Invertebrate Biology

Invertebrate Biology 133(4): 340–353.

DOI: 10.1111/ivb.12065

The effects of nurse eggs and sibling interactions on the larval development of the poecilogonous annelid *Boccardia proboscidea* (Spionidae)

Fernanda X. Oyarzun[1,2,3,a] and Antonio Brante[1]

[1] Facultad de Ciencias, Universidad Católica de la Santísima Concepción, Casilla 297, Concepción, Chile
[2] Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Casilla 160-C, Concepción, Chile
[3] Friday Harbor Laboratories, University of Washington, Friday Harbor, Washington 98250, USA

Abstract. In poecilogony, different types of larvae are produced within the same species. Previous studies have suggested maternal control of the production of larval types; however, it is not clear which factors or mechanisms generate contrasting developmental patterns among siblings. The spionid polychaete *Boccardia proboscidea* produces within the same capsule adelphophagic larvae that eat nurse eggs and siblings and complete all or most of their development inside the capsule (Type A larvae), and larvae with little growth until they hatch as planktotrophic larvae (Type B larvae). In this study, we manipulated capsule content to explore the factors determining larval type in *B. proboscidea* and the role of extra-embryonic maternal nutrition and sib–sib interaction in the developmental fate of offspring. When early larval stages were grown individually *in vitro,* with nurse eggs as the only food source, some of them remained small, while others continue developing into larger pre-competent larvae by feeding on nurse eggs. This suggests that larval types in *B. proboscidea* are determined very early in development and are not solely the product of sib–sib interaction inside the capsule. However, our data also suggest that hatching size variability within larval types of a clutch depends on nurse egg availability. Type B larvae grew normally to metamorphosis when phytoplankton was available, but suffered high rates of cannibalism by Type A larvae. These results are consistent with the hypothesis that individual larval fates are determined very early in development and that once their fate is determined, hatching size and intracapsular survival are affected by maternal food provisioning and sibling interaction.

Additional key words: developmental polymorphism, cannibalism, maternal provisioning, maternal effects, intra-familial conflict

Marine invertebrates show a diversity of developmental modes ranging from those with elaborate larval forms that swim and feed in the plankton for weeks or months, to those that lack a free-living larval stage (Thorson 1946, 1950; McEdward 1995; Allen & Pernet 2007; Collin 2012). Extensive interspecific comparative studies have evaluated the adaptive significance of different developmental modes and their evolution (e.g., Thorson 1946, 1950; Strathmann 1985, 1990, 1993, 1995; Wray & Raff 1991; McEdward 1995; Jeffery et al. 2003; Collin 2004). However, interspecific comparisons may be subjected to confounding factors, given differences in allometric, ecological, and physiological traits, as well as different evolutionary histories of the species (Felsenstein 1985).

Poecilogony, in which diverse kinds of offspring are produced within the same species (Giard 1905), offers the opportunity to comparatively study the processes and factors that influence different developmental modes while avoiding incidental confounding factors, and potentially provides insights into the mechanisms that generate the developmental differences (Levin & Huggett 1990; Chia et al. 1996; Krug 1998; Gibson & Gibson 2004; Ellingson & Krug 2006; Clemens-Seely & Phillips 2011; Knott & McHugh 2012). In some poecilogonous species, the intraspecific differences between developmental modes may be related to how much energy the parent provides directly to the offspring. For example, Krug and collaborators

[a]Author for correspondence.
E-mail: fernanda.oyarzun@udec.cl

have shown that starved females of the poecilogonous gastropod *Alderia willowi* Krug et al. 2007 switched from producing only non-feeding lecithotrophic larvae (low dispersal) to a mix of facultative feeders and feeding planktotrophic larvae (high dispersal) with a concomitant decrease in spontaneous metamorphosis and further increasing dispersal (Krug 1998; Ellingson & Krug 2006; Botello & Krug 2006; Krug et al. 2007; Krug 2009). Their studies support the hypothesis that a female may adjust the developmental mode of offspring in accordance with environmental conditions, controlling yolk provisioning to the egg and dispersing her offspring more broadly if local conditions become unfavorable.

In species with encapsulation, maternal provisioning may be allocated to the offspring not only as embryonic egg yolk, but also as extra-embryonic material such as nurse eggs, nurse embryos, and gel-like intrabrood material. In non-poecilogonous species, this form of maternal provisioning has been shown to reduce time to metamorphic competence, increase hatching size, and increase survival probability of hatchlings. For example, the number of nurse eggs consumed during development determines the size at hatching in some prosobranchs (Spight 1976; Rivest 1983), and when *Nucella ostrina* (Gould 1852) hatchlings were followed in the field, larger hatchlings grew faster and had higher survivorship (Moran & Emlet 2001). Higher concentration of dissolved albumen in the intracapsular fluid favored growth and survival of larvae in *Crepidula fornicata* (Linnaeus 1758) (Brante et al. 2009). In the gastropods *Solenosteira macrospira* (Berry 1957) and *Crepidula coquimbensis* Brown & Olivares 1996, higher levels of intracapsular cannibalism of developing siblings produced significantly larger hatchlings (Kamel et al. 2010a,b; Kamel & Grosberg 2012; Brante et al. 2013). Moreover, in calyptraeid gastropods, a planktonic larval stage is absent in most species that produce nurse eggs or nurse embryos, suggesting that maternal provisioning of extra-embryonic food may play an important role explaining the evolution of developmental modes in this group (Collin 2003).

Similarly, in some poecilogonous species, maternal allocation of extra-embryonic food supplies within capsules could potentially explain how different kinds of larvae are produced or the variation in hatching size within a clutch (Hoagland & Robertson 1988; Gibson 1997; Mackay & Gibson 1999). In some poecilogonous polychaete worms, two different types of offspring coexist within capsules in which nurse eggs are also available: one type of larva (Type A) always feeds on nurse eggs and hatches close to metamorphosis, and the other type (Type B) does not eat nurse eggs and hatches as a smaller planktonic larva (Gibson 1997; Smith & Gibson 1999; Duchêne 2000; Oyarzun et al. 2011; Gibson et al. 2012; David et al. 2014). Experimental observations show that both types of larvae have the capacity to feed on phytoplankton; a differential capacity to consume nurse eggs inside the capsule would then explain the two kinds of larvae (Smith & Gibson 1999; Duchêne 2000). Still, it is not clear if this differential consumption of nurse eggs is the result of a polymorphism, a polyphenism, or a combination of both. Sibling competition for nurse eggs could result in different larval sizes at hatching, but different larval sizes could also be the result of different feeding behaviors or capacities (e.g., feeding structures, digestive enzymes), which could be determined genetically by the male or female, by maternal factors, by sibling inhibition, or by environmental factors. In addition to food supply, two other factors have been suggested as possible mechanisms determining different developmental modes in poecilogonous species. First, Rice & Rice (2009) raised females of the poecilogonous spionid *Polydora cornuta* Bosc 1802 in isolation over a period of 16 weeks. They found that as time progressed, the number of fertilized embryos declined, that the remaining larvae ate the unfertilized embryos, and therefore that hatching size of larvae increased. Given that females of *P. cornuta* store sperm similarly to other poecilogonous species, they suggest that the variable size of offspring reported for poecilogonous species might be simply the consequence of stored sperm limitation. Secondly, Gibson & Chia (1995) reported that the rate of metamorphosis of the poecilogonous opisthobranch *Haminaea japonica* Pilsbry 1895 (Gibson & Chia reported the species as *Haminoea callidegenita*, but it is now considered to be a synonym of *H. japonica*) depended on the presence of a metamorphic inducer produced by the mother that existed within the egg mass. After testing for the direct response of embryos to the inducer, they concluded that the variation in metamorphosis before or after hatching was a consequence of variation in larval sensitivity to such inducer.

The spionid polychaete *Boccardia proboscidea* Hartman 1940 reproduces with a type of poecilogony. Some females produce within the same capsule (1) many nurse eggs, (2) adelphophagic larvae that complete all or most of their development inside the capsule while eating nurse eggs and siblings and reaching an approximate maximum of 16 setigers (segments with setae) at hatching (Type A larvae),

and (3) offspring that remain at an early stage of development, between 3 and 5 setigers, until hatching (Type B larvae; Fig. 1A–D) (Hartman 1940, 1941; King 1976; Woodwick 1977; Gibson 1997; Oyarzun & Strathmann 2011). Gibson & Carver (2013) found no evidence of significant morphological differences between Type A and Type B larvae at the same developmental stage, and no other study has shown evidence that suggests that these two larval types are significantly different in any aspect except for the developmental stage they reach at hatching. Type B larvae provide the potential for long-distance dispersal and increased population connectivity because they hatch as planktotrophic larvae and require ~15 d to complete their development (Gibson 1997; Gibson & Gibson 2004; Oyarzun & Strathmann 2011; Oyarzun et al. 2011; Gibson & Carver 2013). It remains to be explained why some larvae stop or slow their development until hatching and if the mother determines or influences their fate. Therefore, this species presents an ideal opportunity to study the potential contribution of maternal provisioning and sibling interactions in determining the two different developmental modes.

Specifically, four factors related to maternal provisioning and sibling interactions could greatly modify the outcome of this mixed reproductive strategy in *B. proboscidea*: cannibalism on Type B larvae by Type A siblings inside the capsule (Hartman 1941), length of incubation, regulated by the capsule opening behavior of females and affecting the size at hatching and the amount of cannibalism that takes place inside the capsule (Oyarzun & Strathmann 2011), nurse egg/larvae ratio (Gibson 1997; Oyarzun et al. 2011), and the ratio of Type B/Type A larvae in a capsule. Here, we test the hypotheses that larval type is determined early in development and that variability in hatching size in *B. proboscidea* is explained by the differential capacity of larvae to use extra-embryonic nutrition and by sibling interactions inside the capsule. Specifically, we tested if the type of development is predetermined from early stages by isolating individual larvae and growing them *in vitro*, and we manipulated composition of clutches to examine the effect of nurse egg number and the presence of other larvae on the growth rates of both larval types and the occurrence of intracapsular competition for food and cannibalism.

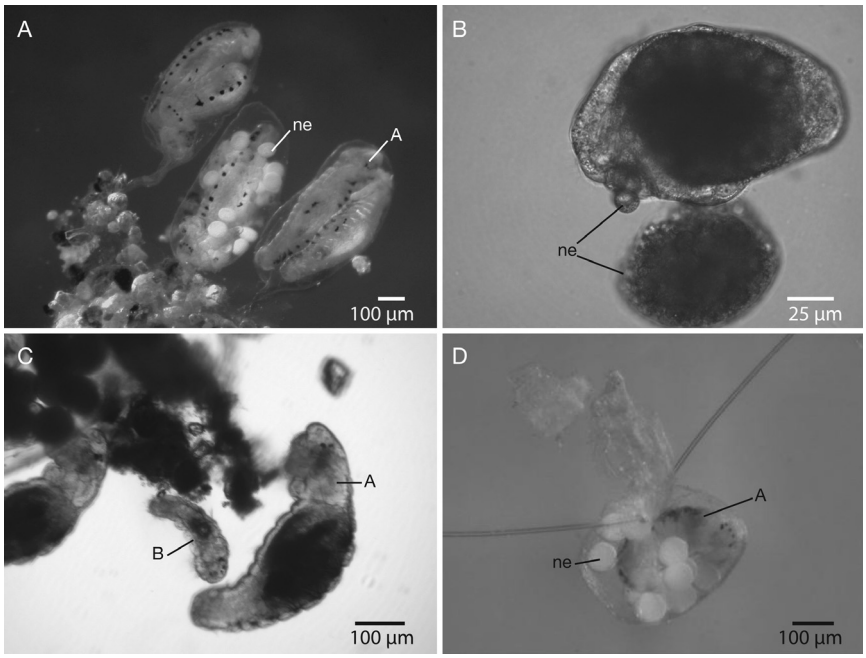


Fig. 1. Capsules and larvae of *Boccardia proboscidea*. **A**. Capsules of females that produce two larval types and nurse eggs in the same capsule. **B**. Presetiger larva feeding by eroding the surface of a nurse egg with the help of the active cilia of prototroch and telotroch. **C**. Siblings released from a capsule show the difference in sizes. In this case, the Type B larva has three setigers and the Type A larva eight setigers. **D**. Manipulated capsule where one Type A larva and nurse eggs are visible; a knotted filament of dental floss has closed the capsule. A, Type A larva; B, Type B larva; ne, nurse egg.

Methods

Collection

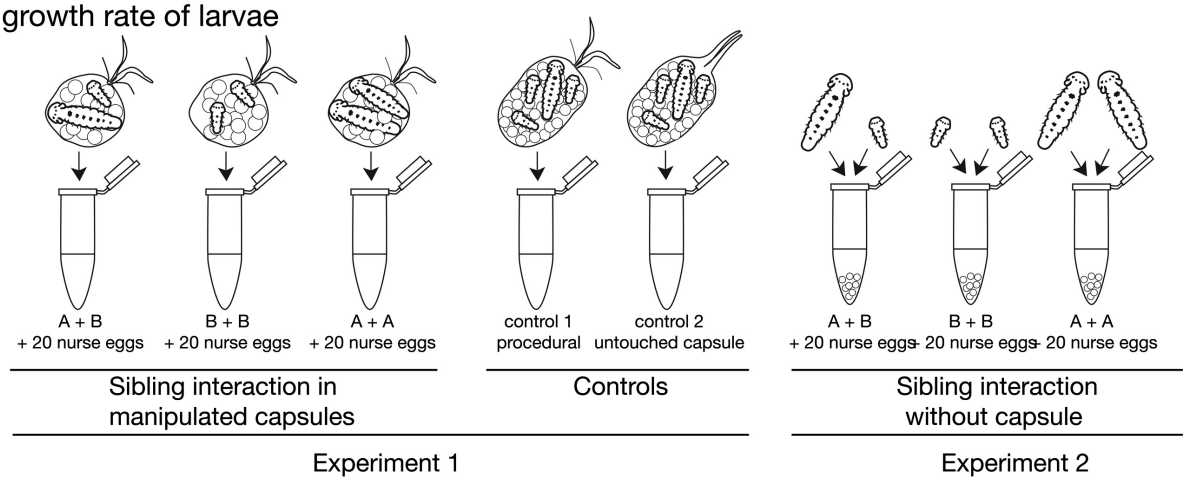
Adults of *Boccardia proboscidea* were collected in August and September 2005 at False Bay (48.45°N, 123.07°W) on San Juan Island, WA, USA where they establish tubes in sand between barnacles on rocks in the intertidal. Sediment was taken to the laboratory and egg capsules were removed from inside the tubes using a dissecting microscope. Capsules were rinsed and kept in 0.45 μm filtered seawater plus antibiotics (streptomycin sulfate and penicillin G, each 50 μg mL^{-1} seawater; Strathmann 1987). All experiments were conducted at ambient light/dark cycles (16 h:8 h for the time of the year that the experiment was performed). In our study, setiger number and body length of larvae were correlated (Pearson correlation: n=84, r=0.93, $p<0.001$). Because setiger number is unaffected by muscle contractions, it is commonly used in polychaete studies as a measure of length. In addition, setiger number provides a more accurate measurement of the stage of development of the larvae, and so we used setiger number to report size and stage of development, and setiger/day to report growth rate in all our experiments.

Preliminary experiments

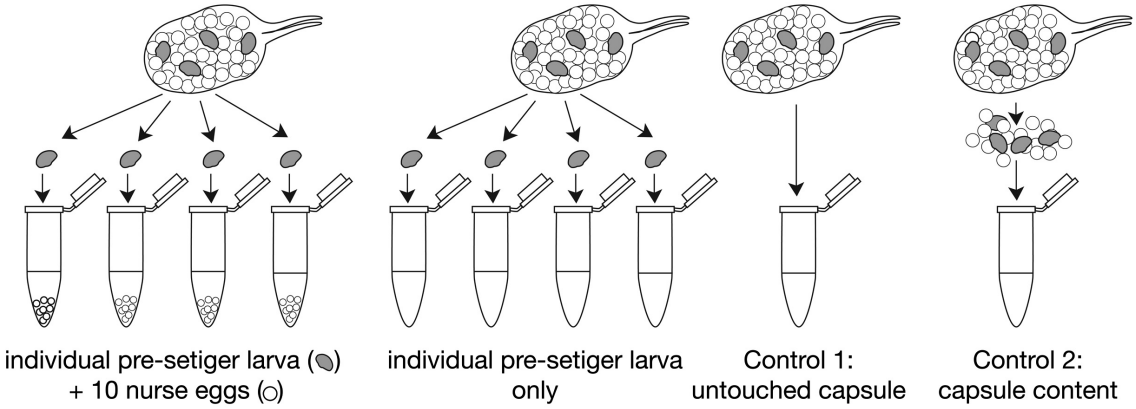
Effect of dye on larval survival and growth rate. In several of the experiments that we performed in this study, we used neutral red to dye Type A or B larvae to identify and follow specific individuals. A preliminary experiment tested the effect of dyeing larvae with neutral red on their survival and growth rate. Seven Type A and seven Type B larvae were dyed with neutral red by placing them for 1 min in a 0.1% solution of neutral red in seawater (Strathmann 1987). Another seven Type A and seven Type B larvae were placed for 1 min in seawater without dye. The average number of setigers of Type A larvae at the beginning of the experiment was of 5.7±0.3 (standard error), and the average number of setigers of Type B larvae was of 4.4±0.2; all of them belonged to the same clutch. These larvae were subsequently grown individually in Eppendorf tubes that contained 20 nurse eggs and 0.45 μm filtered seawater plus antibiotics (streptomycin sulfate and penicillin G, each 50 μg mL^{-1} seawater, as above and in all other experiments). After 4 d, we measured the growth and calculated the growth rate.

Effect of capsule manipulations and larval type on feeding behavior and growth rate of larvae. To validate the *in vitro* methodology of subsequent experiments, we tested if larvae inside intact capsules, manipulated capsules, and artificially excapsulated larvae grown in Eppendorf tubes showed similar feeding behaviors and growth rates. Capsules that already had two distinctive larval types, small larvae of 5.0±0.2 setigers and larger larvae of 8.6±0.5 setigers on average, were selected to test the effect of different larval types on the development of other larvae and the occurrence of sibling cannibalism. Juveniles have 16 setigers or more (Gibson 1997), so selecting Type A larvae that had 8.6±0.5 setigers allowed growth to occur during the experiment. Capsules from five clutches that came from five different females were collected from the field, and were assigned to treatments randomly. Capsules were opened with fine forceps at the capsule stalk area and, with a micropipette, the contents were modified to leave one of three treatments: (AB) 1 Type A larva and 1 Type B larva with 20 nurse eggs, (BB) 2 Type B larvae with 20 nurse eggs, and (AA) 2 Type A larvae with 20 nurse eggs (Figs. 1D, 2A). A general control consisted of untouched capsules, and a procedural control consisted of capsules that were opened and closed without removal of contents. A knot was made with one filament of dental floss to close the manipulated capsules (Freeman 1993; Fig. 1D). Each capsule was placed in a 0.5 mL Eppendorf tube with 0.2 mL FSW and antibiotics and kept at 11°C for a week. This temperature was chosen because it was the average temperature that month in the sediment where *B. proboscidea* were collected (temperature data were obtained by burying two Stowaway TidbiT −5 to 37°C range temperature loggers [Onset Computer Corporation, Massachusetts] in the sediment). Later experiments were performed at 20°C, after confirming that that was the average temperature that the embryos actually experience inside the mother's tube during summer time in the high intertidal zone (Oyarzun 2010). We also used 1.5 mL Eppendorf tubes in subsequent experiments, as it was easier to manipulate and recover embryos in them. We do not have evidence suggesting that size of Eppendorf tubes affected the finding of this particular experiment. In addition, to test the effect of being contained in a capsule, we repeated the same combinations of Type A and B larvae with 20 nurse eggs from the same set of parental capsules, but now swimming free in 0.2 mL FSW plus antibiotics in a 0.5 mL Eppendorf tube (Fig. 2A). We dyed the Type B larvae with neutral red in the AB treatment to confirm ingestion in case

A. Preliminary experiment: Effect of capsule manipulations and larval type on feeding and growth rate of larvae

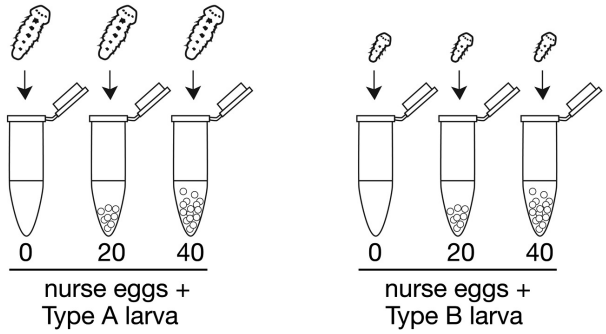


B. Effect of nurse egg provisioning on the determination of larval types

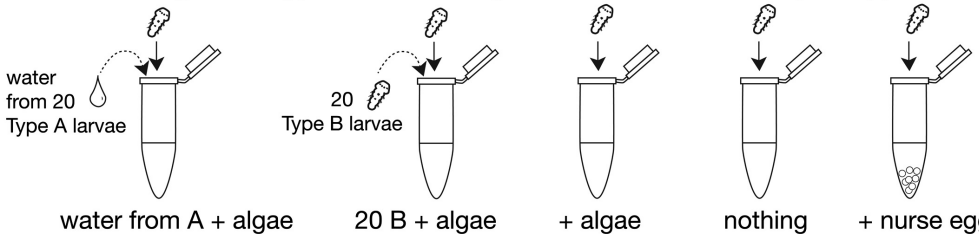


C. Effect of nurse eggs on the growth rate of larvae

C.1. Effect of nurse eggs on the growth rate of larvae



C.2. Effect of siblings and type of food on growth rate and viability of Type B larvae



of cannibalism, and also dyed one of the larvae in the AA and BB treatments to distinguish the identity of the larvae. Each treatment in both experiments was replicated seven times.

Survival of larvae was recorded and growth rates were analyzed with one-way ANOVAs after we tested for normality and homogeneity of variance with Shapiro Wilk W and Brown–Forsythe tests, respectively. When necessary, post hoc analyses with Duncan's Multiple Range test assessed differences among treatments. Transformations were made to meet the assumptions of normality and homogeneity of variance and are reported in the results. All data in this and other experiments were analyzed with the statistical program JMP 8.0.1 (SAS Institute Inc., 1989–2008).

Effect of nurse eggs on the determination of larval types

Capsules that had pre-setiger larvae were selected to test if larval types (A and B) were determined early in development, and to explore the effect of the presence of nurse eggs in larval development. Larvae from capsules from the same female were randomly assigned to one of four treatments: (1) individual larva with nurse eggs; (2) individual larva without nurse eggs or other type of food; (3) larvae staying in an unopened capsule as a control; and (4) larvae staying together with other larvae and nurse eggs coming from an opened capsule (Fig. 2B). In treatments (1) and (2), all larvae from a capsule were isolated and each was placed individually in a 1.5 mL Eppendorf tube with 0.2 mL filtered seawater (FSW) and antibiotics. In treatment (1), ten nurse eggs were added to each tube; the density of nurse eggs was kept constant during the experiment by replacing old nurse eggs and restoring the initial number every 2 d. Larvae in treatment (2) were isolated with no source of food. In treatment (3), an untouched capsule was placed intact in an Eppendorf tube with 0.2 mL FSW and antibiotics. Finally, in treatment (4), the contents of an entire opened capsule were placed in an Eppendorf tube with 0.2 mL FSW and antibiotics. Larvae were kept at 20°C. The experiment was concluded when larvae from the untouched capsules had depleted their nurse eggs. The number of setigers was measured at days 3, 7, 11, and 15 with a compound microscope at 100× final magnification. The four treatments were replicated for larvae coming from four capsules each, and the whole experiment was replicated for two females. Because no differences were detected between larvae from the two females after the experiment concluded, the data were pooled. A total of 78 larvae were followed through development in this experiment. A frequency distribution of setiger number was constructed for each day. At the end of the experiment, the number of setigers was compared between the two groups that resulted from the nurse egg treatment (Type A and B larvae; see Results) with a one-way ANOVA. Also, a one-way ANOVA compared final number of setigers among Type A larvae of the different treatments. We used a post hoc Student's t-test analysis to assess differences among treatments. We had previously checked normality and homogeneity of variance by Shapiro Wilk W and Brown–Forsythe tests, respectively.

Effects of siblings and food on larval growth

Effect of nurse eggs on the growth rate of larvae. Single Type A and Type B larvae were placed in Eppendorf tubes containing 0, 20, or 40 nurse eggs with 0.2 mL of FSW and antibiotics to test the effect of extra-embryonic nutrition on growth rate of larvae. No obvious external morphological features differentiate the two types of larvae except number of setigers, so we waited until we could observe two distinct body size groups of larvae inside capsules (larvae with few setigers and larvae with many setigers) before starting the experiment. At the beginning of the experiment, Type A larvae had an average of 5.5±0.27 setigers, and Type B larvae from the same capsules had an average of 3.7±0.18 setigers. Larvae were haphazardly picked from a pool of larvae from seven different clutches that belonged to seven different females. The nurse eggs were haphazardly picked from a common pool of nurse eggs obtained from the same set of capsules. Each treatment was replicated five times. Tubes were kept at 20°C for 2 d. The number of setigers for each larva was recorded at the beginning and at the end of the experiment and the percentage of missing nurse eggs was documented.

Data were analyzed with a two-way ANOVA (fixed factors: larval type and nurse egg provisioning)

Fig. 2. Schematic representations of the experiments. **A**. Preliminary experiments on the effect of capsule manipulations and larval type on feeding behavior and growth rate of larvae. **B**. The effect of nurse egg provisioning on the determination of larval types. **C**. Effect of nurse eggs on the growth rate of larvae, and effect of siblings and type of food on growth rate and viability of Type B larvae. A, Type A larva; B, Type B larva.

after we tested for normality and homogeneity of variance with Shapiro Wilk W and Brown–Forsythe tests, respectively. We used Duncan's Multiple Range Test to assess differences among treatments. Transformations made to meet the assumptions of normality and homogeneity of variance are reported in the results.

Effects of siblings and type of food on growth rate and viability of Type B larvae. Presence of siblings and food type were manipulated to test if these factors could limit or influence the growth of Type B larvae. The influence of chemical signals from siblings and the type of food available for larvae (i.e., nurse eggs) were tested as potential factors determining Type B larva development. Given that Type B larvae are capable of using phytoplankton as food, *Rhodomonas* sp. was used in the chemical signal treatments to discriminate between feeding inhibition and food type (nurse eggs) effects. Thus, individual Type B larvae all from the same clutch were grown in Eppendorf tubes with 0.2 mL of FSW and antibiotics, plus either (1) "Type A-FSW+algae," filtered seawater that come from another tube where 20 Type A larvae were kept plus *Rhodomonas* sp. at 5000 cells mL^{-1} (in this treatment, Type A larvae could not be placed in the same tube as Type B larvae, as they would have cannibalized them), (2) "Type B-FSW+algae," filtered seawater plus 20 other Type B larvae plus *Rhodomonas* sp. at 5000 cells mL^{-1}, (3) "FSW +algae," filtered seawater plus *Rhodomonas* sp. at 5000 cells mL^{-1}, (4) "FSW+nurse eggs," filtered seawater plus 20 nurse eggs, and (5) "FSW," filtered seawater and no food as a control. The average number of setigers of Type B larvae at the beginning of the experiment was of 5 (±0.2 SE). Each treatment was replicated seven times, kept at 20°C, with water changed every second day. The concentration of *Rhodomonas* sp. was estimated with a hemocytometer. The experiment lasted 7 d, after which larval growth was measured. Type B larvae were dyed with neutral red in each treatment to distinguish them when they were mixed with other larvae as in the "Type B-FSW+algae" treatment. To check for viability of Type B larvae and also the capacity of Type A larvae to grow with phytoplankton as their only food source, we separately grew 50 Type A and 50 Type B larvae from five different clutches from five different females in jars with FSW and fed them *Rhodomonas* sp. at 5000 cells mL^{-1}. We kept larvae at 20°C and followed them until metamorphosis.

Two different one-way ANOVAs tested the effect of food supply and the presence of siblings (fixed factors with five treatments), and tested the viability of Type A and Type B larvae to grow with phytoplankton (fixed factors with two treatments). Normality and homogeneity of variance were checked with Shapiro Wilk W and Brown–Forsythe tests, respectively. Post hoc analyses with Duncan's Multiple Range Test were used to assess differences among treatments. Transformations made to meet the assumptions of normality and homogeneity of variance are reported in the results.

Results

Preliminary experiments

Effect of dye on larval survival and growth rate. The average growth rate of Type A and Type B larvae was not statistically different between the treatments in which larvae were dyed versus the ones in which they were not, validating the usage of dye in the rest of the experiments (two-way ANOVA with fixed factors: dye effect (dye, no dye): $F_{1,15}$=0.019, p=0.89; larval type effect (Type A, Type B): $F_{1,15}$=15.34, p=0.0014; larval type x dye effect: $F_{1,15}$=0.18, p=0.68). There was no mortality of larvae in any of the treatments.

Effect of capsule manipulations and larval type on feeding behavior and growth rate of larvae. Type A larvae showed no significant difference in growth rate between treatments with Type A or Type B larvae present, or between the encapsulated treatment versus the treatment where larvae had been artificially removed from the egg capsule (Table 1, Fig. 3). In this experiment, Type A larvae cannibali-

Table 1. Results of 2-way ANOVA for Type A larvae for the capsule manipulation experiment. Type A and Type B larvae were grown in pairs in Eppendorf tubes with filtered seawater and antibiotics plus 20 nurse eggs. There are two fixed factors: encapsulation (encapsulated or following removal from the egg capsule) and combination of larval types (1 Type A and 1 Type B larvae, or 2 Type A larvae). Larval growth was measured after 1 week and reported as number of setigers per day. Each treatment was replicated seven times. There were no significant differences among treatments.

Source	df	SS	F	p-value
Encapsulation	1	0.824	3.791	0.069
Combination of larvae	1	0.033	0.152	0.702
Encapsulation× combination of larvae	1	0.079	0.362	0.556
Total	19	4.323	—	—

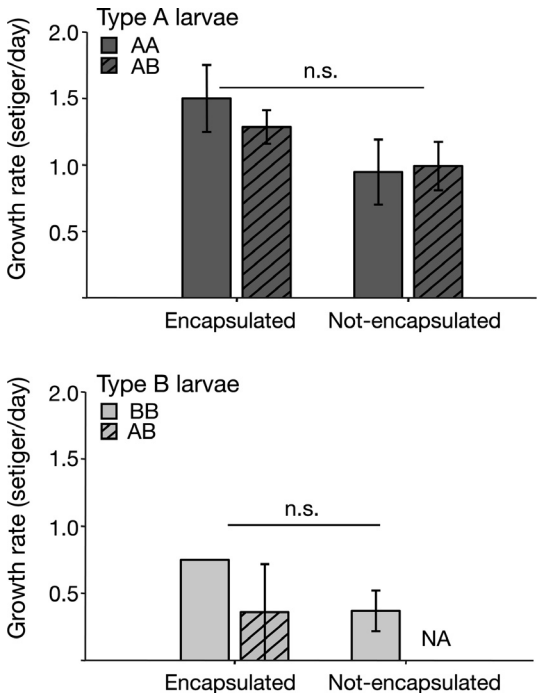


Fig. 3. Results of the capsule manipulation experiment for Type A larvae (upper panel, dark gray) and Type B larvae (lower panel, light gray). For Type A larvae, growth rates are for Type A larvae that grew encapsulated or following removal from the egg capsule in Eppendorf tubes. These Type A larvae grew with another Type A larva (AA) or with a Type B larva (AB). For Type B larvae, growth rates are for Type B larvae that grew encapsulated or following removal from the egg capsule in Eppendorf tubes. These Type B larvae grew paired with another Type B larva (BB) or with a Type A larva (AB). Treatments were replicated seven times. NA indicates a treatment for which no data were available due to 100% cannibalism. Temperature in this experiment was 11°C, and the experiment lasted 1 week. Non-significant differences are indicated as n.s. Error bars indicate standard error.

zed smaller Type B larvae, but never other Type A larvae. A higher proportion of cannibalized Type B larvae occurred in the treatment where larvae had been removed from their capsule (100%, all of them) versus the treatment where larvae were encapsulated (50%, exactly half).

Due to the high rate of cannibalism, there were not enough replicates to perform a two-way ANOVA for Type B larvae, so two separate one-way ANOVAs were performed: one ANOVA compared the two treatments of combinations of larval types (B/B and A/B), and the other compared the two treatments of encapsulation (encapsulated and artificially excapsulated larvae). The growth rate of Type B larvae was not significantly different between the treatment inside a manipulated capsule with Type A or with another Type B larva (one-way ANOVA: $F_{1,10}$=0.01, p=0.91). There was also no significant difference in their growth rate when they were encapsulated versus when they had been removed from an egg capsule (1-way ANOVA: $F_{1,10}$=0.21, p=0.65; Fig. 3). Data from Type B larvae were (log+1) transformed to fulfill the assumption of normality.

Effect of nurse eggs on the determination of larval types

The development of larvae was as described in previous studies (Fig. 1; King 1976; Woodwick 1977; Gibson 1997; Gibson & Gibson 2004). The first three setigerous segments developed simultaneously at the metatrochophore stage, which had two black eyespots. Provisional chaetae (i.e., chaetae that are only present in larva) were present in each segment. Further growth occurred by the addition of setigers terminally. Because there were no significant differences in number of setigers between larvae from the two females at the end of the experiment (one-way ANOVA: $F_{1,68}$=0.03, p=0.87), data were pooled, and larvae were treated as independent units for all comparisons. A total of 78 larvae were followed through development in this experiment.

In all larvae maintained individually in Eppendorf tubes, development was synchronous and indistinguishable among individuals until the third setiger stage. Then, individual larvae in isolation but with nurse eggs available developed into two significantly different size groups. By day 15, a total of 31 larvae had grown significantly faster (Type A) while eating nurse eggs; these had reached an average number of setigers of 13.6±0.1. Five larvae had grown more slowly (Type B) and reached an average number of setigers of only 6.3±1.8 (one-way ANOVA: $F_{1,34}$=492.9, p<0.0001; Fig. 4). Type A larvae started feeding at the metatrochophore stage by eroding the surface of nurse eggs with the help of the active oral and ventral cilia patches (Fig. 1B). Once they had grown more, they were able to ingest the nurse eggs whole. All Type A larvae assigned to the treatment with no nurse eggs developed to only a small size, equivalent to the size of Type B larvae of other treatments (average number of setigers of 5.3±0.3). The final size of individual Type A larvae was different among treatments (one-way ANOVA: $F_{2,41}$=3.45, p=0.041). Post hoc comparisons showed that Type A larvae that grew in isolation with nurse eggs (average number of setigers 13.6±0.1 SE) were not significantly different in final size from the Type

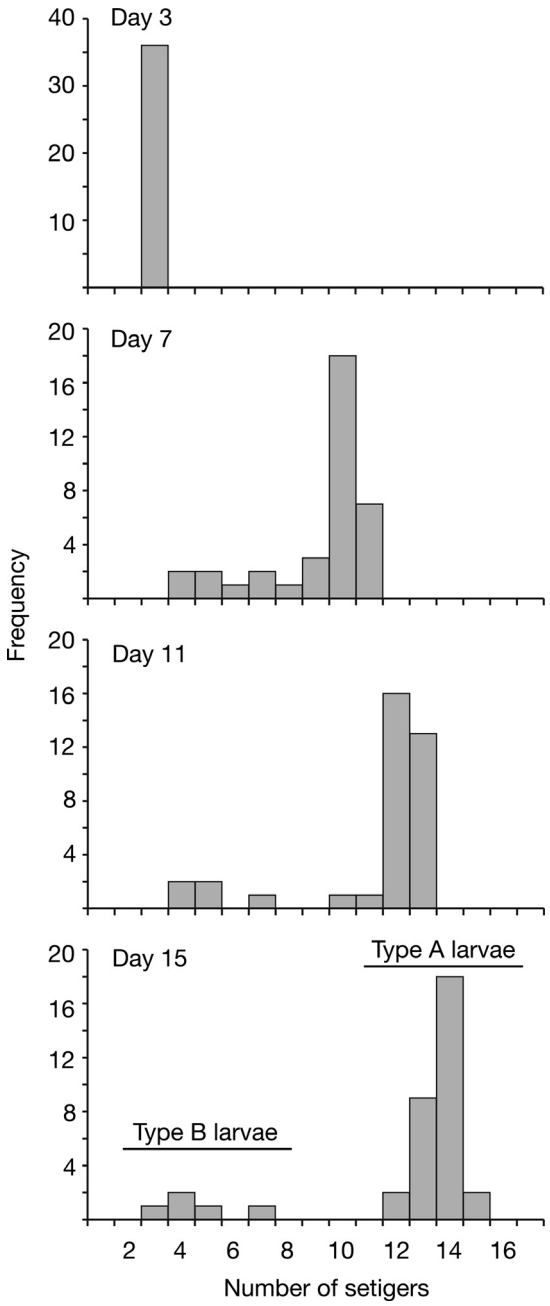


Fig. 4. Results of the experiment on the effect of nurse egg provisioning on the determination of larval types. Each plot shows the frequency distributions of the number of setigers of 39 experimental larvae growing individually in Eppendorf tubes, with nurse eggs as their only food source. The larvae come from two clutches from two females; they were not significantly different at the beginning or the end of the experiment, so data were pooled. Graphs show the distributions of larval size on days 3, 7, 11, and 15. The two larval types, Type A and B, are not evident at the beginning of the experiment, but become progressively apparent over time. Temperature in this experiment was 20°C.

A larvae that grew together with other larvae (average number of setigers of 13.7±0.2 SE) in the capsule content control ($p>0.05$), but they were slightly smaller than the Type A larvae coming from the untouched capsule control (average number of setigers of 14.5±0.4; $p<0.05$). This result suggests that Type A larvae at a very early developmental stage (pre-setiger) might have difficulty finding food in the Eppendorf tube. The mortality during this experiment was 18.1%, but it was evenly distributed among treatments.

Effects of siblings and food on larval growth

Effect of nurse eggs on the growth rate of larvae. Growth rates of Type A and Type B larvae differed significantly in the nurse egg manipulation experiment (Table 2, Fig. 5). The growth rate of Type A larvae (3.0±3.9 setigers/day) was twice the growth rate of Type B larvae (1.5 ± 0.8 setigers/

Table 2. Results of 2-way ANOVA for the effect of nurse eggs on the growth of Type A and Type B larvae. There are two factors: larval type (Type A or Type B) and nurse egg treatment (0, 20, 40). Treatments were replicated 5 times. Type A larvae grew larger than Type B larvae, and both larval types grew larger when given nurse eggs.

Source	df	SS	F	p-value
Larval type	1	3.221	8.382	0.009*
Nurse egg treatment	2	2.795	3.637	0.046*
Larval type×nurse egg treatment	2	1.503	1.956	0.169
Total	24	15.177	—	—

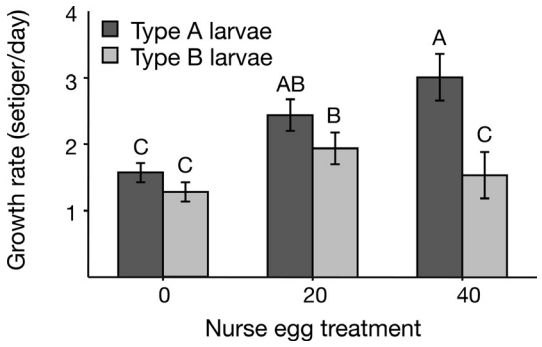


Fig. 5. Results of the nurse egg manipulation experiment for Type A larvae (dark gray) and Type B larvae (light gray). The graph shows the growth rates of Type A and Type B larvae after 48 h with 0, 20, and 40 nurse eggs. Each treatment was replicated five times. Temperature in this experiment was 20°C. Significantly different results are shown as different letters. Error bars indicate standard error.

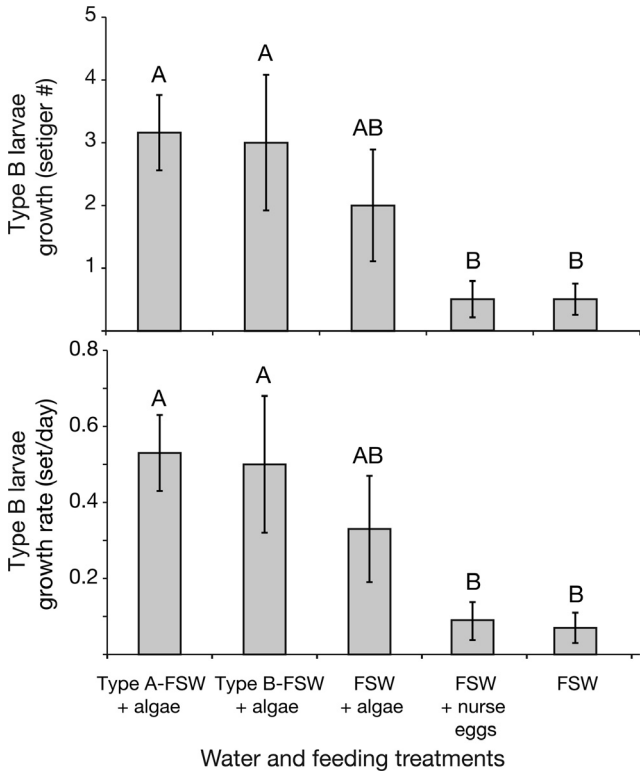


Fig. 6. Results of the experiment on the effects of siblings and food type on the growth rate of Type B larvae. Each of the five treatments consists of one Type B larva plus either of the following: Type A-FSW+algae (FSW from another tube where 20 Type A larvae were kept plus *Rhodomonas* sp. at 5000 cells mL^{-1} [in this treatment Type A larvae could not be in the same tube as they would have cannibalized the Type B larva]); Type B-FSW+algae (FSW plus 20 other Type B larvae plus *Rhodomonas* sp. at 5000 cells mL^{-1}); FSW+algae (FSW plus *Rhodomonas* sp. at 5000 cells mL^{-1}); FSW+nurse eggs (FSW plus 20 nurse eggs); or FSW (FSW and no food). Size is indicated as total number of setigers and growth as setigers per day. Treatments were replicated seven times. Temperature in this experiment was 20°C, and the experiment lasted 1 week. Results of one-way ANOVA are reported in the results section. Growth in treatments without the same letter was significantly different. Error bars indicate standard error.

day) in the treatment with the greater number of nurse eggs. As a general trend, Type A larvae ate more nurse eggs when there were more nurse eggs available, on average, 12.6±3.5 nurse eggs in the 20 nurse egg treatment, and 24.6±3.9 nurse eggs in the 40 nurse egg treatment. Unexpectedly, some Type B larvae were observed ingesting small particles of nurse eggs by eroding the surface, but we did not see Type B larvae ingesting whole nurse eggs. Furthermore, Type B larvae grew more in the treatment with 20 nurse eggs than in the control treatment with no nurse eggs.

Effect of siblings and type of food on growth rate and viability of Type B larvae. Growth rates of Type B larvae were significantly different among treatments (Fig. 6; one-way ANOVA by setiger number $F_{4,19}$=3.71, p=0.02; by setiger/day $F_{4,19}$= 3.68, p=0.02). Duncan's post hoc test showed that when Type B larvae were fed algae, they grew more than when they had no food or nurse eggs as a food source ($p<0.05$). There was no significant difference in their growth rate when they were in the presence of other Type B larvae, in the water coming from Type A larvae, or by themselves ($p>0.05$; Fig. 6). Additionally, Type A and type B larvae developed to metamorphosis and settlement when grown in jars and fed *Rhodomonas* sp., confirming the capacity of both types of larvae to reach metamorphosis with phytoplankton as food after they are removed from capsules.

Discussion

We experimentally manipulated capsules, nurse eggs, and larvae to evaluate the roles of extra-embryonic nutrition and sib–sib interaction as determinant factors of poecilogony in the polychaete *Boccardia proboscidea*. Results showed that (1) experiments *in vitro* are feasible and there was no detectable effect of manipulating the egg capsules or using neutral red as larval marker on the growth rate of the larvae; (2) Type B larvae are viable as previously shown in other studies, and they can complete their development if provided with phytoplankton as a food source; (3) Type A larvae eat more nurse eggs than Type B larvae when they are available; (4) Type A larvae cannibalize Type B larvae when available; (5) larvae of Types A and B can grow in the presence of phytoplankton as their food source, but the growth rate of Type B larvae is slower than that of Type A when feeding on nurse eggs; and (6) the slowing growth of Type B larvae after they have reached five setigers is not the result of inhibition by siblings. Our results support the hypothesis that the two larval types in *B. proboscidea,* which use or do not use nurse eggs as extra-embryonic nutrition inside the capsule, are determined completely genetically or early in development. While the hatching size of Type A larvae is mediated by intracapsular availability of nurse eggs, nurse eggs have little effect on the growth and hatching size of Type B larvae. Additionally, we found high levels of intracapsular cannibalism,

which could reduce the variability of hatching sizes of larvae by eliminating the smaller Type B larvae.

In *B. proboscidea*, larval type and hatching size variability seem to be explained by two mechanisms: larval specialization by early differentiation of capacity of larvae to feed on nurse eggs, and the maternal effect of extra-embryonic nutrition supplied by females, which favors faster development in Type A larvae. In our experiments, we found that when larvae were isolated at a pre-setiger stage, all larvae grew at a similar rate when fed phytoplankton. However, in the presence of nurse eggs, two different larval size classes developed. Most larvae had the capacity to use nurse eggs as a nutritional source. However, Type A larvae grew faster when nurse eggs were available than when they fed on phytoplankton. Moreover, Type A larvae grew faster when there were more nurse eggs. In contrast, a smaller percentage of larvae (Type B) ceased growth when in the presence of nurse eggs, suggesting the inability to either ingest or digest this type of food. A few Type B larvae ingested small pieces of nurse eggs, and we have contradictory evidence that the growth rate of Type B larvae does or does not differ significantly when nurse eggs are available (Figs. 5, 6), indicating variability among larvae that deserves further exploration. In addition, the presence of other larvae did not inhibit or stimulate the growth rate of Type B larvae that have reached the five setiger stage. Overall, these results demonstrate that the larvae differ in ability to eat nurse eggs, and that these differences are determined very early in their development (pre-setiger stage).

We do not yet know if this early differentiation in feeding capacities is triggered by maternal effect, by intracapsular environmental conditions that affect larvae before setiger development, or if it is completely genetically determined. In *Streblospio benedicti* Webster 1879, in which females differ in the sizes of eggs they produce and the development of the resulting larvae, experimental crosses between females and males coming from populations that produce only facultative planktotrophic (initially described as lecithotrophic) versus only planktotrophic larvae recovered substantial additive genetic variance in such life-history traits as ovum diameter, larvae per brood, and the presence of larval swimming setae, but not on larval type, larval length, or larval survivorship (Levin et al. 1991). Their results suggest that, although the relative importance of paternal and maternal influence on the determination of larval type is unclear, poecilogony in *S. benedicti* has a strong genetic component. In the case of poecilogonous species in which different larval modes occur within the same female (e.g., the mixed strategy in *B. proboscidea*, *Boccardia wellingtonensis* Read 1975, *Boccardia polybranchia* (Haswell 1885), *Polydora cornuta, Polydora hoplura* (Claparède 1869), and *Alderia willowi*), there is not yet experimental evidence of a genetic or developmental mechanism associated with the production of two or more larval types. Gibson & Gibson (2004) proposed that poecilogony in *B. proboscidea* has evolved as a sequence heterochrony in morphogenesis, but a close look at the two larval types of this species has not revealed the mechanism. Gibson & Carver (2013) reported some differences in gut and coelom formation in *B. proboscidea* when both larval types had developed together inside a capsule. However, according to the authors, these morphological differences were not sufficient to explain why Type B larvae do not ingest nurse eggs at the same rate as their adelphophagic (Type A) counterparts, and why Type B larvae are better at eating phytoplankton than at eating nurse eggs.

A second mechanism that could regulate larval growth rate and larval hatching sizes within a clutch is maternal allocation of nurse eggs. Although in *B. proboscidea*, Type A and Type B larvae are determined early in development, our experiments also support the hypothesis of a significant role of nurse egg allocation on the developmental rate, cannibalism, and hatching size of larvae. In addition to the differences that we found on feeding capacities among larvae, which allow Type A larvae to advance in development inside a capsule and hatch as juveniles, the presence of greater numbers of nurse eggs could potentially reduce the probabilities of cannibalism on Type B larvae, increasing their chances of hatching as planktotrophs. According to Smith & Gibson (1999) and Gibson et al. (2012), production of nurse eggs in *B. proboscidea* and *P. cornuta* form through an active process that resembles apoptosis (but see Rice & Rice 2009). Females of *B. proboscidea* therefore have the potential to adjust the proportion of larvae that will hatch as advanced larvae or juveniles versus larvae that will hatch as planktotrophs by manipulating the number of nurse eggs inside capsules, or by limiting the ingestion of nurse eggs and siblings by adjusting the hatching time (Oyarzun & Strathmann 2011). In field and common garden conditions with females of *B. proboscidea* of similar size, females from Washington state (USA) provided their larvae with more nurse eggs than did the females from California, suggesting local adaptation (Oyarzun & Strathmann 2011). Also, when females from Washington changed the hatching time from an average of 26 d at 11°C to an average of

15 d at 25°C, they liberated larvae with fewer setigers (i.e., developmentally younger and therefore smaller individuals), showing that females have a mechanism of regulating nurse egg allocation and size of hatching larvae after capsules have been laid (Oyarzun & Strathmann 2011). Females from higher latitudes also brooded their offspring for a longer period in common garden conditions, suggesting that local adaptation of this trait also occurs (Oyarzun et al. 2011). Chia et al. (1996) proposed that poecilogony could have evolved as a bet-hedging strategy in unstable environments, producing offspring with different dispersal potential. Under this scenario, we hypothesize that females of *B. proboscidea* should be able to sense the environment and adjust the number of nurse eggs available for larvae inside capsules, or the hatching time, to change the proportion of different larval types according to environmental quality.

Although we provide evidence that Type B larvae are viable and can complete development to metamorphosis when fed phytoplankton, the actual number that hatch as planktotrophs are few and suffer the disadvantages of intracapsular sibling cannibalism. Moreover, Type B larvae hatch at a small size after which prolonged planktonic growth is necessary, and therefore experience high mortality without the compensation of large numbers (compared with a reproductive strategy in which females produce only planktotrophic larvae). Finally, Type B larvae that hatched after a long period in the capsule look thinner and in poorer condition than those that hatch earlier, which suggests that there also might be a cost of staying in the capsule for longer periods (Gibson 1997; F. unpubl. data). We expect that if these larvae play a role in the dynamics of populations of this species, they would be important in highly variable environments, where they could favor population survival by the export of at least a few offspring to another location at the event of a local catastrophe.

In conclusion, our results demonstrate that both the number of nurse eggs consumed during development and some type of specification that occurs early on in development explain the variability of larval sizes observed in *B. proboscidea*. *Boccardia proboscidea* is a good system with which to study the evolution of larval types, maternal provisioning, and sibling competition. Most previous studies have hypothesized roles of different factors in the development of poecilogonous species, but have not experimentally manipulated capsule contents and larvae to investigate their roles. This study presents simple and useful techniques to manipulate the amount and type of food and sibling interaction to further explore this system. Further experimentation with this and other poecilogonous species could provide insights into the evolution of different modes of development.

Acknowledgments. We thank Richard Strathmann, Billie Swalla, Martha Groom, and Daniel Grünbaum for discussion of ideas and help through this research. Thanks to Alex Primus, Federico Brown, Caroline Faria, Bruno Pernet, Paul Bourdeau, and Melissa Romero for discussion of ideas, help in laboratory, statistical analyses, or proofreading of this manuscript. We thank two anonymous reviewers for feedback. Thanks to Friday Harbor Larval Biology Class of 2005 for early input in this research. NSF grants OCE 0217304 and OCE 0623102, Postdoctoral Project Fondecyt # 3120202, and the Friday Harbor Laboratories of the University of Washington supported this research.

References

Allen JD & Pernet B 2007. Intermediate modes of larval development: bridging the gap between planktotrophy and lecithotrophy. Evol. Dev. 9: 643–653.

Botello G & Krug PJ 2006. Desperate larvae revisited: age, energy and experience affect sensitivity to settlement cues in larvae of the gastropod *Alderia sp*. Mar. Ecol. Prog. Ser. 312: 149–159.

Brante A, Fernández M, & Viard F 2009. Limiting factors to encapsulation: the combined effects of dissolved protein and oxygen availability on embryonic growth and survival of species with contrasting feeding strategies. J. Exp. Mar. Biol. Ecol. 212: 2287–2295.

——— 2013. Non-random sibling cannibalism in the marine gastropod *Crepidula coquimbensis*. PLoS One 8: e67050. doi:10.1371/journal.pone.0067050.

Chia FS, Gibson G, & Qian PY 1996. Poecilogony as a reproductive strategy of marine invertebrates. Oceanol. Acta 19: 203–208.

Clemens-Seely K & Phillips NE 2011. Effects of temperature on hatching time and hatchling proportions in a poecilogonous population of *Haminoea zelandiae*. Biol. Bull. 221: 189–196.

Collin R 2003. Worldwide patterns of development in calyptraeid gastropods. Mar. Ecol. Prog. Ser. 247: 103–122.

——— 2004. Phylogenetic effects, the loss of complex characters, and the evolution of development in calyptraeid gastropods. Evolution 58: 1488–1502.

——— 2012. Nontraditional life-history choices: what can "intermediates" tell us about evolutionary transitions between modes of invertebrate development? Integr. Comp. Biol. 52: 128–137.

David AA, Matthee CA, & Simon CA 2014. Poecilogony in *Polydora hoplura* (Polychaeta: Spionidae) from commercially important molluscs in South Africa. Mar. Biol. 161: 887–898.

Duchêne JC 2000. Effects of poecilogony and delayed larval emission on recruitment of subantarctic population of *Boccardia polybranchia* (Polychaeta: Spionidae). Bull. Mar. Sci. 67: 311–319.

Ellingson RA & Krug PJ 2006. Evolution of poecilogony from planktotrophy: cryptic speciation, phylogeography, and larval development in the gastropod genus *Alderia*. Evolution 60: 2293–2310.

Felsenstein J 1985. Phylogenies and the comparative method. Am. Nat. 125: 1–15.

Freeman G 1993. Regional specification during embryogenesis in the articulate brachiopod Terebratalia. Dev. Biol. 160: 196–213.

Giard A 1905. La poecilogonie. Comp. Rend. Six. Congr. Internationale de Zool, Berne, pp. 617–646.

Gibson GD 1997. Variable development in the spionid *Boccardia proboscidea* (Polychaeta) is linked to nurse egg production and larval trophic mode. Invertebr. Biol. 116: 213–226.

Gibson GD & Carver D 2013. Effects of extra-embryonic provisioning on larval morphology and histogenesis in *Boccardia proboscidea* (Annelida, Spionidae). J. Morphol. 274: 11–23.

Gibson GD & Chia FS 1995. Developmental variability in the poecilogonous opisthobranch *Haminoea callidegenita:* life-history traits and effects of environmental parameters. Mar. Ecol. Prog. Ser. 121: 139–155.

Gibson GD & Gibson AJF 2004. Heterochrony and the evolution of poecilogony: generating larval diversity. Evolution 58: 2704–2717.

Gibson GD, Hart C, Coulter C, & Xu H 2012. Nurse egg form through an active process of apoptosis in the spionid *Polydora cornuta* (Annelida). Integr. Comp. Biol. 52: 151–160.

Hartman O 1940. *Boccardia proboscidea*, a new species of spionid worm from California. J. Wash. Acad. Sci. 30: 382–387.

——— 1941. Some contributions to the biology and life history of Spionidae from California. Allan Hancock Pac. Exped. 7: 289–324.

Hoagland KE & Robertson R 1988. An assessment of poecilogony in marine invertebrates: phenomenon or fantasy. Biol. Bull. 174: 109–125.

Jeffery CH, Emlet RB, & Littlewood DTJ 2003. Phylogeny and evolution of developmental mode in temnopleurid echinoids. Mol. Phylogenet. Evol. 28: 99–118.

Kamel SJ & Grosberg RK 2012. Exclusive male care despite extreme female promiscuity and low paternity in a marine snail. Ecol. Lett. 15: 1167–1173.

Kamel SJ, Grosberg RK, & Marshall DJ 2010a. Family conflict in the sea. Trends Ecol. Evol. 25: 442–449.

Kamel SJ, Oyarzun FX, & Grosberg RK 2010b. Reproductive biology, family conflict, and size of offspring in marine invertebrates. Integr. Comp. Biol. 50: 619–629.

King K 1976. The life history of *Boccardia proboscidea* Hartman (Polychaeta: Spionidae). Master's thesis, Department of Biology, California State University, Long Beach, California. 118 pp.

Knott K & McHugh D 2012. Introduction to symposium: poecilogony—a window on larval evolutionary transitions in marine invertebrates. Integr. Comp. Biol. 52: 120–127.

Krug PJ 1998. Poecilogony in an estuarine opisthobranch: planktotrophy, lecithotrophy, and mixed clutches in a population of the ascoglossan *Alderia modesta*. Mar. Biol. 132: 483–494.

——— 2009. Not my "Type": larval dispersal dimorphisms and bet-hedging in ophistobranch life histories. Biol. Bull. 216: 355–372.

Krug PJ, Ellingson RA, Burton R, & Valdés Á 2007. A new poecilogonous species of sea slug (Opisthobranchia: Sacoglossa) from California: comparison with the planktotrophic congener *Alderia modesta* (Lovén, 1844). J. Molluscan Stud. 73: 29–38.

Levin LA & Huggett DV 1990. Implications of alternative reproductive modes for seasonality and demography in an estuarine polychaete. Ecology 71: 2191–2208.

Levin LA, Zhu J, & Creed E 1991. The genetic-basis of life-history characters in a polychaete exhibiting planktotrophy and lecithotrophy. Evolution 45: 380–397.

Mackay J & Gibson G 1999. The influence of nurse eggs on variable larval development in *Polydora cornuta* (Polychaeta: Spionidae). Invertebr. Reprod. Dev. 35: 167–176.

McEdward LR 1995. Ecology of Marine Invertebrate Larvae. CRC Press, Boca Raton, FL. 480 pp.

Moran AL & Emlet RB 2001. Offspring size and performance in variable environments: field studies on a marine snail. Ecology 82: 1597–1612.

Oyarzun FX 2010. Brooding mothers, sibling rivals, and population structure in Boccardia proboscidea, a polychaete with alternative reproductive modes. PhD thesis, University of Washington, Seattle, Washington. 139 pp.

Oyarzun FX & Strathmann RR 2011. Plasticity of hatching and the duration of planktonic development in marine invertebrates. Integr. Comp. Biol. 51: 81–90.

Oyarzun FX, Mahon AR, Swalla BJ, & Halanych KM 2011. Phylogeography and reproductive variation of the poecilogonous polychaete *Boccardia proboscidea* (Annelida: Spionidae) along the West Coast of North America. Evol. Dev. 13: 489–503.

Rice SA & Rice KA 2009. Variable modes of larval development in the *Polydora cornuta* complex (Polychaeta: Spionidae) are directly related to stored sperm availability. Zoosymposia 2: 397–414.

Rivest BR 1983. Development and the influence of nurse egg allotment on hatching size in *Searlesia dira* (Reeve, 1846) (Prosobranchia, Buccinidae). J. Exp. Mar. Biol. Ecol. 69: 217–241.

SAS Institute Inc 1989-2008. JMP, Version 8.0.1. SAS Institute Inc, Cary, North Carolina.

Smith HL & Gibson GD 1999. Nurse egg origin in the polychaete *Boccardia proboscidea* (Spionidae). Invertebr. Repr. Dev. 35: 177–185.

Spight TM 1976. Hatching size and the distribution of nurse eggs among prosobranch embryos. Biol. Bull. 150: 491–499.

Strathmann RR 1985. Feeding and nonfeeding larval development and life-history evolution in marine invertebrates. Annu. Rev. Ecol. Syst. 16: 339–361.

Strathmann MF 1987. Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast: Data and Methods for the Study of Eggs, Embryos, and Larvae. University of Washington Press, Seattle, Washington. 670 pp.

Strathmann RR 1990. Why life histories evolve differently in the sea. Am. Zool. 30: 197–207.

——— 1993. Hypothesis on the origins of marine larvae. Annu. Rev. Ecol. Syst. 24: 89–117.

——— 1995. Peculiar constraints on life histories imposed by protective or nutritive devices for embryos. Am. Zool. 35: 426–433.

Thorson G 1946. Reproductive and larval development of Danish marine bottom invertebrates. Medd. Kom. Dan. Fisk. Havunders, ser. Plankton 4: 1–523.

——— 1950. Reproduction and larval ecology of marine bottom invertebrates. Biol. Rev. 25: 1–45.

Woodwick KH 1977. Lecithotrophic larval development in *Boccardia proboscidea* Hartman. In: Essays on Polychaetous Annelids in Memory of Dr. Olga Hartman. Reish DJ & Fauchald K, eds., pp. 347–371. University of Southern California, Los Angeles, California.

Wray GA & Raff RA 1991. The evolution of developmental strategy in marine invertebrates. Trends Ecol. Evol. 6: 45–50.