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Variable modes of larval development in the *Polydora cornuta* complex (Polychaeta: Spionidae) are directly related to stored sperm availability

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Abstract

Reproductive crosses between geographically separated populations of the nominal species, *Polydora cornuta*, support the hypothesis that the Florida/ Gulf of Mexico populations represent a single, potentially interbreeding lineage that is reproductively isolated from West Coast (California) and East Coast (Carolinas to Maine) populations. Previous research has indicated that California populations are reproductively compatible with worms from North Carolina but reproductively isolated from Maine populations. In spite of these species-level differences, all populations of this nominal species deposit egg capsules inside the female's tube that usually develop into three-chaetiger planktonic larvae measuring about 200 µm in length. Although adelphophagy (feeding upon unfertilized eggs within an egg capsule) has been reported in some populations of *P. cornuta* and in numerous other spionid polychaetes, the relationship between stored sperm in the female parent and the size of larvae within capsules has not been explored. We raised isolated female *P. cornuta* from three genetically and reproductively distinct populations (Florida, California and Maine) over a period of about 16 weeks and determined percent fertilization and larval size in successive spawnings over time until the females ran out of stored sperm. As each female used up stored sperm during successive spawnings, the percent of fertilized eggs per capsule declined and larval size at release increased. In some cases, the largest larvae produced by an isolated female were 114% larger than the smallest larvae produced by the same female. Larvae inside capsules containing unfertilized eggs fed upon these eggs and grew larger than larvae that did not have unfertilized eggs to feed upon. The effects of producing larger larvae following stored sperm depletion were completely reversed by transfer of fresh spermatophores to the isolated females. Variable larval size produced by a single female worm (poecilogony) may therefore be a result of stored sperm limitations rather than a genetically determined reproductive strategy in this species complex.

Key words: variable larval development, reproductive isolation, cryptic species, sperm storage

Introduction

Cryptic species complexes are well known among the polychaetes (Hoagland & Roberts 1988) and continue to be discovered through studies of comparative genetics and reproductive biology (Rice et al. 2008). Here we report the results of reproductive crosses between populations of the widely distributed cryptic species complex, *Polydora cornuta* Bosc and summarize the known distributions of these species in North America. In addition, we have documented the occurrence of variable modes of larval development in several North American populations and present results identifying a contributing proximal cause of variable larval size within single broods of larvae.

Reproductive isolation between North American populations of the morphological species, *P.*

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cornuta was first reported by Rice & Simon (1980). Additional populations were tested for reproductive compatibility by Rice (1991) and genetic data combined with laboratory crosses was reported by Rice et al. 2008. The combined results suggest that there are at least three separate, reproductively and genetically distinct lineages of this morphologically indistinguishable species complex in North America. The present report summarizes the known distributions of these lineages including additional population crosses from Florida and the Northern Gulf of Mexico.

Reproduction in spionid polychaetes has been studied extensively and has been recently reviewed by Blake & Arnofsky (1999) and Blake (2006). A wide range of plasticity has been reported among species in this group with regard to processes of gametogenesis, spawning behavior, brood protection, and larval development (Blake & Arnofsky 1999). Several examples of developmental plasticity have been reported within a single species involving differences, for example, in egg and sperm morphology, larval morphology, larval trophic mode, and larval planktotrophy or lecithotrophy (Blake & Arnofsky 1999, Table 4). The possibility that some of these reports may involve cryptic species remains to be fully explored.

In many spionid polychaetes, females deposit their eggs inside some sort of brooding structure within their tube and tend to the embryos as they pass through early development (reviewed in Blake 2006). In species with small eggs, larvae are released at a relatively small size and feed for some time in the plankton prior to settling and undergoing metamorphosis. In species that produce larger eggs, however, larvae may grow to much larger sizes and be ready to settle and metamorphose close to the time of release from the brood structures (see Levin & Bridges 1995; Jaeckle 1995 for reviews). Occasionally, unfertilized eggs or eggs that have stopped developing are present within brood structures along with developing embryos. These non-developing eggs may be consumed by developing embryos in a process called adelphophagia (Simon 1967; Blake 1969b; Blake & Arnofsky 1999). Some species within the Subfamily Spioninae routinely deposit non-developing eggs within brood structures along with developing embryos. In some of these species, the non-developing eggs are morphologically identical to the pre-cleavage fertilized eggs (Woodwick 1977; Blake 1969b) while in other species, the non-developing eggs are morphologically different from developing zygotes and may break up into small yolk granules within the brood structure (Rasmussen 1973; Blake & Woodwick 1975). In either case, the non-developing eggs may be consumed by developing embryos and thus provide an additional source of nutrition resulting in large yolky larvae that may have different trajectories toward settlement and metamorphosis than smaller larvae following release. The proximal mechanisms resulting in the presence of non-developing eggs within brood structures along with developing embryos have not been fully identified although Blake (1969b) speculated that sperm availability may be a contributing factor.

The ability of a single, sexually reproducing species to utilize two or more different modes of development has been referred to as poecilogony (Giard 1905) and its occurrence among marine invertebrates has been reviewed by Hoagland & Robertson (1988). Some of the reported cases of poecilogony in spionid polychaetes involve geographically separated populations of what appear to be a single species based upon morphological criteria. Closer examination of these populations has indicated that separate but morphologically similar species are involved (Hoagland & Robertson 1988). In few cases of suspected poecilogony has the reproductive compatibility or genetic divergence been assessed between populations exhibiting different modes of development. Among the eight examples of suspected poecilogonous species in the Spionidae listed by Blake & Arnofsky (1999) only a few have been well studied and appear to be true cases of this phenomenon; these include *Streblospio benedicti* Webster, *Boccardia proboscidea* Hartman, *Pygospio elegans* Claparéde and *Polydora cornuta* (this last taxon was not included by Blake & Arnofsky 1999, but see below).

In *S. benedicti*, different modes of larval development (planktotrophy or lecithotrophy) have been reported in different populations and even within some sympatric populations (Levin 1984; Levin & Creed 1986; Levin & Bridges 1994; 1995). Specimens from the Pacific Coast, the western Gulf of Mexico, and parts of the Atlantic Coast displayed lecithotrophic larval development while some populations from the Gulf of Mexico and the Atlantic Coast produced planktotrophic larvae (Levin 1984). Only at one site in North Carolina were both planktotrophic and lecithotrophic forms present at the same location. Laboratory crosses between individuals from planktotrophic and lecithotrophic populations from different geographic regions indicated that these different forms were reproductively compatible (Levin 1984). Populations from Florida and the northern and eastern Gulf of Mexico with only planktotrophic larval development turned out to be a separate species, *S. gynobranchiata* (Rice & Levin 1998; Schulze et al. 2000). Analysis of mtDNA sequence data from North American populations of *S. benedicti* and *S. gynobranchiata* indicated that lecithotrophic and planktotrophic forms of *S. benedicti* represent a single species that is distinct genetically and reproductively from *S. gynobranchiata* (Schulze et al. 2000).

Boccardia proboscidea has a wide geographical range and has been reported to display up to three different developmental patterns (Petch 1988; Gibson 1997). Populations from California (Hartman 1940; Gibson 1997), British Columbia (Gibson et al. 1999) and Australia (Petch 1988; Blake & Kudenov 1981) have been studied and found to exhibit variable modes of larval development from planktotrophy to lecithotrophy to direct development. Lecithotrophic larvae feed upon unfertilized eggs within their brood capsule and grow to substantially larger sizes than planktotrophic larvae. Gibson (1997) reported that offspring from planktotrophic forms were interfertile with offspring from lecithotrophic forms in the laboratory and that maternal developmental mode was retained in subsequent broods. Gibson et al. (1999) compared planktotrophs and lecithotrophs from southern California with lecithotrophs from British Columbia using RAPD-PRC haplotypes and adult morphology. They reported that no significant differences in adult morphology were detected and that genetic variance was more related to geographic origin than to developmental mode.

Three modes of reproduction have been reported in *Pygospio elegans* (Gudmundsson 1985; Blake & Arnofsky 1999). These include asexual reproduction via fragmentation in some populations as well as sexual reproduction with planktotrophic or lecithotrophic larval development in other populations (Gudmundsson 1985). This species occurs throughout Europe as well as some parts of North America. Comparative adult morphology and molecular genetic analyses have not been reported with the exception of Morgan et al. (1997) who compared French and English populations using allozymes and found no significant genetic differentiation.

Polydora cornuta (formerly *P. ligni* Webster, see Blake & Maciolek 1987) has a wide geographical distribution and has been considered to be a single species with broad dispersal capabilities (Radashevsky 2005). Other assessments of this species have reported significant reproductive isolation between geographically separated populations (Rice 1991) and large genetic differences coupled with reproductive isolation in three North American populations (Rice et al. 2008). Each of these separate lineages of *P. cornuta* in North America has been found to produce variable types of larvae (planktotrophs without adelphophagy and lecithotrophs with adelphophagy). Further, these different larval types were produced by the same female worms in response to the availability of stored sperm. In this report, we present the details of this larval transition phenomenon in populations of the nominal species, *P. cornuta* from Florida, California, and Maine. Using isolated female worms in the laboratory, we have been able to reversibly control the type of larvae produced by individual females over time. We believe that this represents the first documentation of a proximal cause for variable larval size within an individual polychaete.

Materials and methods

Worms were collected from Tampa Bay as planktonic larvae and allowed to settle in the laboratory. *Polydora* larvae are available in Tampa Bay and adjacent waters throughout the year with peaks in abundance during the early spring (January–March). The Tampa Bay worms used in these experiments were collected between 2001 and 2005. The individual worms used in the variable larval development studies were collected in spring 2005. The metamorphosed worms were transferred to monocultures and raised to sexual maturity. Larvae produced from these laboratory cultures were raised and used in reproductive experiments. Specimens from California were collected as adults (by Dr. B. Pernet) from Anaheim Bay and shipped to Florida via overnight express arriving in Tampa on June 1, 2005. These worms were maintained in the laboratory and their offspring used in reproductive experiments. Two separate collections were made by Dr. S. Lindsay from Lowes Cove near the Darling Marine Laboratory in Maine. The first collection was made in June 2001 and the second in June 2003. Intertidal mud cores were collected at the site and returned to the lab at the University of Maine, Orono, where they were sieved and *Polydora* were separated from other worms before overnight shipping to Tampa. Upon arrival, *P. cornuta* were isolated and placed into Petri dish cultures for experiments. Reproductive crosses were also carried out between worms from Tampa Bay and specimens collected by Katie McGhee in 2004 near Wakulla, Florida (Florida Panhandle); specimens collected in Chesapeake Bay, Virginia by Linda McCann in 1995; specimens collected from Fort Pierce Inlet (Florida East Coast) by us in 1998 and 2003; and specimens collected from Mobile Bay, Alabama, by Stefan Schulze in 1998.

Culture procedures. Adult worms were maintained as separate populations in physically separated portions of the laboratory. Stock cultures of adult worms were kept in 100 x 25 mm plastic Petri dishes with 10–50 worms per dish. Isolated males and females were maintained in 12-well and 6-well tissue culture dishes (Corning Cell Wells) for gamete studies and experimental crosses. All adult cultures were fed every 2–3 days along with water changes. Adult food consisted of natural sediment from the mouth of Tampa Bay that had been blended in a blender and frozen at –20°C for at least four weeks followed by a second blending prior to use. Sediment was added to culture containers with a plastic pipette and water changed after gently washing loose sediment and fecal matter out of the dishes. In addition to sediment, 10–30 drops of cultured *Tetraselmis* and *Nanochloropsis* (both from Florida Aqua Farms) were added to the adult cultures depending upon the size of the dish and density of adult worms. Seawater was collected from Buncess Pass near the mouth of Tampa Bay, transported to the laboratory at the University of Tampa and filtered through Qualitative P5 filter paper (Fisher Scientific). Filtered seawater was adjusted to 25‰ salinity with deionized water and stored in Nalgene carboys. Swimming larvae were collected from adult cultures by pouring the water from the adult culture dish through a 35-µm-mesh screen (separate screens were used for each population to prevent contamination) and gently washing the larvae into a clean 100 x 25 mm Petri dish. Larvae were fed and water changed every 1–2 days. Larval food consisted of cultured *Tetraselmis* and *Nanochloropsis* supplemented with diluted Rotirich (Florida Aqua Farms). Larval cultures were maintained on an orbital shaker (Daigger) rotating at 44 rpm to keep the larvae and food in suspension. Larval cultures were provided with a final concentration of 6.5 x 10^6 cells per ml of phytoplankton. As larvae grew and began to settle on the bottom of the dishes, they were removed from the orbital shaker, provided with adult food (sediment) and maintained as above for adult cultures. Laboratory temperature was maintained as close as possible to a range of 22–25°C.

Reproductive crosses. Selected juvenile worms were transferred from larval cultures into 12-well tissue culture dishes at metamorphosis, one worm per well. At maturity, female worms were

transferred into 6-well tissue culture dishes, one worm per well and monitored daily for egg capsule production by inverting the dish on the stage of a dissecting microscope and viewing the worm through the bottom of the dish. Male worms were transferred into 60-mm Petri dishes and monitored for spermatophore production. Spermatophores were collected daily from the male culture dishes of each of the three populations. Female worms were observed daily until egg capsules were deposited and determined to be entirely unfertile (confirming that the females contained no stored sperm). Females in 6-well dishes were then given spermatophores by hand from another population using a fine-tip glass pipette. Following spermatophore transfer, the worms were examined daily for egg capsule production. Newly spawned eggs were observed daily for 1–3 days to determine if larval development was occurring. Each interpopulation cross was repeated with a fresh batch of spermatophores following each deposition of egg capsules for a target total of two or three separate spawnings. Following the last interpopulation spermatophore transfer and subsequent egg capsule deposition, each female was given spermatophores from her population of origin and monitored for new egg capsules. If an individual female worm failed to produce fertilized eggs and larvae following this intrapopulation cross, she was excluded from the data set. All combinations of male/female crosses were attempted for each population.

Histology and electron microscopy. Adult specimens for scanning electron microscope (SEM) studies were fixed at room temperature in either 10% buffered formalin or 2.5% glutaraldehyde in 0.2M PO_4 plus 0.14M NaCl buffer, postfixed in 2% OsO_4 in 0.15M $NaHCO_3$ and dehydrated in ethanol to acetone for critical point drying. Dried specimens were mounted and coated with gold-palladium and viewed on a Novascan 30 SEM (Zeiss). Specimens used for histological studies and transmission electron microscopy (TEM) were fixed in glutaraldehyde and osmium as above, dehydrated to propylene oxide and embedded in Embed 812 epoxy (Ted Pella, Inc). One micron sections were cut on a Reichert-Jung Ultracut E ultramicrotome, stained with methylene blue and azure II, and photographed with an Olympus BH2 photomicroscope. Thin sections were cut with a diamond knife, stained with aqueous uranyl acetate and lead citrate, and viewed on a Hitachi H500 TEM. One micron epon sections were prepared for SEM by removal of epon using KOH, methanol, and propylene oxide (Maxwell 1978) followed by critical point drying and coating as above.

Variable larval development. Worms used in variable larval development experiments were selected from culture dishes upon settlement and transferred to 12-well tissue culture dishes, one worm per well. At sexual maturity, female worms were transferred to 6-well tissue culture dishes, one worm per well, and given spermatophores from male worms cultured separately. Female worms were observed for reproductive state through the bottoms of the 6-well dishes. When egg capsules were produced, a sample of eggs (usually ±300) was removed from the brood and examined for signs of development at 100× on a compound microscope. The presence of cleavage furrows or the appearance of polar bodies was taken to be a sign of development following fertilization. The remaining egg capsules in each brood were allowed to remain inside the female's tube until the larvae were released. Released larvae were collected, relaxed in isotonic magnesium chloride and measured for total length at 100x on a compound microscope. The mean percent fertilization and mean larval size were determined for successive broods of eggs for each isolated female until stored sperm were nearly or completely depleted and fertilized eggs were rare. Following depletion of stored sperm, additional spermatophores were transferred to the female and the entire process repeated.

Data analysis. The variance of larval size distributions between broods was not homogeneous so comparisons were made using the Kruskal-Wallis test (Statview 5.0, SAS 1999). The correlation between mean larval size and percent fertilization was made with Spearman Rank Correlation Coefficient (Statview 5.0, SAS 1999). Pairwise significance testes between population means were

done using Fisher's Protected Least Significant Difference (Fisher PLSD) method as modified in StatView to use unequal as well as equal sample sizes.

Results

Reproductive crosses

The results of laboratory crosses between North American populations of *Polydora cornuta* clearly indicate the existence of at least three separate lineages (Table 1). The reproductive compatibility data are incomplete since not all populations were available in the laboratory at the same time and thus crosses between some combinations of populations were not attempted. Reproductive compatibility between populations from Mobile Bay (MB), Wakulla (WK), & Fort Pierce (FP) with worms from Tampa Bay (TB) suggests that the Northern Gulf of Mexico/ Florida populations represent a single biological species. Based upon crosses with TB individuals, the California (CA), North Carolina (NC) & Chesapeake Bay (CB) populations appear to represent a different species from the Gulf of Mexico/ Florida populations. Reproductive isolation between CA and Maine (ME) as well as between TB and ME populations suggest that ME represents a third species while reproductive compatibility between CA and NC suggests a recent common ancestor for the latter two populations. The known distributions of these lineages are depicted in Fig. 1. Insufficient data are available to identify the precise species boundaries between these lineages. We will therefore refer to them as the Florida/Gulf of Mexico lineage, the Central Atlantic Coast/Pacific Coast lineage, and the Northern Atlantic Coast lineage.

TABLE 1. Results of laboratory crosses between North American populations of *Polydora cornuta*, including data from Rice (1991) and Rice et al. (2008). TB, Tampa Bay Florida; WK, Wakulla Florida; FP, Fort Pierce Florida; CA, Southern California; ME, Maine; NC, North Carolina; CK, Chesapeake Bay Virginia; MB, Mobile Bay Alabama. All within-population crosses were successful with high levels of fertilization and viable larvae. Each cross was replicated at least six times with different female worms.

Populations	Fertilization	Larvae	Comments
TB x WK	yes	yes	Full reproductive compatibility
TB x MB	yes	yes	Full reproductive compatibility
TB x FP	yes	yes	Variable results among crosses
TB x NC	no	no	Complete reproductive isolation (Rice 1991)
TB x CB	no	no	Complete reproductive isolation
TB x ME	yes	no	Low % fertilization, no larvae (Rice et al. 2008)
TB x CA	yes	no	Asymmetric results (Rice et al. 2008)*
CA x ME	yes	no	Low % fertilization, no larvae (Rice et al. 2008)
NC x CA	yes	yes	Full reproductive compatibility (Rice 1991)

*CA females x TB males (42.0 ± 10.7 percent fertilization); CA males x TB females (4.2 ± 8.4 percent fertilization)

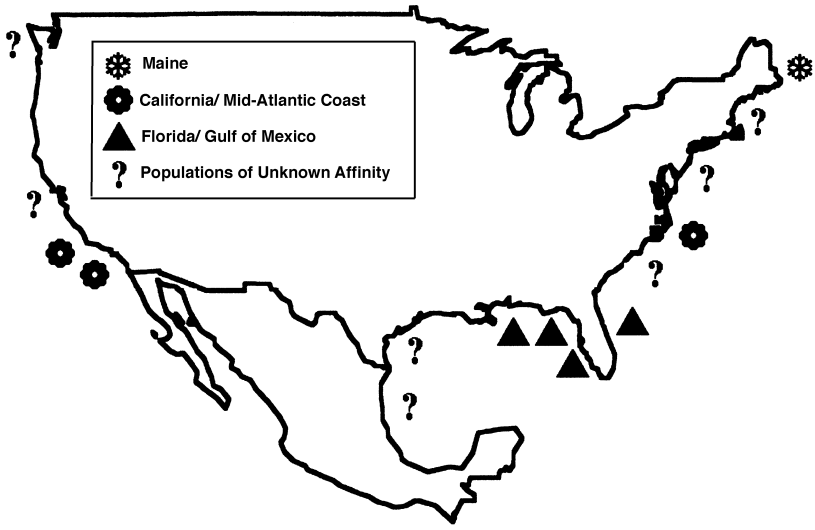

FIGURE 1. Distribution of *Polydora cornuta* lineages in North America based upon reproductive cross results and molecular genetics. Locations with the same symbol exhibit reproductive compatibility and genetic similarity (legend on figure). Question marks identify known populations but genetic and reproductive data are not complete.

Sperm storage

Female *Polydora cornuta* from each population studied are capable of storing sperm in seminal receptacles for later use during spawning. The seminal receptacles are paired, segmental sacs that open dorsally on each gamete-bearing chaetiger. Sperm were also observed inside blind pockets located along the middle sections of the paired, segmental nephridia in females (Fig. 2). Since the eggs exit the female's body through the nephridial canal, this is likely the site where fertilization occurs. The means by which the stored sperm travel from the dorsal seminal receptacles into the nephridial canal is unknown. By documenting percent fertilization in successive broods for isolated females, we were able to estimate the duration of sperm storage following a single transfer of spermatophores. For the CA females, several worms were still producing larvae after ±40 days following a single transfer of spermatophores. For six CA females observed over successive broods, the mean percent of fertilized eggs remained high for the first three broods (±97%) then decreased over successive broods reaching ±15% fertilized eggs after 8–9 broods (Fig. 3). Similar results were obtained for the ME and FL populations (data not shown).

Variable larval development

Each population of *Polydora cornuta* cultured in the laboratory displayed a tendency toward increasing mean larval size produced by isolated females as supplies of stored sperm were depleted. Data are presented for the California population which represented the most complete data set. A sample of six isolated female worms from the California population was monitored daily (with a few exceptions) from early August through mid-December, 2005. The reproductive state of each worm was recorded and a sample of egg capsules removed from the female's tube for analysis as soon as possible following spawning (embryos were typically in cleavage stages and easily distinguished from unfertilized eggs). Each brood was then monitored until the larvae were released and a sample of larvae was measured for total length. A compromise had to be made between collections of newly

deposited egg capsules for percent fertilization and the need to have adequate numbers of larvae at release for larval size measurements. Typically, 4–8 capsules (~300 eggs/embryos) were removed from a brood for percent fertilization counts and the remaining egg capsules left undisturbed to develop. Under the experimental conditions of isolation, female worms continue to grow and add gamete-bearing segments that do not contain any stored sperm. During spawning, these new segments produce egg capsules containing all unfertilized eggs while older segments may produce egg capsules with most or all eggs fertilized. If the capsules derived from new segments are collected after spawning and used in percent fertilization counts, this can introduce errors into the data (underestimate of fertilization percent). Typically, we collected egg capsules from the middle of the string of capsules in order to avoid this problem. As female worms depleted their supplies of stored sperm, the number of released larvae available for measurements decreased resulting in smaller sample sizes for broods with low initial fertilization percents.

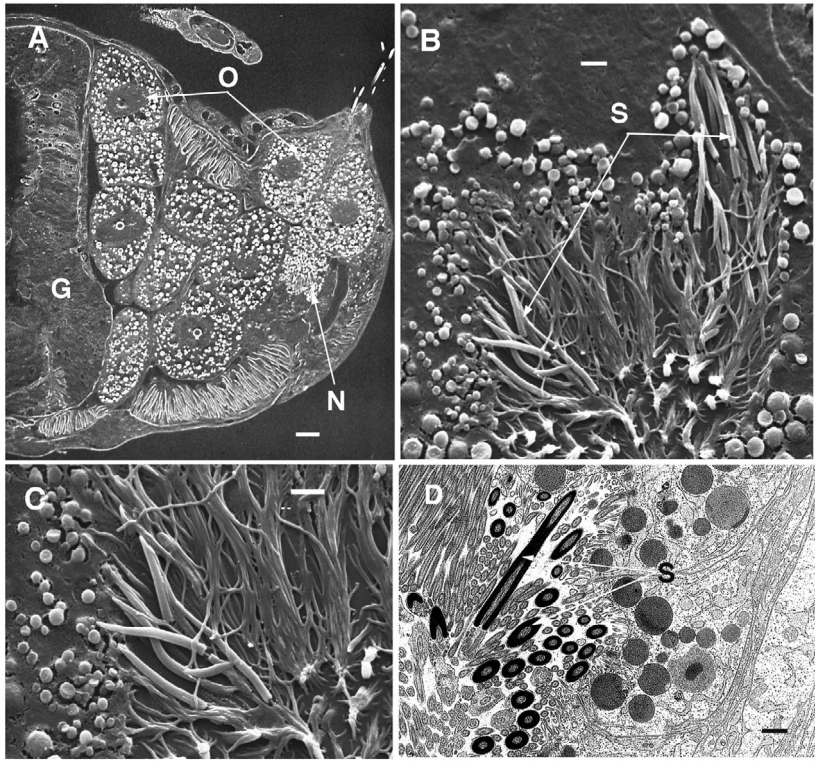

FIGURE 2. Sperm storage in *Polydora cornuta* from Florida. A–C are SEM micrographs of 1-µm-thick sections with epon removed. A, cross-section of female showing mature oocytes (O), gut (G), and a portion of the nephridium (N) scale bar = 20µm; B, portion of female nephridium containing pockets of stored sperm, scale bar = 2µm; C, close-up of stored sperm in nephridial pockets, scale bar = 2µm; D, TEM micrograph through a portion of the female nephridium showing stored sperm in section, scale bar = 1µm.

Isolated female worms were capable of producing at least some fertilized eggs over extended periods of time in the laboratory following a single transfer of spermatophores to the female worm. For the six CA females followed daily, the mean percent fertilization in successive broods remained very high (±97%) for the first three broods, then began to decrease in subsequent broods reaching

very low mean values (±15%) after nine broods (Fig. 3). Female worms were not given additional spermatophores during this period of observation but were rather utilizing sperm stored within their bodies. The time elapsed from brood No. 1 to brood No. 9 (Fig. 3) was approximately six weeks.

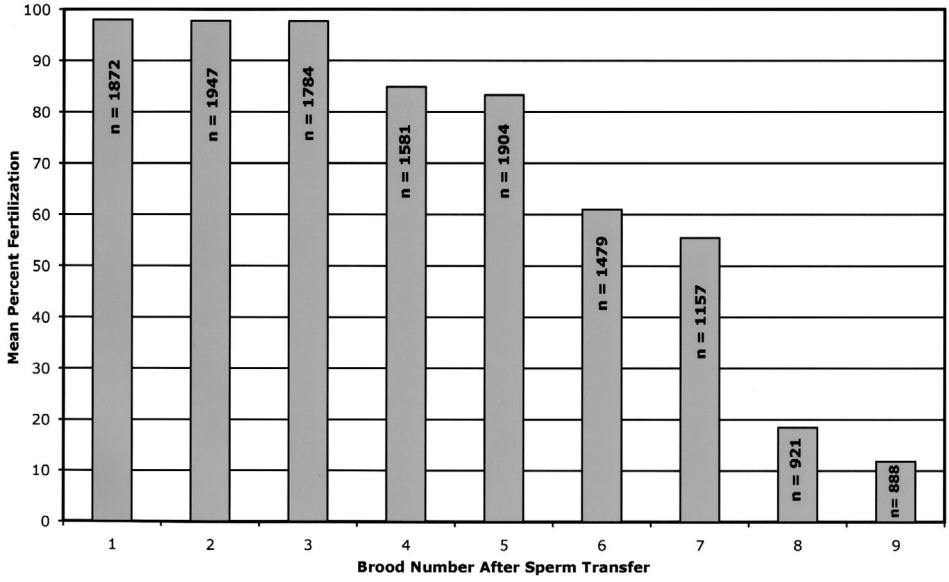

FIGURE 3. Mean percent fertilized eggs for six California females over nine successive spawnings in isolation from males. Sample sizes (number of eggs/larvae counted) are displayed on bars. The total time period for these nine successive spawnings was about six weeks.

Broods were produced at a mean rate of one per 5.2 days (n=40 broods, Standard Deviation = 1.07 days). During this same time period, the mean size of hatching larvae increased as percent fertilization decreased (Fig. 4). During the first five broods following sperm transfer, mean larval size remained small and relatively consistent but by brood No. 6, mean larval size was significantly larger than brood No. 1 (217±17.5µm, n=168 for brood #1; 246±34.6µm, n= 139 for brood No. 6; $P<0.0001$). Mean larval size for broods 7, 8 & 9 were likewise significantly larger than brood No. 1 ($P<0.001$). Larvae within brood capsules containing unfertilized eggs often consumed these eggs or the yolk material released from them. This lead to "large" larvae containing abundant additional yolk material within their guts. Not all developing larvae inside capsules with unfertilized eggs fed upon these eggs. This resulted in large size differences between larvae within and between brood capsules at the time of release. Fig. 4 demonstrates this discrepancy in size between larvae as an increasing standard deviation around the means for broods 6–9. The distributions of larval sizes for each of the nine broods represented in Fig. 4 are presented in Fig. 5. Each bar on the graphs in Fig. 5 represents the number of larvae that fell into 10µm size categories. As brood number increases, the spread (range) of the larval size data broadens and the number of larvae in each 10µm category decreases. This indicates that the size difference between the largest and smallest larvae is increasing through successive broods, an effect previously noted by the increasing standard deviations in Fig. 4. Some of the largest larvae obtained from brood No. 7 (Fig. 5) were 114% larger than the smallest larvae from brood No. 3. Fig. 5 also shows that through all nine successive broods, some small larvae were

produced. This suggests that not all larvae were feeding upon unfertilized eggs even if the latter were present within their capsules.

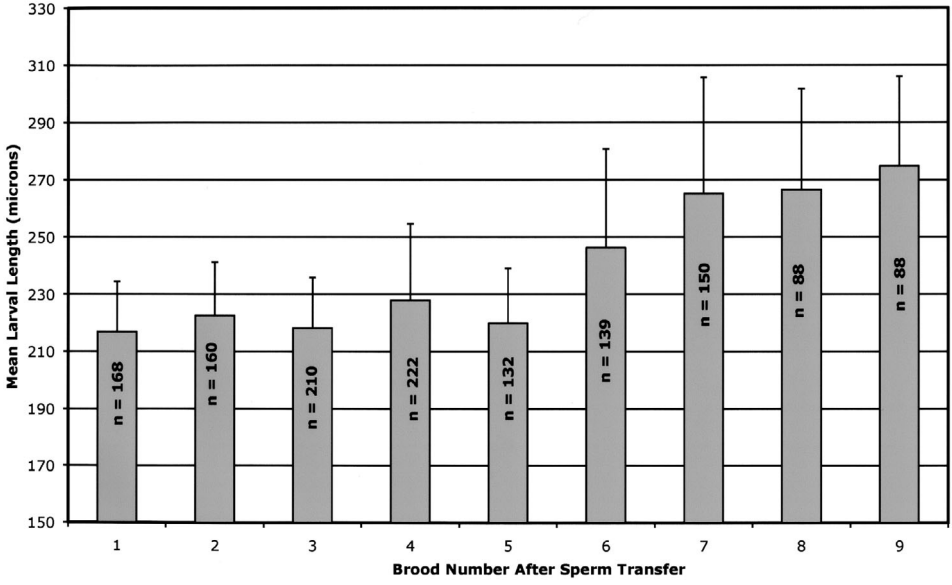

FIGURE 4. Mean larval size over nine successive broods for six isolated California female *Polydora cornuta*. Sample sizes (number of larvae measured) are indicated on each column. The bar at the top of each column represents one standard deviation. The smallest mean brood sizes (broods 1–5) are significantly smaller than the largest mean brood sizes (broods 7–9) in a Kruskal-Wallis Rank test ($H = 446.3$, $P<0.0001$).

The effect of increasing larval size through successive broods by isolated female worms could be completely reversed by addition of fresh spermatophores from a compatible male worm. Fig. 6 presents data for one of the six California females followed over 19 broods (106 days) in the laboratory. The female worm was given spermatophores prior to brood No. 1 in the graph. The following six broods contained mostly small larvae but mean larval size began to increase after brood No. 6. Mean larval size reached a maximum at brood No. 8 as percent fertilization reached a minimum. The addition of spermatophores to the culture dish containing this isolated female resulted in subsequent broods (broods 9–14, Fig. 6) similar in mean size to initial broods (broods 1–6). Percent fertilization likewise increased following spermatophore transfer indicating that the female had accepted and stored the sperm. Mean larval size remained low through the next five broods and then began to increase as percent fertilization decreased (broods 15–17). The addition of fresh spermatophores following brood No. 17 resulted in a repeat of the previous pattern. The standard deviations for mean larval size in Fig. 6 increased as larval size increased and percent fertilization decreased as noted in Fig. 4. These data collectively demonstrate that larval size is dependent upon the availability of unfertilized eggs within the brood capsule which in turn is dependent upon the availability of stored sperm within the female parent. Comparable data collected from laboratory cultured *Polydora cornuta* from Florida and Maine demonstrated this same relationship between percent fertilization and larval size in isolated females (data not shown).

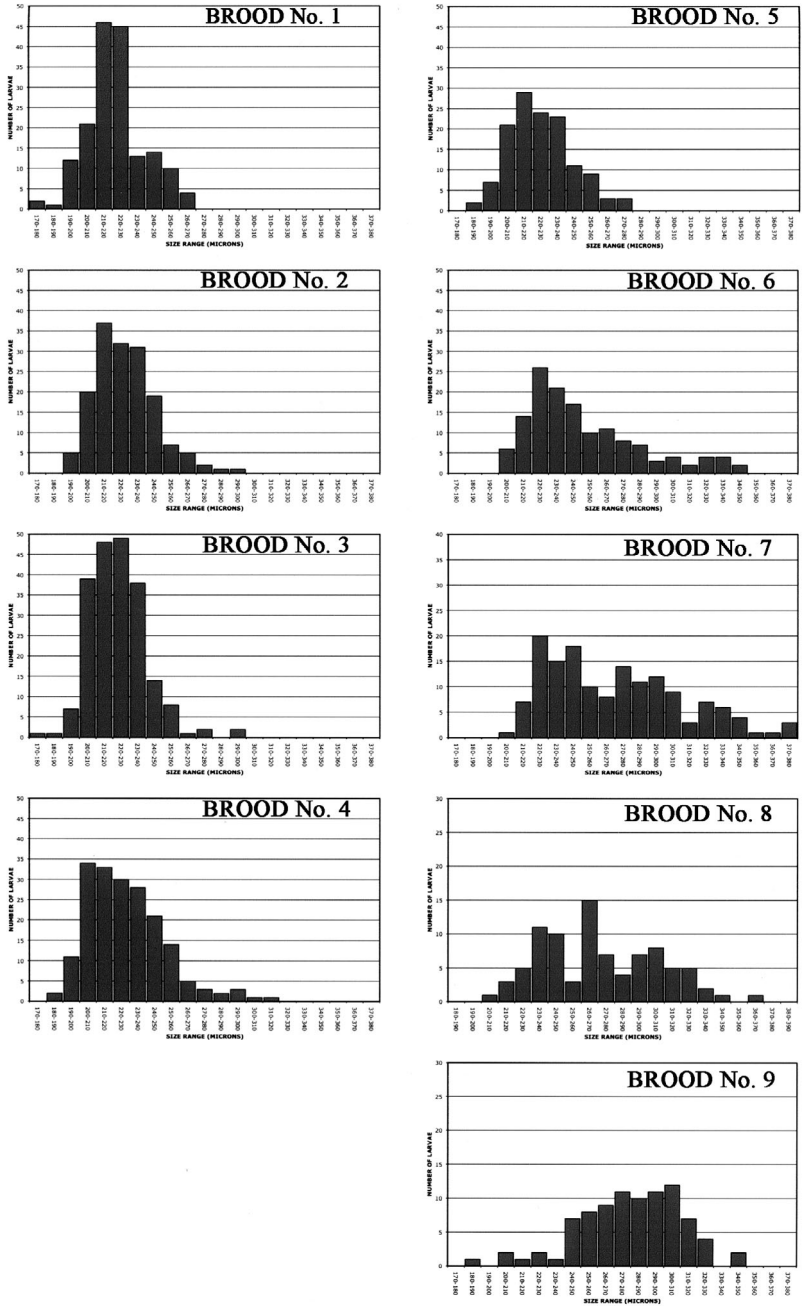

FIGURE 5. Frequency distributions of larval size for each of nine successive broods of larvae produced by six California females. Mean larval sizes are reported in Fig. 4. Size range (x-axis) is presented in increments of 10 microns, from 170 to 380 microns for all but brood 8 (180 to 390 microns); number of larvae (y-axis) is presented in increments of 5, up to 50 for broods 1–6, up to 40 for brood 7, up to 30 for broods 8–9. Brood numbers are indicated on each frequency diagram.

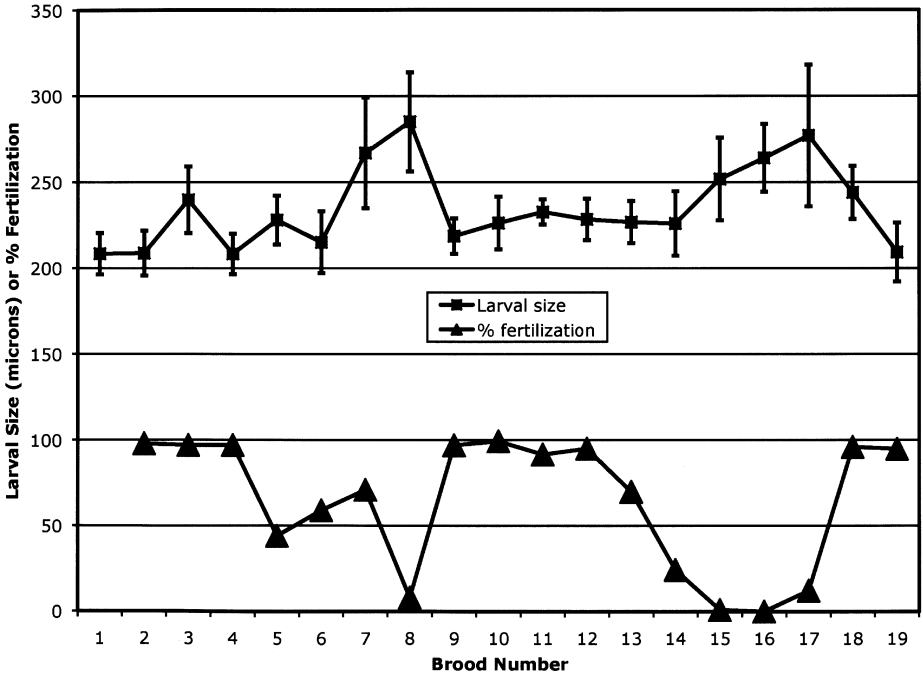

FIGURE 6. Percent fertilization and mean larval size through 19 successive broods (106 days) for a single isolated California female. Fresh spermatophores were provided following brood No. 8 and brood No. 17. Note the rapid increase in percent fertilization and decrease in mean larval size following each spermatophore transfer. Bars on larval size data points represent ± one standard deviation. The average number of larvae measured for each data point was 32 (range 10–49) and the number of eggs/embryos counted for each percent fertilization mark was ±300.

Fig. 7 represents a combined scatter plot of 92 separate broods produced by six California females in which both initial percent fertilization and mean larval size at release were measured. The Spearman Rank Correlation analysis shows a significant ($r = -0.624$, $P<0.0001$) relationship between decreasing percent fertilization and increasing mean larval size at release.

Discussion

The possibility that the morphological species, *Polydora cornuta* (*P. ligni* prior to 1987), was actually a cryptic species complex rather than a cosmopolitan single species was first suggested by Rice & Simon (1980) based upon experimental crosses, allozyme patterns, physiological responses, and adult morphology. Further evidence for a cryptic species complex was presented by Rice (1991) who showed that the Florida population of *P. cornuta* was reproductively isolated from California and North Carolina populations of the same morphological species. Rice (1991) also showed that population parameters including gamete distributions, sperm and egg dimensions, and some chaetal characteristics differed between reproductively incompatible populations. Definitive gene sequence

data (mtDNA, CO1) along with additional experimental crosses and reproductive data confirmed the suspected separate species status of the Florida, California, and Maine populations (Rice et al. 2008). Additional reproductive crosses presented in this report suggest that North America contains at least three distinct species of *P. cornuta:* (1) the Central Atlantic coast/ Pacific coast population, (2) the Florida/Gulf of Mexico population, and (3) the Northeast Atlantic coast population. With large gene sequence differences (0.15–0.86 maximum likelihood distance, Rice et al. 2008) and complete reproductive isolation between Florida, California, and Maine populations, it is difficult to support an argument that *P. cornuta* is a single species worldwide as suggested by Radashevshy (2005). Further genetic and reproductive assessments of worldwide populations are necessary to determine the true number of morphologically similar lineages of *P. cornuta* (*sensu latu*) that exist.

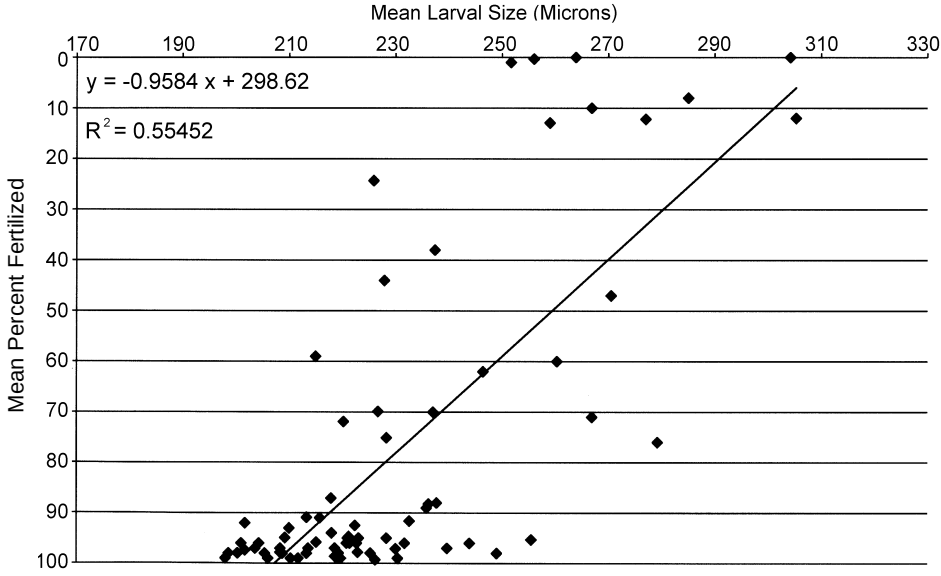

FIGURE 7. Correlation analysis (Spearman rank correlation) for six California female *Polydora cornuta* each producing multiple broods in isolation over a period of 106 days in the laboratory. Each of the 92 points on the graph represents a single brood with initial percent fertilization estimates and corresponding mean larval size at release. Initial percent fertilization was based upon a sample of ±300 eggs/embryos and mean larval size was based upon a sample of 12–42 larvae. (r-value = -0.62, P< 0.0001).

It is interesting that a similar distribution of reproductive isolation and genetic differentiation to the one described above for *P. cornuta* was likewise reported for populations of *Streblospio benedicti* (Schulze et al. 2000). Reproductive crosses between Florida worms and those from the central Atlantic coast (Norfolk, VA and Charleston, SC) were unsuccessful and mtDNA CO1 sequence analysis revealed differences of about 20% (Schulze et al. 2000). In a previous report, the Florida population of *Streblospio* was shown to be reproductively isolated from California worms but California individuals were reproductively compatible with mid-Atlantic coast worms (Rice 1991). Genetic analysis of mtDNA CO1 sequences (Schulze et al. 2000) from California and mid-Atlantic populations revealed high degrees of similarity (0.0–4.0%) concomitant with reproductive

compatibility. The Florida/ Gulf of Mexico *Streblospio* populations were described as a new species, *Streblospio gynobranchiata* by Rice & Levin (1998) based upon reproductive isolation from other geographical regions and distinct morphological features of the mature females of the new species. The similarities between the distribution patterns of *Streblospio* and *Polydora* populations are likely not coincidental. Schulze et al. (2000) presents a plausible explanation for the observed distribution of *Streblospio* populations based upon historical glacial maxima and minima over the past 10 million years. Since *Polydora* and *Streblospio* share similar habitat preferences and commonly co-occur in present day estuaries, the proposed historical scenario for *Streblospio* divergence could also apply to *Polydora*. More extensive genetic and reproductive studies on *Streblospio* and *Polydora* populations throughout North America may lead to resolution of the proposal that *S. benedicti* and *P. cornuta* were introduced into California habitats from the East Coast (Cohen & Carlton 1995)

Sperm storage organs in spionid polychaetes have not been fully explored. Söderström (1920) presented a histological micrograph showing stored sperm in dorsal seminal receptacles in *Polydora cornuta* (as *P. ligni*) but the sperm were difficult to see in the figure. Blake (1969a) presented a similar micrograph of a histological section illustrating the dorsal seminal receptacles in female *P. cornuta* (as *P. ligni*) but the low magnification of the figure made details difficult to see. Rice (1979) first reported sperm storage in the nephridia of female *P. cornuta* (as *P. ligni*) but the details of the relationship between the dorsal seminal receptacles and the nephridial storage sites has not been reported. The mechanism of sperm transfer in *P. cornuta* was described by Rice (1978; 1980) and the location of sperm storage sites appears to be consistent among all North American populations examined (personal observation). In any event, the ability of female worms to store sperm following a single spermatophore transfer can be viewed as an adaptive advantage since continuous contact with males becomes unnecessary. In the laboratory, stored sperm can last at least five weeks and still be used to produce viable larvae. As demonstrated here, mean larval size increases as stored sperm supplies are depleted and it is possible that the largest larvae may settle and metamorphose in close proximity to their female parent. This local recruitment could conceivably provide males capable of producing spermatophores within 2–3 weeks based upon laboratory maturation rates reported by Rice et al. (2008).

The ability of a single species to engage in variable modes of larval development has been reported in the literature for over 100 years and has been debated as to its validity and adaptive significance (Levin et al. 1987; Levin & Huggett 1990; Levin & Bridges 1995). This phenomenon, termed poecilogony (or poecilogonie, Giard 1892; 1905) has been reviewed for marine invertebrates by Hoagland & Robertson (1988) and specifically for spionid polychaetes by Blake & Arnofsky (1999). In many cases of reported poecilogony, cryptic species have been suspected or confirmed leaving few well studied examples of this process. In spite of numerous reports of poecilogony over the years, there are few cases within the Polychaeta where single species status has been demonstrated through reproductive crosses and genetic analysis. One such case involves the mid-Atlantic coast populations of *Streblospio benedicti* mentioned above. Levin (1984) documented the occurrence of two types of larval development (planktotrophic and lecithotrophic) at a single site in North Carolina and determined that larval type was a heritable trait (Levin et al. 1991). The two forms of this species were shown to be reproductively compatible (Levin 1984) and genetically similar (Schulze et al. 2000). Levin et al. (1991) speculate that the planktotrophic and lecithotrophic forms may have evolved in allopatry and recently reunited in several geographic locations in North America. Blake & Arnofsky (1999) agree that the two forms of *S. benedicti* are likely in the early stages of speciation.

Populations of *Boccardia proboscidea* have been reported along much of the Pacific coast of North America as well as from Australia & Japan (Blake & Arnofsky 1999; Gibson et al. 1999).

Three different types of larval development have been observed among female worms from a single population in Southern California (Gibson 1997). Individuals producing the two most extreme types of larvae (Type 1, planktotrophic, and Type 3, lecithotrophic) were shown to be interfertile with retention of larval type in offspring (Gibson 1997). Comparative genetic analyses (RAPD) between Type 1 and Type 3 females from Southern California and between Type 3 females from Southern California and British Columbia revealed no significant variation due to larval type and a low proportion of variance (6.91%) due to geographic origin (Gibson et al. 1999). An assessment of adult morphological characters between reproductive types and geographical locations produced no significant differences in taxonomically important characters (Gibson et al. 1999). The evidence collected to date for *B. proboscidea* suggests a true case of poecilogony but additional genetic analysis using other genetic markers (like mtDNA CO1 sequences) may reveal previously undetected variation between reproductive modes or geographical locations. Genetic analysis of the Australian population of *B. proboscidea* may be useful in identifying the population of origin for this suspected Australian introduction as was done for New Zealand *Polydora cornuta* by Rice et al. (2008).

In each of the above examples of poecilogonous species, female worms seem to be programmed to produce a specific type of larva (except hybrids) and do not vary their type of larval production over time. This is very different from the condition reported here for *Polydora cornuta* populations where each female can produce planktotrophic or lecithotrophic larvae over time depending upon supplies of stored sperm. In addition, each of the demonstrated genetically distinct and reproductively isolated populations of *P. cornuta* (Florida, California, Maine) is equally capable of variable types of larval development by individual females. We believe that this is the first report of a proximal mechanism associated with variable larval production. The significant correlation between initial percent fertilization of eggs within a brood and the corresponding increasing mean larval size at release for the same brood coupled with the reversibility of this trend by addition of spermatophores, strongly suggest a cause and effect relationship. Whether sperm availability contributes to variable larval size in other species of spionid polychaetes, remains to be determined.

Much discussion in the literature has been directed toward the ecological and evolutionary significance of variable modes of larval development (Levin & Bridges 1994; 1995). In general, lecithotrophic larvae are thought to spend less time (if any) in the plankton and to have higher survival probabilities than planktotrophic larvae. Lecithotrophic larvae would be expected to be better colonizers of the local environment while planktotrophic larvae would be more valuable in dispersal of the species (Thorson 1950, but see Levin & Huggett 1990). It has been suggested that nutritional state of the female parent may be correlated with larval type with well-nourished parents producing yolky, lecithotrophic larvae while under-nourished parents would produce planktotrophic larvae as demonstrated in opisthobranch mollusks (Krug 1998). This was shown not to be the case in *Streblospio benedicti* populations (Levin & Bridges 1994) where food level and composition did not affect egg size or larval trophic mode. Rather, reproductive characteristics such as egg diameter, fecundity, larval planktonic period and larval swimming chaetae were shown to exhibit a strong genetic basis and/or maternal influence (Levin et al. 1991).

The developmental consequences in *Polydora cornuta* larvae of being released from brood structures as large lecithotrophic or as small planktotrophic individuals remains to be determined. The ability and occurrence of larval suspension feeding in large yolky larvae following release needs to be explored. Pernet & McArthur (2006) reported that lecithotrophic larvae of *Streblospio benedicti* are capable of ingesting and assimilating suspended food material and that this additional nutrition resulted in faster growth than in sibling lecithotrophic larvae not provided with suspended food. They concluded that "lecithotrophic" *S. benedicti* larvae are actually facultative planktotrophs

and likely supplement their stored yolk with ingested material in the field. It is possible that large yolky *P. cornuta* larvae in the field feed upon suspended material and may have higher survival potential than smaller larvae, especially under conditions where suspended food material is scarce. The comparative survival rates, time to metamorphosis, size at metamorphosis, and post-metamorphic growth rates remain to be determined for large and small sibling larvae in *P. cornuta*.

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