gender roles to enhance the

418 individual's fitness (Charnov *et al*., 1976; Charnov, 1979, 1982). However, a caveat must be
419 placed upon this. Such arguments are often based upon assumptions of a fixed resource
420 budget for reproduction and a trade-off purely between sex functions within this, which may
421 not always be the case (Schärer, 2009). While it is tempting to infer male allocation from
422 assessment of female function alone, it is possible that the total allocation to reproduction
423 may change (Schärer, 2009), with reallocation to other uses such as growth or storage
424 products (possibly benefitting future reproduction). Time and resource constraints precluded
425 measurement of male function and hence total reproductive allocation in the present study.
426 Estimations of sperm production per clone (or per ramet) would address this limitation.
427 Clones of *Tubulipora plumosa* acted as both sperm donors and recipients as expected in
428 functioning simultaneous hermaphrodites, with a single exception (Clone 4). The
429 reproductive activity of Clone 4 suggested that investment was solely in sperm production, or
430 that female investment was rare. This clone did not routinely produce female zooids—only a
431 single gonozooid developed across all 16 ramets—and showed no evidence of production of
432 progeny. However, Clone 4 apparently acted as a sperm donor as suggested by the
433 increased gonozooid production observed in its companion (recipient) clones in the mixed-
434 clone treatment
435
436 In contrast, results presented here for *Filicrisia geniculata* suggest the existence of colonies
437 of separate sexes. Complete gonozooids developed only in one of the two colony types
438 identified (Type 2) and only when in the presence of the alternative colony form composed
439 solely of autozooids (Type 1), evoking female and male colony forms respectively. Robertson
440 (1903) first suggested the presence of dioecious colonies in crisiids (*Crisia occidentalis* and
441 *C. franciscana*, the latter as *C. eburnea*). However, further investigations are required to
442 confirm the findings regarding *F. geniculata*, and will be pursued in the future.
443
444 In conclusion, our findings suggest that, although restrained female investment in the
445 absence of allosperm occurs in the two divergent orders Cyclostomata and Cheilostomata,

substantial diversity exists in the detail of female allocation patterns amongst bryozoans. Inferences from the most comprehensive investigations to date (i.e. with the cheilostome *Celleporella hyalina*) were not matched entirely in the cyclostomes studied here. Furthermore, differences were revealed between the two cyclostomes themselves. Future investigations may uncover further variations, at both familial and ordinal levels, within the phylum as a whole. This will be of importance for understanding the maintenance of universal hermaphroditism amongst Bryozoa and also for interpreting both past and present life-history patterns. The growing number and taxonomic diversity of known cases in which female investment is restrained until outcrossing opportunity is assured by reception of allosperm suggest an adaptive allocation strategy similar to one typical of many flowering plants. Restraint of female investment until outcrossed male gametes become available therefore may prove to be of general adaptive significance among outcrossing modular organisms, sensu Harper (1977).

**Acknowledgements**

We are grateful to Christine Wood (MBA) for her assistance in maintaining lab cultures and to Dr Paul D. Taylor (NHM) for the SEM imaging of specimens (Figures 1B-C, E-F and 2A). Many thanks also to Prof. Beth Okamura who advised on data analysis and read and reviewed the related PhD thesis chapters. We thank the four anonymous reviewers for their helpful comments. This research was funded by a NERC Open CASE PhD Studentship (NE/G012644/1) to Bangor University (RNH), NHM (Prof. Beth Okamura) and MBA (JDDB), and awarded to HLJ.

**References**

**Avise JC. 2011.** *Hermaphroditism: a primer on the biology, ecology, and evolution of dual sexuality.* New York: Columbia University Press.

**Bernasconi G, Ashman TL, Birkhead TR, Bishop JDD, Grossniklaus U, Kubli E. *et al*. 2004.** Evolutionary ecology of the pre-zygotic stage. *Science* **303**: 971-975.

**Bishop JDD. 1998.** Fertilization in the sea: are the hazards of broadcast spawning avoided when free-spawned sperm fertilize retained eggs? *Proceedings of the Royal Society London Series B, Biological Sciences* **265**: 725-731.

**Bishop JDD, Jones CS, Noble LR. 1996.** Female control of paternity in the internally fertilizing compound ascidian *Diplosoma listerianum*. II. Investigation of male mating success using RAPD markers. *Proceedings of the Royal Society London Series B, Biological Sciences* **263**: 401-407.

**Bishop JDD, Manriquez PH, Hughes RN. 2000.** Water-borne sperm trigger vitellogenic egg growth in two sessile marine invertebrates. *Proceedings of the Royal Society London Series B, Biological Sciences* **267**: 1165-1169.

**Bishop JDD, Pemberton AJ. 2006.** The third way: spermcast mating in sessile marine invertebrates. *Integrative and Comparative Biology* **46**: 398-406.

**Bishop JDD, Pemberton AJ, Sommerfeldt AD, Wood CA. 2001.** Laboratory studies of mating in the aplousobranch *Diplosoma listerianum*. In: Sawada H, Lambert CC, Yokosawa H, eds. *Biology of Ascidians*. Tokyo: Springer-Verlag, pp. 305-310.

**Bishop JDD, Ryland JS. 1991.** Storage of exogenous sperm by zooids of the ascidian *Diplosoma listerianum*. *Marine Biology* **108**: 111-118.

**Borg F. 1926.** Studies on recent cyclostomatous Bryozoa. *Zoologiska bidrag från Uppsala* **10**: 181-507.

**Calvet L. 1900.** Contribution a l'histoire naturelle des bryozoaires ectoproctes marins. *Travaux de l'Institut de Zoologie de l'Universite de Montpellier et de la station zoologique de Cette* **8**: 1-488.

**Charnov EL. 1979.** Simultaneous hermaphroditism and sexual selection. *Proceedings of the National Academy of Sciences of the United States of America* **76**: 2480-2484.

**Charnov EL. 1982.** *The Theory of Sex Allocation.* Princeton: Princeton University Press.

**Charnov EL, Maynard Smith J, Bull JJ. 1976.** Why be an hermaphrodite? *Nature* **263**: 125-126.

**Craig SF, Slobodkin LB, Wray G. 1995.** The 'paradox' of polyembryony. *Trends in Ecology and Evolution* **10**: 371-372.

**Darwin C. 1876.** *The effects of cross- and self-fertilisation in the vegetable kingdom.* London: John Murray.

**Harmer SF. 1893.** On the occurrence of embryonic fission in Cyclostomatous Polyzoa. *Quarterly Journal of Microscopical Science* **34**: 199-241.

**Harmer SF. 1896.** On the development of *Lichenopora verrucaria*, Fabr. *Quarterly Journal of Microscopical Science* **39**: 71-144.

**Harmer SF. 1898.** On the development of Tubulipora, and on some British and northern species of this genus. *Quarterly Journal of Microscopical Science* **41**: 73-157.

**Harmer SF. 1929.** Polyzoa. *Proceedings of the Linnean Society London* **141**: 68-118.

**Harper JL. 1977.** *Population Biology of Plants*. London: Academic Press.

**Hayward PJ, Ryland JS. 1985.** Cyclostome Bryozoans. Keys and notes for the identification of the species. *Synopses of the British Fauna* (n. s.) **34**. EJ. London & Leiden: Brill/Backhuys for the Linnean Society & Estuarine & Brackish-water Sciences Association.

**Heath DJ. 1977.** Simultaneous hermaphroditism: cost and benefit. *Journal of Theoretical Biology* **64**: 363-373.

**Hoare K, Hughes RN. 2001.** Inbreeding and hermaphroditism in the sessile, brooding bryozoan *Celleporella hyalina. Marine Biology* **139**: 147-162.

**Hoare K, Hughes RN, Goldson AJ. 1999.** Molecular genetic evidence for the prevalence of outcrossing in the hermaphroditic brooding bryozoan *Celleporella hyalina*. *Marine Ecology Progress Series* **188**: 73-79.

**Hughes DJ, Hughes RN. 1986.** Life history variation in *Celleporella hyalina* (Bryozoa). *Proceedings of the Royal Society London Series B, Biological Sciences* **228**: 127-132.

**Hughes RN. 2005.** Lessons in modularity: the evolutionary ecology of colonial invertebrates. *Scientia Marina* **69**: suppl. 1, 169-179.

**Hughes RN, Cancino JM. 1985.** An ecological overview of cloning in Metazoa. In: Jackson JBC, Buss LW, Cook RE, eds. *Population Biology and Evolution of Clonal Organisms*. New Haven, CT: Yale University Press, pp. 153-186.

**Hughes RN, Manriquez PH, Bishop JDD. 2002.** Female investment is retarded pending reception of allosperm in a hermaphroditic colonial invertebrate. *Proceedings of the National Academy of Sciences USA* **99**: 14884-14886.

**Hughes RN, Manriquez PH, Bishop JDD, Burrows MT. 2003.** Stress promotes maleness in hermaphroditic modular animals. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 10326-10330.

538 **Hughes RN, Wright PJ. 2014.** Self-fertilisation in the *Celleporella angusta* clade and a
539 description of *Celleporella osiani* sp. nov. In: Rosso A, Wyse Jackson PN, Porter J, eds.
540 *Bryozoan Studies 2013: proceedings of the 16th International Bryozoology Association*
541 *conference*, Studi Trentini di Scienze Naturali **94**: 119-124.

542 **Hughes RN, Wright PJ, Carvalho GR, Hutchinson, WF. 2009.** Patterns of self
543 compatibility, inbreeding depression, outcrossing, and sex allocation in a marine bryozoan
544 suggest the predominating influence of sperm competition. *Biological Journal of the Linnean*
545 *Society* **98**: 519–531.

546 **Hunter E, Hughes RN. 1993.** Effects of diet on life-history parameters of the marine
547 bryozoan, *Celleporella hyalina* (L.). *Journal of Experimental Marine Biology and Ecology* **167**:
548 163-177.

549 **Jarne P, Auld JR. 2006.** Animals mix it up too: the distribution of self-fertilisation among
550 hermaphroditic animals. *Evolution* **60**: 1816-1824.

551 **Jenkins HL. 2013.** *Polyembryony, brood chamber development and gender specialization in*
552 *cyclostome bryozoans*. PhD thesis. Bangor University, UK.

553 **Johnson CH. 2010.** Effects of Selfing on Offspring Survival and Reproduction in a Colonial
554 Simultaneous Hermaphrodite (*Bugula stolonifera*, Bryozoa). *Biological Bulletin* **219**: 27–37.

555 **Knowlton N, Jackson JBC. 1993.** Inbreeding and outbreeding in marine invertebrates. In:
556 Thornhill NW ed. *The Natural History of Inbreeding and Outbreeding: Theoretical and*
557 *Empirical Perspectives* . Chicago: Chicago University Press, pp. 200-249.

558 **Lloyd DG, Bawa KS. 1984.** Modification of the gender of seed plants in varying conditions.
559 *Evolutionary Biology* **17**: 255-338.

560 **McDonald JH. 2009.** *Handbook of Biological Statistics*. 2nd edn. Baltimore, MD: Sparky
561 House Publishing.

562 **Manríquez PH. 1999.** *Mate choice and reproductive investment in the cheilostome bryozoan*
563 *Celleporella hyalina (L.).* PhD thesis. Bangor: University of Wales, Bangor.

564 **Manríquez PH, Hughes RN, Bishop JDD. 2001.** Age-dependent loss of fertility in water-
565 borne sperm of the bryozoan *Celleporella hyalina*. *Marine Ecology Progress Series* **224**: 87-
566 92.

567 **Michiels NK. 1998.** Mating conflict and sperm competition in simultaneous hermaphrodites.
568 In: Birkhead TR, Moller AP, eds. *Sperm competition and sex selection*. San Diego, CA:
569 Academic Press, pp. 219-254

570 **Nielsen C. 2012.** *Animal Evolution: interrelationships of the living phyla.* 3rd edn. Oxford:
571 Oxford University Press.

572 **Ostrovsky AN. 2013b.** *Evolution of Sexual Reproduction in Marine Invertebrates. Example*
573 *of gymnolaemate bryozoans.* Dordrecht: Springer.

574 **Ostrovsky AN, Gordon DP, Lidgard S. 2009.** Independent evolution of matrotrophy in the
575 major classes of Bryozoa: transitions among reproductive patterns and their ecological
576 background. *Marine Ecology Progress Series* **378**: 113-124.

577 **Ostrovsky AN, Lidgard S, Gordon DP, Schwaha T, Genikhovich G, Ereskovsky AV.**
578 **2015.** Matrotrophy and placentation in invertebrates: a new paradigm. *Biological Reviews*.
579 DOI: 10.1111/brv.12189

580 **Reed CG. 1991.** Bryozoa. In: Giese AC, Pearse JS, Pearse VB, eds. *Reproduction of Marine*
581 *Invertebrates*. The Boxwood Press, Pacific Grove, pp. 85-245.

582 **Richards AJ. 1997.** *Plant Breeding Systems.* London: Chapman & Hall.

583 **Robertson A. 1903.** Embryology and embryonic fission in the genus *Crisia*. *University of*
584 *California Publications in Zoology* **1**: 115-156.

585 **Ryland JS, Bishop JDD. 1990.** Prevalence of cross-fertilisation in the hermaphroditic
586 compound ascidian *Diplosoma listerianum*. Marine Ecology Progress Series **61**: 125-132.

587 **Ryland JS, Bishop JDD. 1993.** Internal fertilisation in hermaphroditic colonial invertebrates.
588 *Oceanography and Marine Biology Annual Review* **31**: 445-477.
589
590 **Schärer L. 2009.** Tests of sex allocation theory in simultaneously hermaphroditic animals.
591 *Evolution* **63**: 1377-1405.

592 **Silén L. 1972.** Fertilisation in the Bryozoa. *Ophelia* **10**: 27-34.

593 **Stebbins GL. 1950.** *Variation and Evolution in Plants.* New York: Columbia University Press.

594
595
596
597
598
599
600
601
602
603
604
605
606
607

## Tables

Table 1: The total number of completed gonozooids produced by all *Filicrisia geniculata* clones in each treatment (from total of six ramets per clone per treatment).

| Clone | Single-clone | Mixed-clone |
|---|---|---|
| A | 0 | 0 |
| B | 0 | 24 |
| C | 0 | 79 |
| D | 0 | 0 |

Table 2: Total number of completed gonozooids and progeny produced by *Filicrisia geniculata* Type 2 clones (bold) in the mixed-clone treatment (crosses involving both colony types). Number of progeny recorded over the duration of experiment. Note: 'Rep' = replicate, 'GZ' = gonozooid, Type 1 colonies = Clones A & D, Type 2 colonies = Clones B & C.

| Cross | Tank | No. of completed GZ | No. of progeny |
|---|---|---|---|
| **B** x A | Rep 1 | 3 | 0 |
| | Rep 2 | 10 | 1 |
| **B** x D | Rep 1 | 2 | 0 |
| | Rep 2 | 9 | 0 |
| **C** x A | Rep 1 | 33 | 0 |
| | Rep 2 | 20 | 116 |
| **C** x D | Rep 1 | 16 | 9 |
| | Rep 2 | 10 | 0 |

631

632 **Figure legends**

633

634 Figure 1: Images of *Tubulipora plumosa* and *Filicrisia geniculata* from light microscopy and
635 scanning electron microscopy. *T. plumosa* (A) colony in culture, (B) part of colony with
636 gonozooid, (C) primary disc (left) and ancestrula (right). *F. geniculata* (D) colony in culture,
637 (E) ancestrula, (F) complete gonozooid.

638

639

640 Figure 2: Gonozooids of similar developmental stage in *Filicrisia geniculata* from light
641 microscopy and scanning electron microscopy: (A) incomplete gonozooids, (B) transitory
642 lophophore in funnel-shaped gonozooid.

643

644

645 Figure 3: The mean number of gonozooids produced by *Tubulipora plumosa* clones in the
646 two experimental treatments (from a total of eight ramets per clone per treatment). The range
647 of gonozooid production among clonal ramets in each treatment is also shown.

648

649 **Supporting Information**

650 Table S1: Estimated progeny production in *Tubulipora plumosa*.