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Host use and diversification in symbiotic polychaetes

by

Bruno Pernet

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

1998

Approved by ______________________________
Chairperson of Supervisory Committee

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Program Authorized
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University of Washington

Abstract

Host use and diversification in symbiotic polychaetes

by Bruno Pernet

Chairperson of the Supervisory Committee
Professor Alan J. Kohn
Department of Zoology

I studied development and larval biology, host use, and gamete interactions in three obligately symbiotic polychaetes in the genus *Arctonoe* (Family Polynoidae). The three polychaetes are ectosymbionts of other marine invertebrates, and within the Puget Sound region, their host ranges do not overlap. Except for *A. fragilis*, which feeds on host tissue, they are probably commensals. The three species have similar ontogenies, with long-lived planktotrophic larvae; benthic juveniles seek out hosts soon after metamorphosis.

Surveys of allozyme variation confirmed that the three *Arctonoe* species were genetically distinct. Populations of *A. pulchra* on two host species were also strongly differentiated, and should be considered distinct species. Populations of *A. vittata* associated with three host species were weakly differentiated in one of two years. Thus, some "host generalists" are actually comprised of isolated lineages of host specialists. I used transplant experiments to examine the role of physiological incompatibilities in restricting symbiont host range. *A. pulchra* and *A. vittata* died when paired with the seastar *Evasterias troschelii*, the principal host of *A. fragilis*. In other combinations of symbionts and hosts tested, all symbionts survived. While these incompatibilities can partially explain observed patterns of host use in *Arctonoe*, other processes must also be operating.

Gamete incompatibilities among closely-related species may be a consequence of divergence at gamete recognition loci as a by-product of reproductive isolation, selection against hybrids, or a process of sexual selection at polymorphic gamete recognition loci.

The first two hypotheses predict that gamete incompatibility appears after reproductive isolation has arisen, and the third that incompatibility appears simultanously with isolation. Gametes of *Arctonoe* spp. are compatible in all crosses, over a range of concentrations and contact times, despite estimated divergence times of 1-3 million years before present. These data are consistent with the first two hypotheses but allow rejection of the third.

Another common pattern in interspecific gamete interactions is asymmetric gamete compatibility. I use a simple genetic model to show that such asymmetries are predictable, but temporary, consequences of divergence between isolated populations.

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Chapter One

REPRODUCTION AND DEVELOPMENT OF THREE SYMBIOTIC SCALEWORMS (POLYCHAETA: POLYNOIDAE)

Abstract.—The three species of symbiotic polynoid polychaetes in the genus *Arctonoe* have very similar reproductive periods and ontogenies. Free-spawned eggs 80 µm in diameter fuse with sperm and develop into planktonic larvae. Larvae begin feeding after the development of the episphere ciliary bands and an oral brush, consistent with the hypothesis that these structures are involved in particle capture and handling, as suggested by Phillips and Pernet (1996). In the laboratory, metamorphosis occurs in the absence of hosts after 6-12 weeks of planktonic feeding and growth, and sexual maturity is reached 4-6 months after metamorphosis. Geographic differentiation in host preference in *Arctonoe* spp. appears unlikely except over very large spatial scales, because dispersal potential in the larval stage is high. Naive juveniles can now be obtained from laboratory culture to test the hypothesis that inherited preferences are important in determining host use patterns in these symbionts.

INTRODUCTION

The three described species of polynoid polychaetes in the genus *Arctonoe* are obligate symbionts of other marine invertebrates in coastal waters of the north Pacific (Pettibone 1953; Hanley 1991). Like many other symbiotic organisms, aquatic and terrestrial (*e.g.*, Fox and Morrow 1981; Fautin 1991; Thompson 1993, 1994), *Arctonoe* spp. have complex host use patterns over a range of spatial scales. Within a region, a single population of symbionts may be distributed among taxonomically diverse host species. For example, in the San Juan Islands, Washington, *Arctonoe vittata* (Grube) is regularly found associated with a chiton, several gastropods, and several seastars (Pettibone 1953). Despite this apparent lack of host discrimination, however, other

apparently suitable sympatric hosts (*e.g.*, other seastars) are never used. Host use patterns also vary among regions. North of Pt. Conception in central California, *A. vittata* is commonly associated with the seastar *Dermasterias imbricata*, but further south it is replaced on this host by *A. pulchra* (Johnson) (Pettibone 1953; Dimock and Davenport 1971). This geographic shift in host use obtains despite the fact that both symbionts occur north and south of Pt. Conception.

Understanding the ecology and evolution of these host use patterns requires knowledge of the development of *Arctonoe* spp., for two reasons. First, host selection by newly metamorphosed juveniles is likely important in determining patterns of symbiont distribution every generation (Davenport 1950; Britayev 1991; Chapter Two). Direct tests of this hypothesis have not been made because it has not been possible to obtain naive juveniles for experiments (Palmer 1962; Britayev 1991); information about biology of early stages of *Arctonoe* spp. might be of use in solving this practical problem. Second, the length of the planktonic larval period is strongly correlated with dispersal potential in many benthic marine invertebrates (Palumbi 1995). Dispersal potential is one factor important in determining the extent of gene flow, and the likelihood of local adaptation, among geographically isolated populations. Thus, knowledge of larval biology can yield predictions about the likelihood and scale of geographic differentiation in host preferences in *Arctonoe* spp.

The development of polynoids is also of interest because their larvae feed using a mechanism different from that of other polychaete larvae. The best-known feeding polychaete larvae -- *e.g.*, members of the family Serpulidae -- use three equatorial ciliary bands (the prototroch, metatroch, and food groove) to capture particles and transport them to the mouth (Strathmann *et al.* 1972; R. Strathmann 1987). This "opposed-band" or "downstream" feeding mechanism is usually considered to be the ancestral larval feeding mode in polychaetes and related phyla (Strathmann 1978; but see Haszprunar *et al.* 1995).

However, many feeding polychaete larvae -- among them the larvae of polynoids -- lack metatroch and food groove and hence must feed in a different manner. Phillips and Pernet (1996) inferred from videotaped feeding events that larvae of *Arctonoe vittata* capture particles on the episphere (the region of the larva anterior to the prototroch) and transfer them to the mouth using a large, asymmetrically-placed bundle of cilia, the oral brush. These conclusions were based on the movements of particles relative to larvae; putative feeding structures were not directly imaged. Descriptions of the ontogeny and morphology of these structures may be useful in testing hypotheses about their functions in feeding (Hart and Strathmann 1995). Ultimately these data will be important in analyses of the evolutionary history of larval feeding modes in polychaetes, and in studies of functional constraints associated with particular feeding mechanisms.

Some aspects of the reproduction and development of at least 18 species of polynoids, including three symbiotic species, have been described (Wilson 1991; Phillips and Pernet 1996; Giangrande 1997); these include gametogenesis (Sarvala 1971; Daly 1972, 1974; Britayev *et al.* 1983; Clark 1988), embryology (Mead 1897; Dictus and van den Biggelaar, pers. comm.), larval morphology (*e.g.*, Cazaux 1968; Blake 1975; Holborow *et al.* 1969; Holborow 1971), and metamorphosis (Korn 1959; Åkesson 1963). While a few polynoids brood embryos under the elytrae and eventually release feeding planktonic larvae (*e.g.*, *Harmothoe imbricata*: Daly 1972), most species free-spawn gametes and have completely planktonic development (Wilson 1991; Giangrande 1997).

Here I describe the reproduction and development of the three species in the polynoid genus *Arctonoe*. These species occur in sympatry over much of their ranges in the coastal north Pacific, including around San Juan Island, Washington, where this study was carried out (Pettibone 1953; Hanley 1989). Previous work on the reproduction of these species is limited to studies of gametogenesis and early development (Britayev *et al.* 1983) and larval feeding (Phillips and Pernet 1996; Johnson 1998; Johnson and Brink

1998) in *A. vittata*; gamete interactions among the three species are explored in Chapter Three. I focus on the timing of events in embryogenesis and the morphology of larvae. These data are of use in making inferences about dispersal potential and the likelihood of geographic differentiation in host preferences, testing hypotheses on the feeding mechanisms of polynoid larvae, and improving techniques to culture larvae through metamorphosis, so that the hypothesis that juvenile behavior is important in organizing host use patterns can be tested.

MATERIALS AND METHODS

Arctonoe spp. were collected from sites around San Juan Island, Washington, U.S.A., in 1994-1997. Most observations were made in spring and summer, but animals were also studied sporadically throughout the rest of the year. *A. fragilis* (Baird) was collected from its host seastar *Evasterias troschelii* in the intertidal and subtidal (by divers) zones on the east side of San Juan Island. *A. pulchra* was collected from the same sites, from the sea cucumber *Parastichopus californicus*. *A. vittata* was collected from the intertidal zone on the west side of San Juan Island, from the keyhole limpet *Diodora aspera*. Host-symbiont pairs were maintained in tanks of running seawater, without supplementary food, until use. Polychaetes were identified using the morphological criteria listed by Pettibone (1952).

The reproductive condition of live animals relaxed in a 1:1 mixture of 7.5% $MgCl_2$ and seawater was assessed by examination of the coelomic contents through the ventral body wall. The coelomic cavities of females that were ready to spawn contained many circulating oocytes, and those of mature males were swollen and white with sperm. Females were encouraged to spawn by removing them from hosts and isolating them in dishes of seawater. This was not a very reliable method of inducing spawning -- over four years, only ~20% of females so treated spawned within the next two days. Other

treatments (*e.g.*, slow or rapid temperature change, or incubating females in alkaline seawater with hydrogen peroxide: M. Strathmann 1987) were not more effective than simple isolation. As soon as possible after a female had spawned, eggs were washed several times in filtered seawater (FSW; bag filter with mesh size ~5 µm). Eggs can be successfully fertilized for up to 24 hrs after spawning (pers. obs.). Sperm was removed from males through an incision in a parapodium, and added to eggs at concentrations of $\sim 10^5$-10^6 sperm•ml^{-1}.

Larvae were raised in 600 ml beakers at densities of about 1 larva•ml^{-1}. The beakers were partially submerged in running seawater at 9-11° C (near ambient seawater temperatures in spring and summer). Every third day, the larvae were fed a mixture of *Rhodomonas* sp. (maximum dimension 10 µm), *Isochrysis galbana* (6 µm), and *Coscinodiscus radiatus* (40 µm). Early veliger larvae of bivalves (*Mytilus californianus* and *Crassostrea gigas*) or gastropods (*Melibe leonina*) were added as food in some cultures. Beakers were stirred once daily to resuspend settled algal cells. Larval cultures were cleaned weekly by siphoning most of the seawater out of the beaker through a 50-µm mesh, and transferring the larvae to fresh FSW in a clean beaker.

After metamorphosis, juveniles were removed from larval cultures and isolated in 5 ml FSW in small dishes. Dishes were partially submerged in running seawater at 9-11° C. Juveniles were fed newly hatched *Artemia* nauplii daily. For the first week or two after metamorphosis, juveniles were only able to capture immobile nauplii that had been partially crushed with forceps; as they grew larger, however, they could capture intact, swimming nauplii. Their diets were occasionally supplemented with small pieces of polychaete (*Platynereis bicanaliculata*) and unidentified amphipods. Juveniles were transferred to clean dishes containing fresh FSW every few days.

Development was documented using light and electron microscopy. For observations with a light microscope, larvae were placed in a drop of seawater on a glass

slide, dusted with polyethylene oxide to slow their movements, and gently immobilized under a coverslip supported with feet of modeling clay (M. Strathmann 1987). Measurements were made to the nearest 5 µm with a calibrated ocular micrometer. Embryos and larvae to be prepared for scanning electron microscopy were first relaxed in 7.5% $MgCl_2$ and seawater, then fixed in 1% OsO_4 in FSW and dehydrated in ethanol. Dehydrated specimens were infiltrated with hexamethyldisilazane (Nation 1983), air-dried, mounted on stubs, sputter coated, and viewed with a Jeol JSM-35 scanning electron microscope. For transmission electron microscopy, relaxed specimens were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer and post-fixed in 1% OsO_4 in phosphate buffer. After dehydration in ethanol and propylene oxide, they were embedded in epoxy resin (PolyBed 812, Polysciences Inc.). Sections were cut with a diamond knife. Thick sections (~1 µm) were stained with Richardson's stain for light microscopy (Richardson *et al.* 1960); thin sections (~80 nm) were mounted on grids, stained with aqueous uranyl acetate and lead citrate, and viewed with a Phillips EM-300 transmission electron microscope.

RESULTS

My observations did not reveal any differences among the three species in reproductive timing, patterns of early development, or larval morphology. In the rest of this paper I refer to the three species generically as *Arctonoe*.

Reproductive seasonality and spawning.—Reproductive *Arctonoe* were present in San Juan Island populations from about March through August, with most worms longer than 2 cm being mature in the late spring and early summer. Oocytes were clustered on segmental blood vessels in immature females. They were eventually ovulated into the coelom, where they circulated freely for a few days before spawning. Eggs dissected

from the coelomic cavity had a prominent germinal vesicle which did not disappear after prolonged incubation in seawater.

Spawning of *Arctonoe* in the field was not observed. In the laboratory, gametes were spawned through the ventrally directed segmental nephridiopores. Isolated females spawned most frequently at night or in the early morning; isolated males spawned much more readily than females, and did not appear to spawn more frequently at any particular time of day. Spawned eggs of *Arctonoe* had undergone germinal vesicle breakdown, and were opaque, pale yellow in reflected light, and spherical. Eggs bore no jelly coats, were not sticky, and were negatively buoyant. Mean egg diameters ± one standard deviation (n=80 eggs of each species, 20 from each of four females) were 80 ± 0.5 μm (*A. fragilis*), 78 ± 0.6 μm (*A. pulchra*), and 80 ± 0.6 μm (*A. vittata*). Sperm had ovoid heads, with a slight protuberance at the tip, and a midpiece of four mitochondria (Fig. 1.1A). Head and midpiece together were 2-3 μm in length.

Early development.—An outline of the developmental schedule of *Arctonoe* at 9-11° C is shown in Table 1.1. After the introduction of sperm, zygotes showed no obvious morphological signs of fertilization until the formation of the first polar body 30-40 minutes later. The first cleavage, at two hours after insemination, was equal, as were the second and third cleavages (Fig. 1.1B, C). The timing of subsequent cleavages was not followed.

After eight hours embryos were blastulae ~65 μm in diameter with a spacious blastocoel. The primary trochoblasts, arranged in four clusters of four cells each around the equator of the embryo, had started to develop multiple cilia per cell. At ten hours after insemination the primary trochoblasts bore long cilia (Fig. 1.1D). Blastuale also had an apical tuft of several long cilia, and had started to swim. At this stage, and until about 20-30 hr post-insemination, larvae consistently swam upwards, congregating in a thin layer on the top of the beaker.

At 20 hr the embryos had undergone gastrulation (Fig. 1.1E). The primary trochoblast cells had shifted their positions, and with other cells formed a complete and well-defined prototroch of three tiers of cilia. The central tier was densely ciliated with long compound cilia; the anterior and posterior tiers were more sparsely ciliated, with shorter cilia. The posterior tier was incomplete, with at least two substantial gaps in ciliation. By 22 hr, the presumptive mouth had formed adjacent to the blastopore (the presumptive anus; Fig. 1.1F). Over the next 10-15 hrs the mouth gradually moved away from the vegetal pole until it was located just posterior to the prototroch. At 36 hr the mouth and anus were in their definitive positions (Fig. 1.1G). The mouth was large, and it and the surrounding oral region were covered with short cilia. The anus formed a small, ciliated pit posteriorly. Extending towards the mouth from the anus was a short band of neurotrochal cilia.

By 45 hr several novel structures had appeared. On the episphere, the apical tuft remained intact. In addition, however, two pairs of ventro-lateral ciliary bands had appeared (Fig. 1.1H). Each pair consisted of two short straight bands of 5-10 cilia adjacent to each other (Fig. 1.1I). These are similar in arrangement and position to the "akrotrochal" ciliary bands of larvae of *Harmothoe imbricata* (Korn 1959, Holborow *et al.* 1969); I use that name for them here. The akrotrochal cilia beat vigorously towards the ventral margin of the episphere. Slightly posterior to each set of akrotrochal bands, within reach of their cilia, was a small gland opening. Another set of gland openings appeared on either side of the apical tuft (Fig. 1.1H, J). Each of these lateral episphere glands consisted of two adjacent gland openings, with the smaller one located more ventrally. The openings were slightly raised off the surface of the episphere. Immediately lateral to each set of lateral episphere glands was a single black, kidney-shaped eye, with the concavity facing laterally (Fig. 1.2A). Posterior to the eye was a very short band of ocular cilia.

By 50 hr after insemination, a short band of 30-μm long cilia had appeared on the left side of the mouth, just posterior to the prototroch (Fig. 1.2A, B). This group of cilia has been called the "oral brush" (Lacalli 1980, Phillips and Pernet 1996). In addition, the apical tuft had disappeared and was replaced with two transverse bands of episphere cilia (Fig. 1.2A). I refer to these as the apical ciliary bands, after Holborow *et al.* (1969). These larvae had complete guts, with the mouth leading to a heavily ciliated esophagus. The stomach was divided into two compartments by a transverse sheet of tissue (Fig. 1.2A). At this point the larvae began ingesting cells of *Rhodomonas* and *Isochrysis*.

Larvae retained this basic form for the next 4-10 wks. Measurements of larvae of *A. pulchra* and *A. vittata* over the first six weeks of growth showed that larvae reached their maximium body size by about 5 wks (Fig. 1.3A). Larval growth rates of these two species (and of *A fragilis*: pers. obs) were very similar. The length of prototrochal cilia increased from 35 μm at 1 wk to a maximum of 60 μm at 6 wks, and the oral brush cilia reached lengths of 160 μm (Fig. 1.3B). As larvae grew they accumulated red-brown pigment in vesicles in epidermal cells. These vesicles were concentrated in complex linear patterns over the episphere, especially adjacent to the prototroch, and to a lesser degree over the hyposphere. Pigmentation patterns were variable within species, and were not diagnostic for larvae of the three different species. Throughout this period larvae continued feeding. After 1-3 wk, larvae began to consume the 40 μm cells of *Coscinodiscus*, and by 3-4 wk regularly captured and ingested larger particles, including veliger larvae up to 200 μm in maximum shell dimension. Swimming larvae often trailed strands of mucus from their posterior ends. The free ends of these mucus strands frequently adhered to the bottom of the beaker, so that larvae effectively tethered themselves to the beaker. Tethered larvae swam and fed normally, albeit with restricted forward motion.

Morphology of advanced larvae.—Six-week old larvae were similar in shape to the early larvae described above, though they were more flattened in the anterior-posterior

plane, and in particular had strongly flattened epispheres (Fig. 1.4A). No qualitative changes had occurred in external morphology. Sections of the prototroch of 6-wk old larvae confirmed that it was made up of three tiers of cilia (Fig. 1.4B, C). The anterior tier of short prototrochal cilia was derived from a single row of cells. The long cilia of the central tier were derived from two rows of large cells immediately posterior to the anterior row. Finally, the short cilia of the posterior tier were derived from three or four rows of small cells immediately posterior to the central rows. All of these prototrochal cells had large nuclei with prominent nucleoli, and contained many mitochondria. Striated ciliary rootlets penetrated ~5 µm into the cytoplasm of prototrochal cells.

The oral brush was located posterior to the prototroch, on the left side of the mouth only (Fig. 1.4B, C). Between the posterior row of prototrochal cells and the oral brush was one row of unciliated cells. The oral brush was derived from two rows of cells. The anterior row bore long, relatively sparse cilia. The posterior row of cells was larger and densely ciliated. The cilia of both cells had striated rootlets that penetrated ~10-15 µm into the cytoplasm (Fig. 1.4D). The cuticle overlying these cells (and all epidermal cells in the larvae) was slightly raised off the cell membranes and covered with small projections. Mitochondria were densely packed among the rootlets. Transverse sections showed that the cilia of the anterior row were simple, but those of the posterior row were grouped into about 12 bundles of about 200-250 cilia each (Fig. 1.4E, F). The width of the base of the oral brush was about 60 µm at this stage (Fig. 1.4E). The cilia of both rows of cells were of the typical 9+2 arrangement, and there were no apparent connections among adjacent cilia. The microtubule doublets of the upper row were all oriented in the same direction, parallel to the dorsal-ventral plane of the larva. Those of the posterior row were not clearly visualized.

The akrotrochal and lateral episphere gland openings on each side of the episphere were supplied by a common gland cell complex (Fig. 1.4A, 1.5A). In living larvae

viewed from the anterior end, these cells were translucent and visible just ventral to the eyes, and contained numerous vesicles about 1 μm in diameter packed with a granular material (Fig. 1.5B). As vesicles were extruded from the gland openings, their contents became more homogenous.

As noted earlier, the gut was complete (Fig. 1.4A). The esophagus was encircled by several muscle strands near where it joined the stomach. These contracted periodically, squeezing the esophagus closed. The esophagus was heavily ciliated. Near where the esophagus joined the stomach, some of its luminal cilia had extremely expanded cell membranes, as described in *Harmothoe imbricata* by Holborow *et al.* (1969; their Fig. 4B). These enlarged cilia did not fill the entire esophageal lumen. In 6-wk old larvae, two muscular bulbs had budded off postero-laterally from the esophagus (Fig. 1.4A, 1.5C). These bulbs eventually formed the muscular pharynx of juveniles.

The blastocoel contained a few muscle strands that stretched from the anterior to posterior body wall, and several that stretched from the lower lip of the mouth to the posterior body wall. In addition, it contained numerous protonephridia. Transverse sections through the protonephridial terminal cells showed that each was made up of a single central 9+2 flagellum surrounded by a circular array of 13-14 rods, spaced regularly by about 0.05 μm (Fig. 1.5D). Inside of the rods, stretching across the gaps between them, was a thin layer of extracellular matrix. This structure was surrounded by a cellular epithelium of foot processes separated by very small gaps. The terminal cells were presumably connected to a nephridial duct leading out of the blastocoel, but this duct was not seen.

Finally, 6-wk old larvae had developed a pair of lateral bands of thick tissue on the ventral side of the body, stretching from the anus to near the lower lip of the mouth (Fig. 1.4A, 1.5C). These bands, each of which was broken up into 9-12 discrete chunks of tissue, were the rudiments of the juvenile segments. In some larvae, a few setae were

already visible in these segment rudiments, but they had not yet broken through the body wall.

Metamorphosis.—During metamorphosis, which took several days, the hyposphere became elongated in the anterior-posterior plane (Fig. 1.6A). Changes to the episphere included the appearance of two additional pairs of eyes, located posteriorly to the original pair. These new eyes were smaller in diameter than the first pair, and were circular in outline. In addition, a small bulge appeared in the central, most anterior part of the episphere. This would form the median tentacle during metamorphosis. In the hyposphere, the segment rudiments became better defined, and developing structures began to emerge from them. The first segment bore only rudimentary dorsal and ventral cirri. All other segments were setigerous. These bore ventral cirri and elytrae (on segments 2, 4, 5, 7, and 9) or dorsal cirri (segments 3, 6, and 8). Finally, on the pygidium a pair of small bulges marked the developing anal cirri.

A slightly later stage in metamorphosis is shown in Fig. 1.6B, illustrating the continued flattening of the larva in the dorso-ventral plane, and the formation of the juvenile head from the larval episphere. A bulbous median antenna protruded from the central episphere, and small lateral bulges represented the beginnings of the lateral antennae. From the ventral surface of the episphere a pair of large lateral bulges, the presumptive palps, were also forming. All three pairs of larval eyes were still present, but the prototrochal cilia had disappeared. The parapodia of the first, asetigerous segment (the tentacular segment) had turned forward and bore dorsal and ventral cirri. After this segment there were 8 setigerous segments. Setae were simple, spinous capillaries. The larval pharyngeal bulbs had moved towards the midline and elongated along the anterior-posterior axis.

Several days later morphogenesis of the head was complete (Fig. 1.6C). The larval episphere had formed the prostomium, and was well-demarcated from the first segment. The prostomium now bore only two pairs of eyes; whether these were remnant larval eyes

or novel structures is unknown. The final complement of head appendages -- median antenna, lateral antennae, and palps -- were present, as well as the anteriorly directed cirri of the tentacular segment. The pharyngeal jaws did not become visible, and juveniles did not begin to feed, until several days after metamorphosis was completed. Juveniles captured food in the same fashion as adults, everting the pharynx from the mouth, grasping prey with the two pairs of jaws, and drawing it back through the mouth whole. If prey items were too large to fit through the mouth, repeated contractions of the pharynx served to either tear off smaller parts or to suck fluid components of the prey into the mouth.

Growth of these juveniles in cultures was rapid. Three months after metamorphosis, most individuals had reached 6-10 mm in length. Color patterns were not useful in distinguishing juveniles of the three species. Most juveniles were pale yellow in color. Individuals of all three species bore a transverse brown pigment band on the dorsal body wall between segments seven and eight. In many juveniles narrower transverse pigment bands were present on other segments as well. The posterior edges of the first pair of elytrae were often white. By four months after metamorphosis, several individuals had developing gametes visible in their coelomic cavities. After five months, several had spawned viable gametes. One female that spawned at five months, completely emptying her coelom of visible gametes, spawned again one month later. Gametes of both males and females were identical to those of field-collected adults, and produced normal larvae in crosses with both laboratory-raised and field-collected adults. Between six and seven months after metamorphosis, all of the worms died. This sudden mortality was likely due to adverse physical conditions or disease, as the estimated lifespan of *Arctonoe vittata* in the field is several years (Palmer 1968, Britayev 1991).

DISCUSSION

The reproductive periods, early development, and morphology of gametes, larvae, and juveniles in the three species of *Arctonoe* are quite uniform. Patterns of development and larval form in *Arctonoe* are also broadly similar to those described in other polynoids (*e.g.*, Cazaux 1968, Blake 1975).

The oocytes of *Arctonoe* are associated with segmental blood vessels in the ovary until just a few days before spawning, when they are ovulated into the coelomic cavity (Britayev *et al.* 1983). Similar patterns of completely intraovarian oogenesis have been documented in other polychaetes, including the polynoid *Harmothoe imbricata* (Daly 1972, 1974; Eckelbarger 1983). In *Arctonoe*, eggs dissected from the coelomic cavity and incubated in seawater have a persistent germinal vesicle and cannot be fertilized; newly spawned eggs have undergone germinal vesicle breakdown. These observations suggest that oocyte maturation is stimulated by an endocrine signal in the coelom prior to spawning, as described in *Arenicola marina* (Howie 1961; Bentley and Pacey 1992). The timing and mechanism of oocyte maturation may vary among polynoids, however. Simon (1965), for example, found that oocytes dissected from *Lepidonotus sublevis* are fertilizable after brief incubation in seawater.

Embryos of *Arctonoe* begin to swim after the primary trochoblast cells become ciliated, but before an organized prototrochal ciliary band is formed. To my knowledge, this early onset of swimming with respect to prototroch development has not been observed in other polychaete embryos, but this may be a consequence of the difficulty of accurately describing the distribution of cilia in small embryos with the light microscope. A frequent observation in studies of polychaete development is that the earliest swimming stages tend to swim "weakly", with swimming performance improving as development proceeds (*e.g.*, Hermans 1966; Smith 1981). This improvement in swimming performance may be associated with completion of prototroch development.

The mature prototroch of *Arctonoe* is composed of six or seven rows of ciliated cells: an anterior row bears short cilia, two central rows bear long cilia, and three or four posterior rows bear short cilia (Fig. 1.4C). The prototroch of *Harmothoe imbricata* is similar in composition, except that there are only two rows of posterior cells (Holborow 1971). These three groups of cells correspond to the three tiers of prototrochal cilia visible at the light microscope level in *Arctonoe* and other polynoids (Korn 1959, Cazaux 1968). The form of the prototroch varies among and within other polychaete families, in terms of both the number of ciliary tiers visible at the light microscope level, and the number of rows of cells that make up these tiers (*e.g.*, Åkesson 1961, Hermans 1966, Lacalli 1985). The functional significance, if any, of this variation is unknown.

Newly metamorphosed juveniles of *Arctonoe* are similar in morphology to those of other polynoids. In particular, in the three species of *Arctonoe* and eight other species of polynoids, newly metamorphosed juveniles have from 8-10 segments, of which the first is without setae, and the others are setigerous (Korn 1959, Cazaux 1968). This latter group of species includes some with completely planktonic development (*e.g.*, *Acholoe astericola*: Cazaux 1968) and some with maternal brood protection followed by planktonic development (*e.g. Harmothoe imbricata*: Korn 1959). Juveniles of *Arctonoe* do not feed until a few days after metamorphosis. This delay is associated with the organization of the muscular pharynx from the larval pharyngeal bulbs and the formation of the proteinaceous jaws. Korn (1958) noted a similar delay of several days between metamorphosis and the appearance of jaws in three species of *Harmothoe*.

Phillips and Pernet (1996) used the results of feeding experiments and analyses of videotaped feeding events to make inferences about feeding mechanisms in larvae of *Arctonoe vittata*. They hypothesized that these larvae capture particles that come near or strike their epispheres as they swim forward. Captured particles are then dislodged from the episphere by a recoiling motion, and manipulated into the mouth using the oral brush.

The structures thought to be involved in particle capture and transfer -- in particular, the episphere ciliary bands and glands, and the oral brush -- have not previously been described in detail in any polynoid (except for the episphere ciliation of *Harmothoe imbricata*: Holborow *et al.* 1971). This study provides more complete descriptions of these putative feeding structures.

The earliest episphere ciliation in *Arctonoe* is the apical tuft. This is soon augmented by the development of the akrotroch, four short bands of cilia that beat vigorously toward the ventral margin of the prototroch. Just before the onset of feeding, the apical tuft is replaced with two transverse apical ciliary bands. In both *Arctonoe vittata* (Phillips and Pernet 1996) and *Harmothoe imbricata* (Holborow *et al.* 1969; Holborow 1971), these bands beat slowly and irregularly in restrained larvae; how they behave in free-swimming larvae and during particle capture is not known. The apical ciliary bands of *H. imbricata* are slightly different from those observed in *Arctonoe;* in *H. imbricata*, each has a median gap in their ciliation. Otherwise, however, the pattern (and probably the ontogeny) of episphere ciliation described here in *Arctonoe* is similar to that described from larvae of other polynoids (Cazaux 1968; Blake 1975). Apical ciliary bands and akrotroch may function in distributing glandular secretions over the episphere, sensing captured particles, or moving captured particles towards the mouth (Phillips and Pernet 1996). Complex episphere ciliation patterns are also common among the feeding larvae of other members of the order Phyllodocida (Rouse and Fauchald 1997), all of which lack a metatroch (*e.g.*, Chrysopetalidae: Blake 1975; Glyceridae: Cazaux 1967; Phyllodocidae: Cazaux 1969) and capture particles by unknown mechanisms.

Gland openings on the episphere are common among a variety of polychaete larvae, including some that putatively feed with opposed bands (*e.g.*, Hermans 1966; Smith and Chia 1985; R. Strathmann 1987) and some that feed in other ways (*e.g.*, Åkesson 1961, 1968), but their functions are unknown. On each side of the episphere *Arctonoe* bears two

sets of raised gland openings: a single opening associated with the akrotroch, and a pair of lateral gland openings near the eyes. The aktrotrochal and lateral gland openings on each side of the episphere are supplied by a common gland cell complex. These cells contain vesicles filled with a granular material (Fig. 1.5A, B), and are similar to those seen by Holborow (1971; his Fig. 2b) in *Harmothoe imbricata*. The fates of secretions of the episphere glands is unknown, but their association with ciliary bands suggests that they may be spread over parts of the episphere. This may be important in particle capture, as surface properties play an important role in determining how particles adhere to capturing surfaces (Rubenstein and Koehl 1978; Solow and Gallager 1988; Shimeta and Jumars 1991). While glandular secretions of the episphere have not previously been implicated in larval feeding, secretions from glands of the hyposphere are used by larvae of several families of polychaetes to capture planktonic food particles (*e.g.*, Chaetopteridae: Werner 1953, Pisionidae: Åkesson 1961).

The oral brush is a distinctive feature of all polynoid larvae that have been described, and is found in the larvae of some other aphroditaceans as well (Phillips and Pernet 1996). In *Arctonoe*, the oral brush originates just prior to the onset of feeding from two rows of cells located posterior to the prototroch, on the left side of the mouth. It grows continuously throughout larval life until about 5 wk after fertilization. The cilia arising from the more posterior row of cells are compound. The oral brushes of larvae restrained on a sticky surface (*e.g.*, polylysine-coated coverslips: M. Strathmann 1987) can beat rapidly in a relatively planar fashion (pers. obs.), but how they are stimulated to move and how they actually move during particle capture and transport is unknown. Observations of particle captures suggests that particles are manipulated in front of the mouth by the oral brush until they are ingested (Phillips and Pernet 1996). During this time the oral brush may be stationary, held out perpendicular to the anterior-posterior body axis, or it may beat rapidly. It is also possible that the compound cilia of the second tier or oral

brush cells fan out to provide more surface area for manipulating particles (*e.g.*, Sleigh 1972). These possibilities can only be evaluated with direct observation of oral brush movements during particle captures.

Thus the episphere ciliary bands, episphere glands, and oral brush develop before the onset of larval feeding in *Arctonoe* spp., consistent with the hypothesis that they are involved in feeding. Testing this hypothesis by direct observation of the movements of episphere cilia and oral brush during feeding will be difficult, because feeding in *Arctonoe* spp. (and probably other polynoids) involves rotation and forward movement of the whole larva. Even when studying tethered larvae, larval movements enforce the use of relatively low magnification, and structures of interest (*e.g.*, the oral brush) are often hidden behind the body of the larva as it rotates. In contrast, in larvae that feed using opposed-bands of cilia particle capture involves only movement of the cilia, not the larval body; thus feeding can be studied in stationary larvae at high magnification (*e.g.*, Strathmann *et al.* 1972; Emlet and Strathmann 1991).

In this study the plankonic period of laboratory-raised larvae of *Arctonoe* varied from 6-12 wks. This is not unexpected -- all polynoids studied to date have long planktrotrophic larval stages (on the order of weeks: *e.g.*, Cazaux 1968). Such species typically have high dispersal in the larval stage and show little geographic population structure over large spatial scales (see Palumbi 1995 for review). Thus, it seems unlikely that *Arctonoe* spp. will show geographic variation in intrinsic characters related to host use (*e.g.*, host use preferences) except over very large spatial scales. Indeed, observed host use patterns of the three species are relatively homogenous over most of their ranges. *A. fragilis*, for example, which occurs from central Alaska to central California, is found only on several species of forcipulate seastars throughout its range. The other two *Arctonoe* spp. have larger geographic ranges -- *A. pulchra* occurs from central Alaska to Baja California, and *A. vittata* on both sides of the Pacific, with southern range limits in the Sea

of Japan in the northwest Pacific and in Baja California in the northeast Pacific (Pettibone 1953; Hanley 1991). There do appear to be shifts in host use patterns in *A. pulchra* and *A. vittata* north and south of Pt. Conception in central California, a well-known biogeographic transition zone for many invertebrate taxa (Valentine 1973, Briggs 1974). North of Pt. Conception the seastar *Dermasterias imbricata* is host to *A. vittata* (Hartman 1944, Pettibone 1953); south of Pt. Conception *D. imbricata* is host to *A. pulchra* (Dimock and Davenport 1972). This shift in host use obtains despite the fact that *A. pulchra* and *A. vittata* both occur on either side of Pt. Conception. While other explanations are available for this host switch, it is possible that larval dispersal is restricted across Pt. Conception, leading to divergence in host preference and observed host use patterns within these two species. Evidence for restricted gene flow across Pt. Conception has been found in some taxa with planktonic larval stages (*e.g.*, Davis *et al.* 1981; Burton and Lee 1994), but not in others (*e.g.*, Ford and Mitton 1993)

Another geographic shift in host use occurs among populations of *A. vittata* on either side of the north Pacific. In the northwestern Pacific, *A. vittata* is found in association with several species of forcipulate seastars, as well as the sea cucumber *Stichopus japonicus* (Britayev 1991); in the northeastern Pacific neither of these taxa are ever used as hosts (Pettibone 1953; pers. obs.). Indeed, northeastern Pacific *A. vittata* cannot survive on *Evasterias troschelii* (Chapter Two), a forcipulate closely related to those reported as hosts in the northwestern Pacific. Again, this may be an instance of population differentiation over large spatial scales. Open water in the north Pacific may serve as a dispersal barrier to the larvae of other coastal marine invertebrates as well (*e.g.*, Vermeij *et al.* 1990).

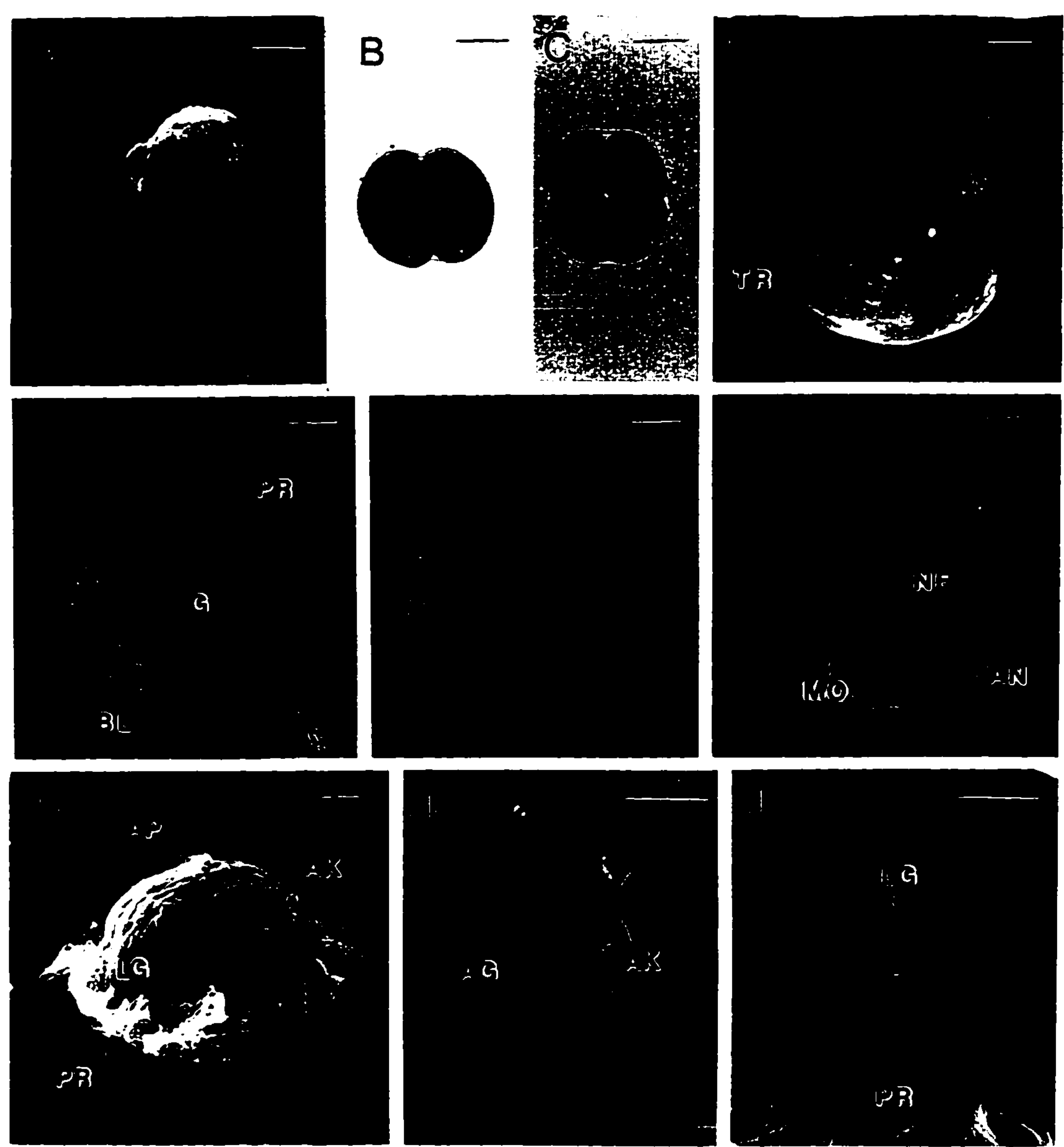
Since the 1950's, *Arctonoe* spp. has been the focus of attempts to understand the role of symbiont behavior in establishing host use patterns (*e.g.*, Davenport 1950; Hickock and Davenport 1952; Dimock and Davenport 1972; Britayev 1991). These studies have

examined the host preferences of adult worms collected from hosts, and have almost invariably found that adult worms prefer the host that they were collected from. However, because an individual's history of host use may have a strong effect on subsequent host preferences (Dimock and Davenport 1972), these results do not explain how host use patterns are initially determined every generation. An important determinant of host use pattern is undoubtedly the behavior of newly metamorphosed, naive juveniles that are seeking out their first host. Juvenile host choice has not been studied because it has not previously been possible to obtain juveniles for experiments (Palmer 1962; Britayev 1991). The results reported here show that it is possible to obtain naive juveniles from laboratory culture. Thus it should be possible to test the hypothesis that inherited preferences are important in organizing host use patterns in *Arctonoe* spp. Further, because hybrids of the three species are viable (Chapter Three), it may also be possible to examine the evolutionary genetics of their host preferences (*e.g.*, Jaenike 1987; Stanhope *et al.* 1991; Thompson 1994).

Table 1.1. Developmental stages of *Arctonoe* spp. at 9-11 °C.

Time	Stage
0	insemination
2 hr	two cells
3 hr	four cells
4 hr	eight cells
8 hr	blastulae; primary trochoblasts ciliated
10 hr	swimming blastulae; apical tuft
20 hr	gastrulae; prototroch complete
22-30 hr	presumptive mouth and anus; mouth migrates to definitive position
36 hr	oral and anal cilia, neurotrochal cilia
45 hr	akrotroch and episphere gland openings; oral brush cilia; one pair of eyes; complete gut
50 hr	apical tuft disappears; apical ciliary bands appear; larval feeding commences
4-10 wk	pharyngeal swellings; segment rudiments
6-12 wk	3 pairs of eyes; metamorphosis

Figure 1.1. *Arctonoe* spp. **A.** SEM of sperm of *A. fragilis* on the surface of an egg. Scale=1 µm. **B.** Light micrograph of a two-cell embryo of *A. fragilis*. Scale=20 µm. **C.** Light micrograph of a four-cell embryo of *A. fragilis*. Scale=20 µm. **D.** SEM of 11-hr old blastula of *A. fragilis*, showing ciliated primary trochoblast cells (TR). Scale=10 µm. **E.** SEM of 20-hr old *A. fragilis* just after gastrulation. Blastopore (BL); gap in posterior band of prototrochal cilia (G); prototroch (PR). Scale=10 µm. **F.** SEM of 23-hr old *A. vittata*, showing division of the blastopore into presumptive mouth and anus. Scale=10 µm. **G.** SEM of 37-hr old *A. fragilis*. Anus (AN); mouth (MO); neurotroch (NE). Scale=10 µm. **H.** SEM of lateral view of 45-hr old *A. vittata*. Akrotroch (AK); apical tuft (AP); lateral gland pores (LG); prototroch (PR). Scale=10 µm. **I.** SEM of 45-hr old *A. vittata* showing akrotrochal cilia (AK) and gland pore (AG). Scale=5 µm. **J.** SEM of 45-hr old *A. vittata* showing lateral gland pores (LG). Scale=5 µm.


B
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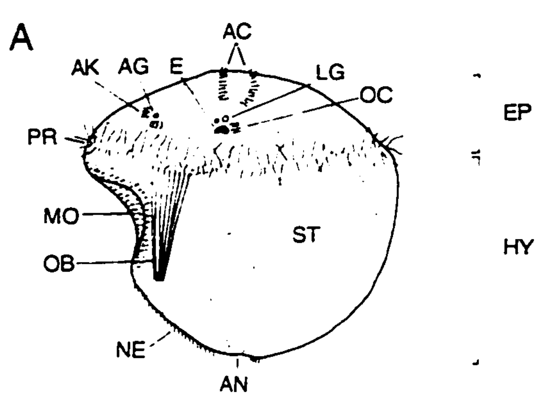
AX
LG
PR

Figure 1.2. *Arctonoe* spp. **A.** Drawing of 50-hr old larva in lateral view. The episphere (EP) is the region of the larva anterior to the prototroch (PR); the hyposphere (HY) is posterior to the prototroch. Akrotrochal cilia (AK); akrotrochal gland pore (AG); anus (AN); apical cilia (AC); eye (E); lateral gland pores (LG); mouth (MO); neurotroch (NE); ocular cilia (OC); oral brush (OB); stomach (ST). **B.** SEM of 48-hr old *A. fragilis* in posterior-lateral view, showing the mouth (MO), anus (AN), prototroch (PR), neurotroch (NE) and oral brush (OB). Scale=10 µm.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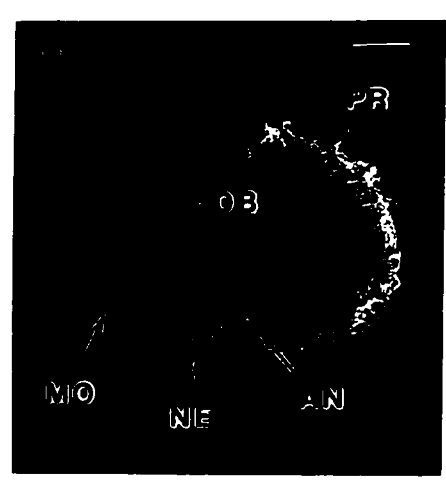
PR
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MO
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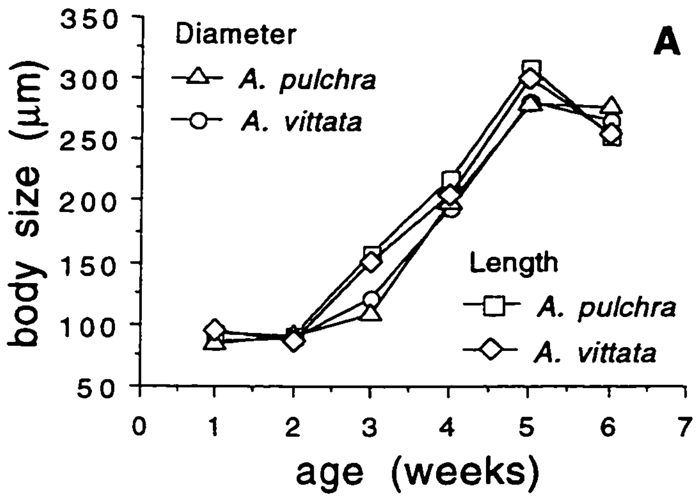
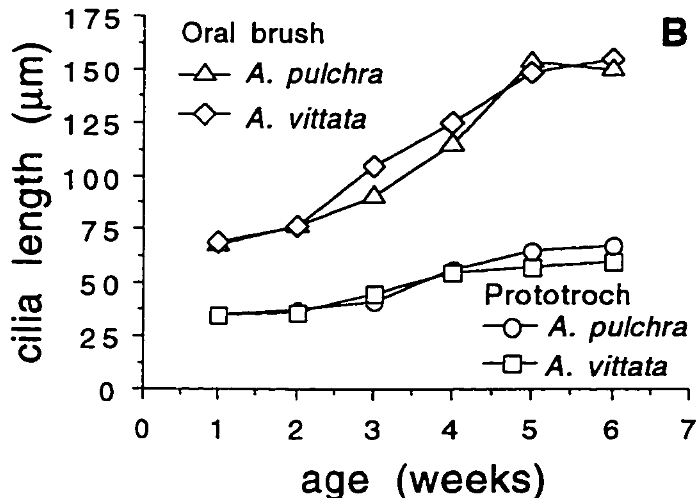
Figure 1.3. Growth of *Arctonoe pulchra* and *A. vittata*. Each point is the mean of measurements of ten larvae, five from each of two separate cultures. **A.** Body diameter (at the level of the prototroch) and body length as a function of age. **B.** Length of prototroch and oral brush cilia as a function of age.



A
Diameter
A. pulchra
A. vittata
Length
A. pulchra
A. vittata
body size (µm)
age (weeks)



B
Oral brush
A. pulchra
A. vittata
Prototroch
A. pulchra
A. vittata
cilia length (µm)
age (weeks)

Figure 1.4. Six-wk old larvae of *Arctonoe* spp. **A.** Drawing of parasagittal section. Akrotrochal cilia (AK); akrotroch gland pore (AG); episphere gland cells (EG); esophagus (ES); intestine (IN); lateral gland pore (LG); ocular cilia (OC); prototroch (PR); segment rudiments (SE); stomach (ST). **B.** Light micrograph of frontal section of *A. vittata*, showing prototroch (PR) and oral brush (OB). Blastocoel (BL); esophagus (ES). Scale=50 µm. **C.** Drawing of frontal section through prototroch (PR) and oral brush (OB). Six rows of cells make up the three tiers of prototrochal cilia (anterior, central and posterior) visible under the light microscope. **D.** TEM of frontal section of oral brush of *A. fragilis*. Cuticle with projections (CU); oral brush cilia of tiers one (OB1) and two (OB2). Long ciliary rootlets are visible in transverse section in each tier of oral brush cilia. Scale=2 µm. **E.** Light micrograph of transverse section posterior to oral brush attachment in *A. vittata*, showing scattered cilia of oral brush tier one (OB1) and groups of cilia of oral brush tier two (OB2). Blastocoel (BL). Scale=20 µm. **F.** TEM of transverse section through oral brush of *A. fragilis*, showing cilia of tiers one (OB1) and two (OB2). Scale=2 µm.

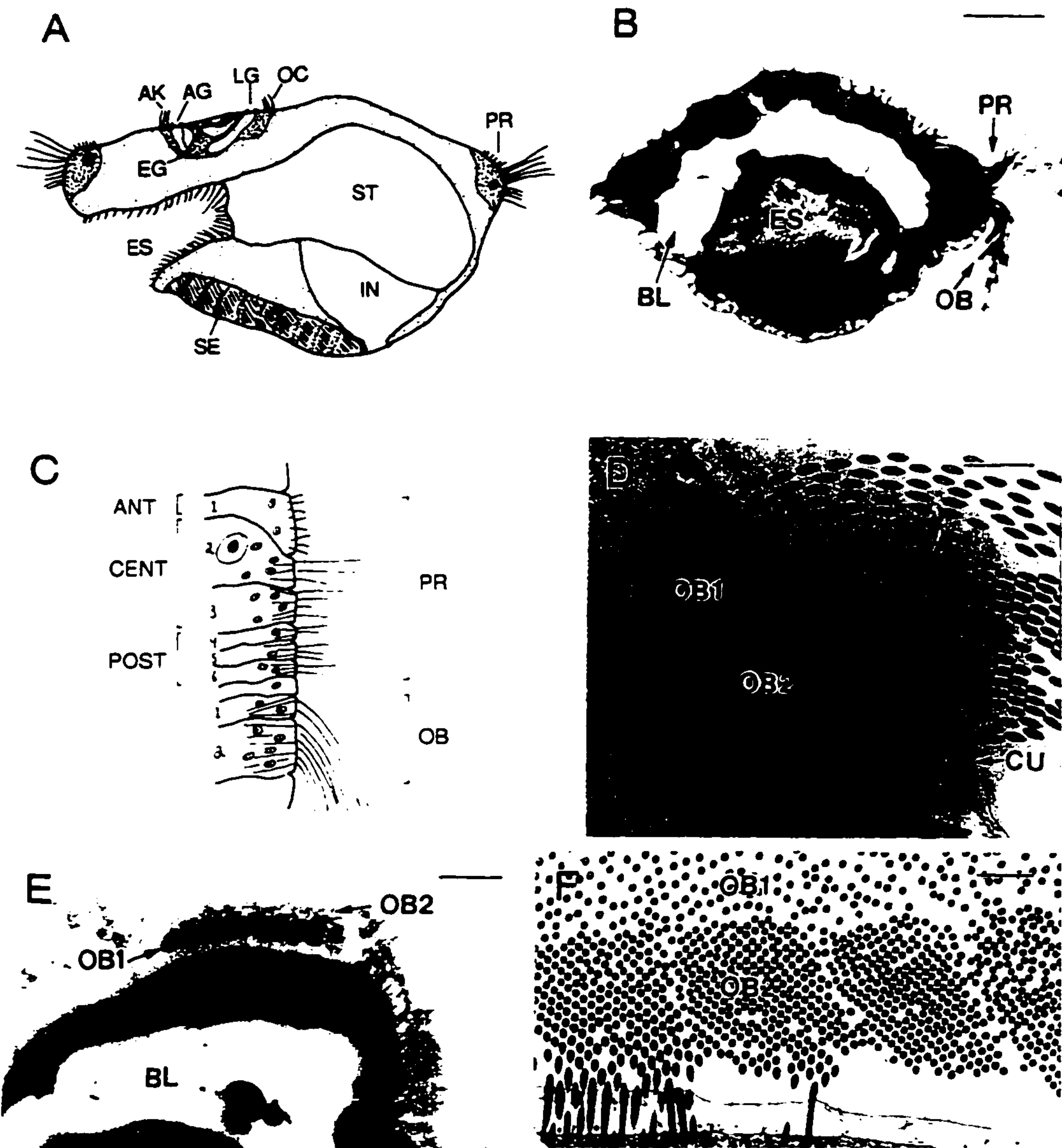


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Figure 1.5. Six-wk old larvae of *Arctonoe vittata*. **A.** Light micrograph of episphere gland cell complex. Akrotrochal cilia (AK); akrotrochal gland pore (AG); apical cilia (AP); lateral gland pore (LG); blastocoel (BL); gland cells (GC). Scale=10 µm. **B.** TEM of sagittal section through lateral gland pore. Lateral gland pore (LG); vesicles of fibrillar material (VE). Scale=2 µm. **C.** Light micrograph of frontal section, showing episphere gland cells (EG), esophagus (ES), pharyngeal bulbs (PB), and lateral bands of segment rudiment tissue (SE). Scale=50 µm. **D.** TEM of transverse section through protonephridial terminal cell. Blastocoel (BL); extracellular matrix (ECM); flagellum (FL); foot processes of terminal cell (FP); rods (RO). Scale=0.5 µm.

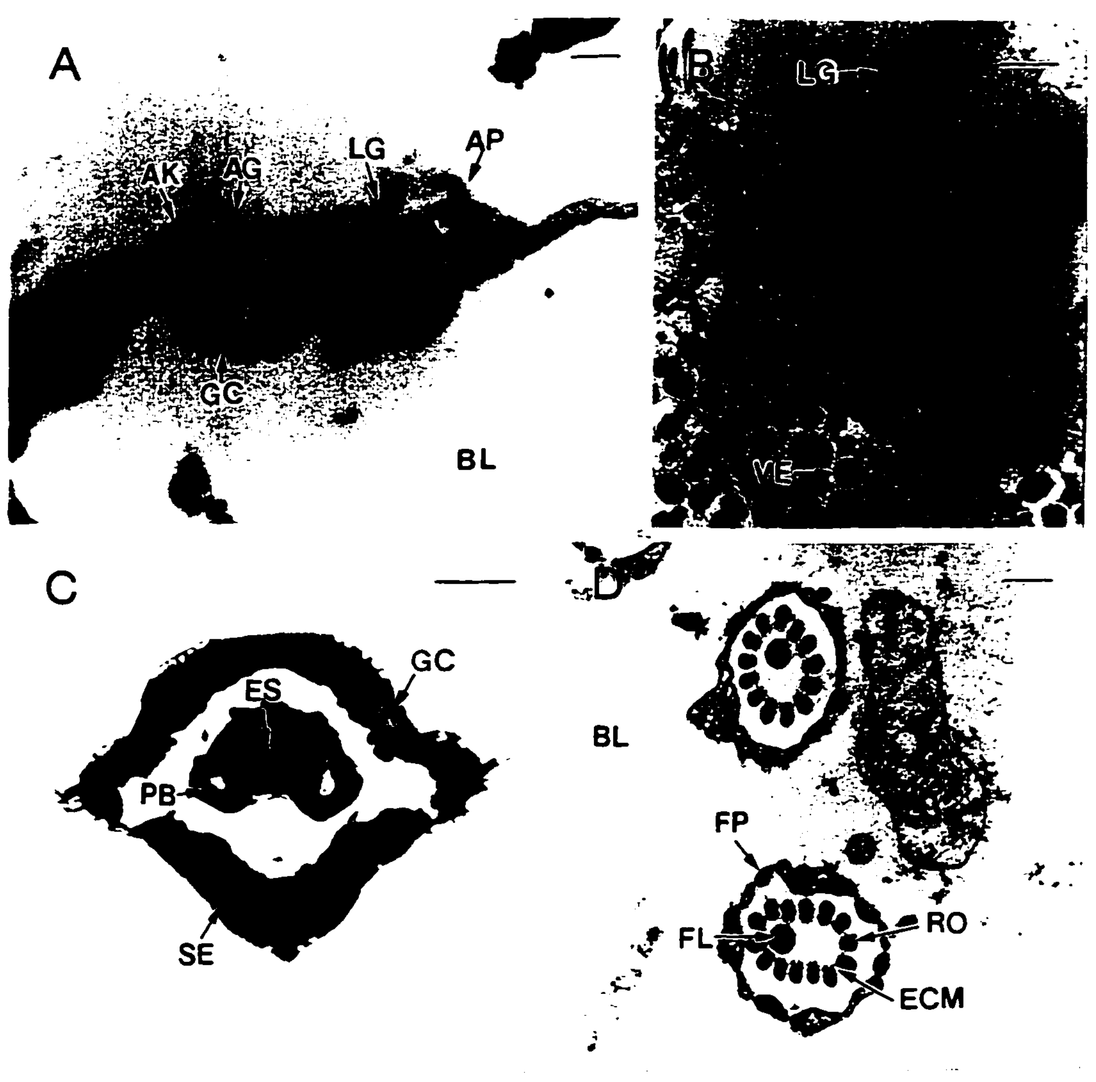

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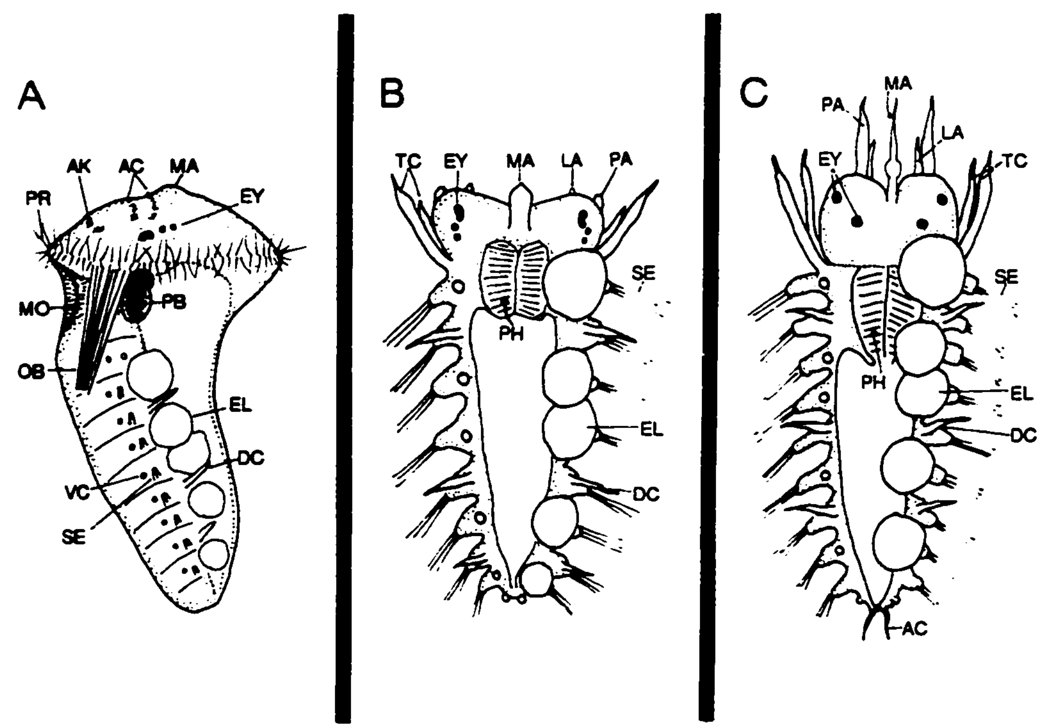
Figure 1.6. Drawings of stages of metamorphosis in *Arctonoe* spp. **A.** Early in metamorphosis, the body elongates, two additional pairs of eyes appear, the rudiment of the median antenna appears, and segmental structures begin to form. Lateral view. Length ~400 µm. Apical cilia (AC); akrotroch (AK); dorsal cirrus (DC); elytron (EL); eyes (EY); median antenna (MA); mouth (MO); oral brush (OB); pharyngeal bulbs (PB); prototroch (PR); setae (SE); ventral cirrus (VC). **B.** Later in metamorphosis the head begins to form. Dorsal view. Length ~450 µm. Dorsal cirrus (DC); elytron (EL); eyes (EY); lateral antenna (LA); median antenna (MA); palp rudiment (PA); pharynx (PH); setae (SE); dorsal and ventral cirri of the first segment (i.e., tentacular cirri: TC). **C.** Dorsal view of juvenile. Length ~500 µm. Anal cirrus (AN); dorsal cirrus (DC); elytron (EL); eyes (EY); lateral antenna (LA); median antenna (MA); palp rudiment (PA); pharynx (PH); setae (SE); dorsal and ventral cirri of the first segment (i.e., tentacular cirri: TC).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Chapter Two

HOST USE AND POPULATION GENETIC STRUCTURE IN SYMBIOTIC SCALEWORMS

Abstract. —In the San Juan Islands, Washington, U.S.A., the three species of symbiotic polychaetes in the genus *Arctonoe* occur in sympatry but use different sets of host species. I examined genetic structure in local symbiont populations to resolve their host use patterns, transplanted symbionts among hosts to test the hypothesis that physiological incompatibilities play a role in limiting host range, and examined symbiont diets to identify direct trophic interactions with hosts. Allozyme electrophoresis confirmed that the three *Arctonoe* spp. were genetically distinct. *Arctonoe pulchra* from two host species also differed substantially at several allozyme loci, and should be considered distinct species; weaker differentiation obtained among populations of *A. vittata* from three host species. Formerly considered generalists, these symbionts may in fact comprise multiple lineages of host specialists. In transplant experiments, *A. pulchra* and *A. vittata* invariably died when paired with the seastar *Evasterias troschelii*, a common host of *A. fragilis*. In other combinations tested, symbionts survived well. Thus, incompatibility can partly account for observed host use patterns in *Arctonoe* spp., but other processes, like host selection behavior by juveniles, or post-settlement interactions with hosts, competitors, and predators, must also influence host use. The ability of *A. fragilis* to survive on the seastar *E. troschelii* allows it to take advantage of an abundant and renewable source of food, the spines and pedicellariae of its host. The other two *Arctonoe* spp. do not regularly consume host tissue but instead feed primarily on annelids and arthropods.

INTRODUCTION

Populations and species of symbiotic organisms may vary in the number and types of host species that they use. These host use patterns result from processes that include

symbiont dispersal, host selection behavior, and interactions with hosts, predators and competitors, and they have important ecological and evolutionary consequences. For example, symbiont (or other habitat specialist) taxa that use multiple host species (or habitats) are expected to have more stable sizes and be less prone to extinction than those that use only one or a few host species (MacArthur 1955; Redfearn and Pimm 1988; Nieminen 1996). The use of only one or a few host species may be correlated with high speciation rates within a taxon of symbionts (Price 1980; Mitter *et al.* 1988; Brooks and McLennan 1993). Further, disjunct patterns of host use appear to play a more direct role in speciation, restricting gene flow and promoting genetic differentiation among closely-related, sympatric populations in a variety of animal groups (*e.g.*, Stevens 1990; Waring *et al.* 1990; Mokady *et al.* 1994; Duffy 1996). Thus, knowledge of how host use patterns are determined is basic to understanding the population biology and evolution of symbionts.

Experimental tests of hypotheses on the determinants of host use patterns can only be made in the context of detailed descriptions of host use. These descriptions require knowledge of symbiont population structure, so that the relevant independent evolutionary units -- reproductively cohesive populations -- can be identified (Fox and Morrow 1978; Thompson 1994). Without adequate resolution of population structure, the results of experiments or observations aimed at untangling the determinants of host use patterns or their evolutionary consequences are difficult to interpret (*e.g.*, Downes 1990; Gwaltney and Brooks 1994; Guo *et al.* 1996). Describing the boundaries of symbiont populations usually requires genetic analysis, because morphological characters are not reliable markers of population structure. In some cases, for example, symbiont morphological characters are affected by the host species used during development. Host-induced morphological plasticity can lead to overestimates of the number of populations involved and the attribution of narrow host ranges to these populations (Jaenike 1981; Downes 1990). On the other hand, symbionts using different host species sometimes show little or no

morphological differentiation even though they are partially or fully reproductively isolated, leading to underestimates of the number of independent populations or species involved and the attribution of broad host ranges to them (Stevens 1990; Mokady *et al.* 1994; Duffy 1996).

Here I begin to describe host use patterns and the processes involved in shaping them in symbiotic polychaetes in the genus *Arctonoe* (Family Polynoidae). The three described *Arctonoe* spp. are obligate ectosymbionts of a diverse array of other marine invertebrates, including echinoderms, molluscs, and polychaetes, in coastal waters of the north Pacific (Pettibone 1953). In the San Juan Islands, Washington, U.S.A., where the three species are found in sympatry, each uses several host species, but their host ranges do not overlap (Table 2.1). Further, each symbiont uses varying numbers of host species. These descriptions of host use may not be adequate for understanding the ecology or evolution of symbiotic interactions in *Arctonoe* spp., for several reasons. First, the three species are separable on the basis of only two morphological traits with few alternative character states (Table 2.1; Pettibone 1953; Hanley 1989); these traits offer little resolution with which to differentiate symbiont populations associated with different host species. One potentially useful character, color pattern, may vary consistently among populations associated with different hosts, but this has not been examined in detail in northeast Pacific populations (Pettibone 1953). Second, gametes of the three species are compatible in all possible crosses, and hybrid offspring are fertile in laboratory culture (Chapter Three). This interspecific reproductive compatibility, unusual among free-spawning marine invertebrates (Palumbi 1994), suggests that the three species may in fact comprise one reproductively cohesive population with polymorphic or phenotypically plastic members. Increased resolution of population genetic structure in *Arctonoe* is needed before their host use patterns can be defined with confidence.

The processes that determine host use patterns in this genus are of particular interest because studies of *Arctonoe* spp. were among the first to demonstrate host location and preference behaviors mediated by waterborne chemicals in marine symbionts (Davenport 1950; Davenport and Hickok 1951; Hickok and Davenport 1957). Almost without exception, these studies and more recent ones (Dimock and Davenport 1971; Britayev 1991) show that adults of all three *Arctonoe* spp. prefer their original host species to alternatives in choice experiments. These results suggest that adult worms, which sometimes migrate among host individuals, will not migrate among host species; these behaviors will tend to maintain whatever host use patterns have already been established. However, other processes must be important in establishing host use patterns earlier in the life histories of symbionts. *Arctonoe* spp. broadcast spawn gametes into the sea, where they are fertilized and develop into long-lived, weakly swimming planktotrophic larvae (Britayev *et al.* 1986; Britayev 1991; Phillips and Pernet 1996; Chapter One). These larvae probably disperse widely in the plankton, so that pre-metamorphosis differences in symbiont distribution are unlikely to affect host use patterns. After metamorphosis (which occurs prior to any interaction with hosts: Chapter One), benthic juveniles seek out hosts (Palmer 1968); host selection behaviors at this stage may be important in determining host use patterns. Finally, a variety of processes that occur after symbionts have paired with hosts -- including physiological interactions of symbionts and hosts, and trophic and competitive interactions -- may also be involved in shaping host use in *Arctonoe* spp.

In this study I use allozyme markers to resolve genetic structure and host use patterns in sympatric populations of *Arctonoe* spp. These data show strong and significant genetic differentiation among populations of *A. pulchra* associated with different host species, and weaker differentiation among populations of *A. vittata* associated with different host species. Hence, host use patterns in the genus are more complex than previously recognized. I also present the results of experiments designed to test the

hypothesis that physiological incompatibilities with hosts may be responsible for restricting host range in *Arctonoe* spp. Previous work indicated that a saponin isolated from the seastar *Evasterias troschelii* is toxic to *A. pulchra,* which is never found on this host, but has no adverse effects on the normal symbiont of *E. troschelii, A. fragilis* (Patterson *et al.* 1978). Laboratory transplant experiments revealed that such incompatibilities might partly explain observed host use patterns -- in particular, *A. pulchra* and *A. vittata* were unable to survive on *E. troschelii.* Finally, analysis of the natural diets of the three species showed that the only symbiont capable of surviving on *E. troschelii, A. fragilis,* was the only one that regularly fed directly on host tissues.

MATERIALS AND METHODS

Population genetic structure.—I used allozyme electrophoresis to describe population structure in all three *Arctonoe* spp. From March-June in 1996 and 1997 I obtained polychaetes from the intertidal and subtidal zones around San Juan Island, Washington, USA (Table 2.2). Worms from the subtidal zone were collected by divers. Because of low sample sizes, collections of *A. vittata* on *Solaster endeca* and *S. stimpsoni* were pooled in analyses. In addition to San Juan Island collections, *A. pulchra* were collected by dredge from the host seastar *Luidia foliolata* in Bellingham Bay, Washington (40 km northeast of San Juan Island) and, in 1997, East Sound, Orcas Island, Washington (15 km northeast of San Juan Island). Worms from these two collections were pooled in analyses of 1997 data. Worms were stored at -80 °C for up to five months before electrophoresis. Tests showed that storage under these conditions had no effect on allozyme patterns.

Samples were prepared for electrophoresis by homogenizing a piece of tissue in an approximately equal volume of grinding buffer (0.05 M Tris-HCl, pH 7.5). Homogenates were loaded on cellulose acetate gels (Helena Laboratories) and analyzed using the methods

of Hebert and Beaton (1989). Twenty-six loci were surveyed initially, and among these four polymorphic loci were consistently resolved: glucose-6-phosphate isomerase (*GPI*, E.C. 5.3.1.9) and phosphoglucomutase (*PGM*, E.C. 5.4.2.2) on tris-glycine buffer, and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*, E.C. 1.2.1.12) and carbonate dehydrogenase (*CD*, E.C. 4.2.1.1) on citric acid-aminopropyl morpholine buffer. Each of the four enzymes encoded a single scorable locus. Alleles were named alphabetically in order of increasing electrophoretic mobility.

I used chi-square tests to identify heterogeneity among allele frequencies at each locus among collections of *Arctonoe* spp., in each case pooling all alleles other than the most common one (this was necessary in most cases so that expected allele frequencies would be at least five in each category). Significance levels were adjusted to account for multiple comparisons by the sequential Bonferroni technique (Rice 1989). Two sets of analyses were carried out. I first assessed heterogeneities in allele frequencies among collections from different host species within the two symbiont species *A. pulchra* and *A. vittata*. I then examined allele frequency heterogeneity among the three described species of *Arctonoe* spp. Because there were significant differences among collections associated with different host species within *A. pulchra* and *A. vittata*, I did not pool data from these collections for the among-species analyses, but instead used allele frequency data from *A. pulchra* on *Parastichopus californicus* and *A. vittata* on *Diodora aspera*. These are the earliest recorded host species from which these symbionts were obtained in the Puget Sound region (Pettibone 1953), and worms from these hosts are presumably representative of *A. pulchra* and *A. vittata* as described by systematists (Pettibone 1953; Hanley 1989).

Physiological incompatibilities with hosts.—I used transplant experiments to identify physiological incompatibilities of symbionts with hosts. Because of the difficulty of obtaining some host species, I tested the three described *Arctonoe* spp. only on their three most common host species (pers. obs.): the keyhole limpet *Diodora aspera*, the

seastar *Evasterias troschelii*, and the sea cucumber *Parastichopus californicus*. Thus, I tested nine combinations of symbionts and hosts.

Symbiont-host pairs were collected from the intertidal and subtidal zones around San Juan Island and maintained in seawater tanks for up to one week before being used in experiments. On the first day of an experiment I removed all worms from hosts, isolating them in bowls of seawater. I then distributed hosts among nine large seawater tanks. Three tanks contained *Diodora aspera*, three contained *Evasterias troschelii*, and three contained *Parastichopus californicus*. I then divided the worms of each of the three species into three groups of similar size distributions, and placed members of each group into one of the host tanks so that each tank contained a unique combination of symbiont and host species (Figure 2.1). For example, the pool of *Arctonoe vittata* was divided into three groups, one of which was placed into a *D. aspera* tank, one into an *E. troschelii* tank, and one into a *P. californicus* tank. When distributing worms among tanks, each worm was placed onto an unoccupied host; the numbers of symbiont and host individuals in each tank were equal.

For the next two weeks, I examined tanks daily. I counted the number of worms remaining in the tank and noted their positions (on or off hosts). If a worm was not on a host, I placed it onto an unoccupied host. Worms were considered dead if I found a body on the bottom of the tank or if I could not locate them for three days in a row. Neither hosts nor symbionts were offered food for the course of the experiment. Starvation by itself was not expected to lead to any mortality in symbionts, as isolated worms held in the laboratory typically survived for more than six weeks without food (pers. obs.).

This experiment was run twice (in May and June 1997), because the tanks used were too small to permit a single run with adequate sample sizes. The results from the two experiments were similar, and were thus pooled to identify heterogeneity in frequencies of survival on different host species within each symbiont species using chi-square tests.

Diet analysis.—Natural diets of *Arctonoe* spp. were determined from contents of fecal pellets of adults collected from the three most common host species. *Arctonoe fragilis* and *A. pulchra* were collected from the subtidal zone on the east side of San Juan Island from *Evasterias troschelii* and *Parastichopus californicus*, respectively; *A. vittata* was collected from the intertidal zone at Deadman Cove, San Juan Island, from *Diodora aspera*. Polychaetes were removed from hosts in the field and placed individually in 20 ml plastic vials, and upon return to the laboratory were isolated in dishes of seawater at 10-13 °C. Once daily for the next three days, fecal pellets were collected from each dish and teased open with fine needles. Their contents were identified under a dissecting microscope at 50X magnification. Three days was sufficient time for all remnants of food items to pass through the gut. After three days, fecal pellets were still produced, but contained only granules 20-30 μm in diameter similar to the "*ballots d'excretion*" described from the gut caecae of another aphroditacean polychaete, *Aphrodite aculeata* (Darboux 1899). Dales and Pell (1971) showed that these are the pinched off tips of caecal epithelial cells, and agreed with Darboux (1899) that they probably contain excretory products.

RESULTS

Population genetic structure.—Staining patterns observed at each of the four loci were consistent with reported enzyme subunit structures (Murphy *et al.* 1996). Allele frequencies for each of the populations are shown in Table 2.3. Chi-square heterogeneity analyses of these data (Table 2.4) showed that the populations of *Arctonoe pulchra* on *Parastichopus californicus* were strongly differentiated from those on *Luidia foliolata* at three of four loci, in both years sampled. One of these differences, at the *G3PDH* locus, was fixed between the two populations. Similar analyses revealed no significant differentiation among host-associated populations of *A. vittata* in 1996, but differentiation at two loci (*CD* and *PGM*) in 1997. There were also significant differences in allele

frequencies at one or more loci in all pairwise comparisons among the three described species of *Arctonoe* (Table 2.4), in both 1996 and 1997.

Physiological incompatibilities with hosts.—When paired with their original host species, symbionts remained on hosts throughout the experiments. However, when paired with alternate hosts their fidelity to hosts varied. *Arctonoe fragilis* rarely left either *Diodora aspera* or *Parastichopus californicus*. *A. pulchra* rarely left *D. aspera*; however, almost all *A. pulchra* paired with *Evasterias troschelii* moved from hosts to the bottom of the tank. Similarly, *A. vittata* rarely left *P. californicus*, but almost always left *E. troschelii*. *A. pulchra* and *A. vittata* on *E. troschelii* were frequently attacked by the seastar's pedicellariae. As these two *Arctonoe* spp. moved around on the seastar, they often left behind elytrae gripped in the jaws of pedicellariae. Damage from pedicellariae was not observed in *A. fragilis* on *E. troschelii*.

The survival of each symbiont species in all combinations with hosts is shown in Figure 2.1. Results from both experiments were identical: survival was 100% for all combinations of symbiont and host species, with the exceptions of *Arctonoe pulchra* and *A. vittata* on *Evasterias troschelii*, where all symbionts died within one week of the start of the experiment. Most of the dead individuals were found intact on the bottom of tanks, indicating that mortality was not the result of ingestion by the host.

Diet analysis.—Fecal pellets of *Arctonoe* spp. were cylindrical, 0.5-1.0 mm in diameter, and 1-15 mm in length. The contents of each pellet were contained in a translucent peritrophic membrane. In the three days of observation, each worm produced from 3-10 fecal pellets. During the first two days of observations, pellets typically contained remants of food items, but on the third day and after pellets contained only excretory granules.

The composition of fecal pellets of *Arctonoe* spp. is shown in Table 2.5. Fecal pellets of *Arctonoe fragilis* contained primarily one type of prey hard part -- ossicles of

pedicellariae and abactinial spines corresponding in size and shape to those of the seastar *Evasterias troschelii* (Fisher 1930; pers. obs.). The fecal pellets of some individuals contained ossicles from up to 20 pedicellariae and spines. Fecal pellets of two individuals also contained barnacle cyprids. Many individuals had not recently fed on any prey with hard parts, however -- 6 of 28 individuals deposited fecal pellets that contained only excretory granules.

Most individuals of *Arctonoe pulchra* produced fecal pellets that contained crustacean parts (mainly harpactacoid copepods and barnacle cyprids), polychaete remains, and sandy sediment. Polychaete parts included a large number of hooded hooks characteristic of spionid polychaetes, unidentified capillary setae, and a few calcareous opercula of small serpulid or spirorbid polychaetes. Four of twenty-nine individuals examined deposited fecal pellets that contained only excretory granules.

The contents of *Arctonoe vittata* fecal pellets typically included large numbers of crustacean parts (mainly harpactacoid copepods), polychaete remains, the larvae of chironomid flies, and sediment. Among the polychaete remains hooded hooks of spionids and unidentified capillary setae were common; jaws and compound setae of nereids and the opercula of small serpulids or spirorbids also occurred occasionally. Chironomid larvae were abundant in fecal pellets, with some worms depositing up to six large larvae. About 50% of the chironomid larvae seen were still alive after passing through the guts of *A. vittata*. All *A. vittata* examined produced fecal pellets that contained prey hard parts.

DISCUSSION AND CONCLUSIONS

The population genetic data reported here reveal unexpectedly complex host use patterns in *Arctonoe* spp. Although the three species are interfertile (Chapter Three), patterns of allozyme variation clearly indicate that they are reproductively isolated in the San Juan Islands (Table 2.4). This conclusion is supported by a few morphological characters

(Pettibone 1953) and data from several other allozyme and DNA loci (Chapter Three). In addition, these data show significant genetic differentiation among populations associated with different host species within two of the described *Arctonoe* spp. In particular, *A. pulchra*, previously considered a generalist symbiont occurring on the sea cucumber *Parastichopus californicus* and the seastar *Luidia foliolata* in the Puget Sound region (Pettibone 1953; Dimock and Davenport 1971), appears to comprise two distinct host-specific taxa. The significant differences in allele frequencies between populations on *P. californicus* and *L. foliolata* at three of four polymorphic allozyme loci, and the absence of shared alleles at one of these loci, *G3PDH*, were consistent over both years sampled (Table 2.4). These differences may be due to geographic isolation, as the two populations were separated by 15-40 km (Table 2.2). This seems unlikely, however, as *Arctonoe* spp. have small, weakly-swimming larvae with a long planktonic period (6-12 wks in the laboratory; Chapter One) probably ensuring widespread dispersal of larvae. Indeed, the allozyme differences between *A. pulchra* from these two hosts in the San Juan Islands are greater than the differences between *A. pulchra* from *P. californicus* in the San Juan Islands and in central California (unpub. data). Hence, these two taxa should be considered separate species. *A. pulchra* was originally described from the hosts *Parastichopus californicus* and *Megathura crenulata* (a fissurellid limpet; Johnson 1897), and thus I consider the symbionts from *L. foliolata* to represent an undescribed species.

Determining whether or not the differences among *Arctonoe vittata* from different hosts detected in 1997 collections represent stable host-associated population structure requires sampling of more individuals and loci over time. That differences were evident in only one year suggests that this may be an example of "chaotic" genetic patchiness caused by spatial and temporal variation in the identity and abundance of recruits (*e.g.*, Johnson and Black 1982, 1984; Watts *et al.* 1990). *A. vittata* adults are estimated to live from 3-10 yr (Palmer 1968; Britayev 1991), so that populations sampled in 1996 and 1997 may have

been composed primarily of members of the same cohorts. However, without additional demographic data it is impossible to reject the hypothesis that different cohorts were sampled. Further, because worms from *Diodora aspera* were collected from the intertidal zone, and those from the other two host species from the subtidal zone, differentiation aong these populations might be associated primarily with habitat, not hosts. Additional differentiation among *A. vittata* using different host species may be detected when symbionts from host species not collected in this study (because of their relative rarity at study sites; Table 2.1) are considered.

These results of this study indicate that there are at least four genetically distinct, reproductively isolated species of *Arctonoe* in the Puget Sound region -- the three described species, and worms formerly considered *A. pulchra* associated with *Luidia foliolata*. There is no overlap in host use among members of these four taxa, with two possible exceptions. Pettibone (1953) recorded both *A. fragilis* and *A. vittata* from the host *Solaster dawsoni*, and both *A. pulchra* and *A. vittata* from *S. stimpsoni*. I did not examine worms from *S. dawsoni* (which was uncommon at the collection sites) in this study, and thus cannot address the first case of overlap in host use. However, it should be noted that *S. dawsoni* is probably the most likely species to serve as host to several species of symbiont. This seastar is a frequent predator of other asteroids, including *S. stimpsoni*, a host of *A. vittata*, and *Evasterias troschelii*, host of *A. fragilis* (Mauzey *et al.* 1968; pers. obs.). Transfer of symbionts from prey to predator during an attack might easily result in overlap in host use on *S. dawsoni*. Because I found no fixed differences between *A. pulchra* and *A. vittata* at the four loci examined here, I cannot exclude the possibility that some worms from *S. stimpsoni* were *A. pulchra*. However, allozyme frequencies at several loci in the population from *S. endeca* and *S. stimpsoni* were not intermediate between those of worms from *Parastichopus californicus* (*A. pulchra*) and *Diodora aspera* (*A. vittata*), suggesting that if *A. pulchra* did occur in the samples, it was uncommon. Further, all

worms examined from *S. endeca* and *S. stimpsoni* had neurosetae characteristic of *A. vittata*. These observations, of course, hold only for populations from the Puget Sound region. *Arctonoe* spp. show some variation in host use patterns over their geographic ranges (Pettibone 1953; Dimock and Davenport 1971). Additional sampling over their ranges would be very useful in clarifying the systematics and biogeography of host use in the group.

What processes are important in determining host use patterns in *Arctonoe* spp.? Not only do larvae of *Arctonoe* spp. spend a long time in the plankton, but hosts of all three described species often co-occur in the same habitats (pers. obs.). Hence, it is unlikely that the spatial distribution of larvae influences host use patterns on the geographic scale considered here. The first life history stage where sorting among host species might occur is when newly metamorphosed juveniles seek out their first host. Because it has been relatively difficult to obtain naive juvenile worms, no experiments have been carried out to see if juveniles are selective, and if so, how well their host choices correlate with observed distributions of adults. Little information is available about the biology and behavior of this important life history stage (but see Chapter One).

After juveniles have selected a host, other factors can also affect observed host use patterns. In this study I examined one such factor, physiological incompatibility with hosts, in the three described species of *Arctonoe*. Results of the transplant experiment show that neither *A. pulchra* nor *A. vittata* are compatible with the seastar *Evasterias troschelii* -- when paired with this host all individuals tested died within one week. One cause of mortality may be saponins produced by *E. troschelii*. Patterson *et al.* (1978) found that a purified saponin ("evasterogen") isolated from *E. troschelii* caused reduction in respiratory rate, and eventually death, in *A. pulchra*. It had no negative effects on *A. fragilis*, which is frequently associated with *E. troschelii* in nature. Asteroid saponins are water-soluble and are often found in high concentrations in epidermis (Mackie *et al.* 1977).

Their toxicity is related to their ability to remove cholesterol from cell membranes, eventually causing cytolysis. Asteroids avoid lysis of their own cells by substituting other sterols that do not interact strongly with saponins in cell membranes (Mackie *et al.* 1977); perhaps this is the mechanism by which *A. fragilis* resists saponin toxicity as well. Several other echinoderm hosts of *Arctonoe* spp. (or their close relatives) contain saponins (Stonik and Elyakov 1988). Whether or not their saponins influence symbiont survivorship or host use patterns might be examined in further transplant experiments.

Another possible cause of symbiont mortality might be damage caused by host pedicellariae. After contact with *Evasterias troschelii*, *Arctonoe pulchra* and *A. vittata* had often lost elytrae to pedicellariae. The basis of immunity to pedicellariae in *A. fragilis* is unknown. It is possible that the symbiont's body surface is coated with substances that inhibit, or simply do not stimulate, the closing of pedicellariae. In addition, the ruffled elytrae of *A. fragilis* may not be as easily gripped by pedicellariae as the straight-edged elytrae of *A. pulchra* and *A. vittata*. Some caprellid ectosymbionts of seastars also have the ability to avoid damage from host pedicellariae (Cuénot 1912; Patton 1968).

The transplant experiments show that while incompatibilities can partly explain observed host use patterns in *Arctonoe* spp., other processes must also be operating, as all three species of symbionts appeared to be compatible with host species on which they are never found in nature. Both *A. pulchra* and *A. vittata* are aggressive towards conspecific worms, and this behavior often results in mortality or at least injury for one worm (Palmer 1968; Dimock 1978; Britayev 1991). It seems likely that resident worms are also aggressive towards non-conspecifics that invade their host. If worms on their normal hosts are more effective aggressors than those on novel hosts, this direct interspecific competition might result in observed host use patterns. Such an explanation might hold for host use on *Diodora aspera*, where in intertidal populations in the San Juan Islands, most hosts (about 85%) bear *A. vittata* (Palmer 1968; unpub. data). However, interspecific competition

cannot explain all host use patterns. In subtidal populations of *Parastichopus californicus*, for example, relatively few individuals (about 20%) bear *A. pulchra* (pers. obs.). Hence, it does not appear that hosts are limiting in this species, and I would not expect interspecific competition to be important in restricting *Arctonoe* spp. distribution on *P. californicus*. It should be noted, however, that individual symbionts are able to move among host individuals (probably only when hosts come into contact with each other: Palmer 1968; Dimock 1974). If *A. pulchra* move among hosts frequently, competitively inferior *Arctonoe* spp. may be prevented from establishing themselves on *P. californicus*. This is similar to the hypothesis of Holmes and Price (1986) that communities of parasites are structured by biotic interactions (*e.g.*, competition) when recruitment rates of parasites are high.

Finally, other factors such as predation and parasitism may also influence host use patterns. The vulnerabilities of *Arctonoe* spp. to predators may vary in a host-specific manner, and may lead to "ecological monophagy" (Smiley 1978; Bernays 1989). Such differential vulnerabilites to predators might be mediated by how well symbiont color matches that of the host; if it does so poorly, symbionts may be vulnerable to visual predators (*e.g.*, fishes). It is not known whether color patterns in *Arctonoe* spp. are genetically or environmentally determined (Dimock 1972). Vulnerability of symbionts to predators might also be affected by the size and location of suitable resting places on the host. For example, on the host *Diodora aspera*, *A. vittata* are usually found within the snail's mantle cavity, underneath its protective shell, but on the seastar *Dermasterias imbricata* the same symbiont is found exposed on the seastar's outer surfaces, perhaps more vulnerable to predators. No natural predators of *Arctonoe* spp. are known. Symbionts on different host species may also be differentially attacked by parasites (*e.g.*, Duffy 1992); these too may influence observed host use patterns.

In *Arctonoe* spp., recently metamorphosed juveniles are the first life-history stage to interact with hosts (Palmer 1968). The initial host selection decisions made by these naive juveniles determine whether or not later processes (*e.g.*, physiological incompatibility or competition) will be important in organizing host use patterns. However, most assessments of host preference in marine symbionts (including *Arctonoe* spp.) have been done with individuals that have already occupied a host (Davenport 1950; Stevens 1990; Guo *et al.* 1994); these experiments are compromised by the real possibility that symbionts are conditioned to prefer a particular host (Dimock and Davenport 1972; Derby and Atema 1980; Gwaltney and Brooks 1993). Experiments with naive juveniles are rarely carried out because many species of symbionts are difficult to culture in the laboratory, and this has been the case with *Arctonoe* spp. (Davenport 1950; Palmer 1962; Britayev 1991). In the few cases where the behaviors of naive larvae or juvenile symbionts have been examined, their preferences tend to correspond closely with observed distributions of adults (*e.g.*, Mokady *et al.* 1991; Mokady *et al.* 1992; Edwards and Dimock 1995; Elliott *et al.* 1995). Recent studies of development and larval feeding mechanisms (Phillips and Pernet 1996; Chapter One) in *Arctonoe* spp. indicate that it is possible to obtain juveniles from laboratory cultures by feeding larvae large, nutritious particles (*e.g.*, bivalve veliger larvae). Ideally the host preferences of these juveniles could be tested in semi-natural field conditions (*e.g.*, Elliot *et al.* 1995), but the small size of recently metamorphosed juvenile *Arctonoe* spp. makes laboratory experiments more feasible. *Arctonoe* spp. may provide an excellent system for studying the genetic basis of host preferences (Stanhope *et al.* 1992, Thompson 1993), because F1 and backcross hybrids, at least, can be obtained from laboratory fertilizations (Chapter Three).

Host use patterns in *Arctonoe* spp. have consequences for their diets. In particular, the ability of *A. fragilis* to tolerate the physiologically-challenging host *Evasterias troschelii* means that it has access to an abundant and renewable source of food -- the tissue-covered

pedicellarie and spines of its host. The aboral surfaces of *E. troschelii* are densely covered with these bite-sized structures; further, they likely regenerate after being grazed (Campbell 1983). *A. fragilis* may also consume papulae of the seastar, but these contain no hard parts and are not detectable in fecal pellets. In contrast, several lines of evidence support the conclusion that *A. pulchra* and *A. vittata* do not feed directly on host tissues. First, these two species inhabit hosts that do not have pedicellariae or other small protruding structures amenable to removal by scaleworm jaws. Second, their fecal pellets contain mainly remnants of other prey items. Most of them also contain sediment, suggesting that these two species capture benthic prey as their hosts move around on the substrate. Fecal pellets of *A. fragilis*, on the other hand, usually contain nothing but ossicles of the host seastar and excretory granules. Finally, if *A. pulchra* fed on its host *Parastichopus californicus*, I would expect to see the relatively large (~100 µm) ossicles of the sea cucumber host (Lambert 1986) in its fecal pellets. While Dimock (1971) reported that sea cucumber ossicles occur occasionally in the gut contents of *A. pulchra* from southern California, I have not seen them in the fecal pellets of symbionts from the San Juan Islands.

Recent genetic analyses have revealed several instances of closely-related (presumably sister-taxa) marine symbionts living in sympatry but using different host species, both in taxa in which larval dispersal potential is low (hydrozoans: Buss and Yund 1989; shrimp: Duffy 1996) and in taxa in which it is high (crabs: Stevens 1990; bivalves: Mokady *et al.* 1994; polychaetes: this study). These examples suggest that divergence in host use patterns may play an important role in the origin of species and the maintenance of diversity in symbiotic taxa. In order to understand how this divergence occurs, additional data on the biogeography, phylogeny, and determinants of host use patterns of marine symbionts like *Arctonoe* are required.

Table 2.1. Morphological characters and host species commonly used by the three described species of *Arctonoe* in the San Juan Islands, Washington, U.S.A. Morphological characters are from Pettibone (1953), and data on host use are based on my personal observations from intertidal and subtidal collections and Pettibone (1953). Pettibone (1953) and Britayev (1991) also give records of host use over a larger spatial scale, the entire range of each species. Classification of asteroids into orders follows Blake (1987). *A. pulchra* is reported from the asteroid *Luidia foliolata* as well as the holothurian *Parastichopus californicus*, but allozyme data reported here (Chapter Two) indicate that populations on the asteroid represent an undescribed species.

	A. fragilis	*A. pulchra*	*A. vittata*
Morphological characters			
blunt, notched neurosetae	--	--	X
frilled elytrae	X	--	--
Host use			
Echinodermata: Asteroidea			
O. Forcipulatida			
Evasterias troschelii	X	--	--
Leptasterias spp.	X	--	--
Orthasterias koehleri	X	--	--
O. Platyasterida			
Luidia foliolata	--	X	--
O. Spinulosida			
Henricia spp.	--	--	X
O. Valvatida			
Dermasterias imbricata	--	--	X
O. Velatida			
Crossaster papposus	--	--	X
Solaster spp.	--	--	X
Pteraster tesselatus	--	--	X
Echinodermata: Holothuroidea			
Parastichopus californicus	--	X	--
Mollusca			
Cryptochiton stelleri	--	--	X
Diodora aspera	--	--	X
Puncturella spp.	--	--	X

Table 2.2. Host and collection locality data for the polychaetes used for allozyme electrophoresis. Unless otherwise noted, all sites are on or adjacent to San Juan Island, Washington, USA. Specimens were collected from each locality in both 1996 and 1997, except for the East Sound collection from *Luidia foliolata*, made only in 1997.

Symbiont	Host	Locality
A. fragilis	*Evasterias troschelii*	Friday Harbor, subtidal
A. pulchra	*Parastichopus californicus*	Friday Harbor, subtidal
A. pulchra	*Luidia foliolata*	Bellingham Bay, WA, subtidal
A. pulchra	*Luidia foliolata*	East Sound, Orcas Island, WA, subtidal
A. vittata	*Dermasterias inbricata*	Eagle Cove, subtidal
A. vittata	*Diodora aspera*	Eagle Cove, intertidal
A. vittata	*Solaster endeca*	Eagle Cove, subtidal
A. vittata	*Solaster stimpsoni*	Eagle Cove, subtidal

Table 2.3. Allele frequencies at four enzyme loci in 1996 and 1997 among populations of *Arctonoe* spp. Numbers of individuals sampled are in bold.

		1996						1997					
		A. fragilis	*A. pulchra*		*A. vittata*			*A. fragilis*	*A. pulchra*		*A. vittata*		
		Evasterias	*Luidia*	*Parast.*	*Derm.*	*Diod.*	*Solast.*	*Evasterias*	*Luidia*	*Parast.*	*Derm.*	*Diod.*	*Solast.*
CD	n	**20**	**20**	**22**	**11**	**25**	**49**	**21**	**22**	**28**	**24**	**28**	**22**
	a	—	.12	.14	.86	.97	.98	—	.09	.16	.73	1.0	.84
	b	1.0	.88	.86	.14	—	.02	1.0	.91	.82	.25	—	.16
	c	—	—	—	—	.03	—	—	—	.02	—	—	—
	d	—	—	—	—	—	—	—	—	—	.02	—	—
G3PDH	n	**20**	**18**	**22**	**9**	**20**	**27**	**21**	**22**	**28**	**24**	**28**	**22**
	a	1.0	—	1.0	1.0	1.0	1.0	1.0	—	1.0	1.0	1.0	1.0
	b	—	.67	—	—	—	—	—	.77	—	—	—	—
	c	—	.33	—	—	—	—	—	.23	—	—	—	—
GPI	n	**24**	**33**	**44**	**12**	**25**	**51**	**21**	**21**	**28**	**24**	**27**	**22**
	a	—	—	.28	.21	.32	.24	.02	—	.38	.23	.20	.25
	b	1.0	1.0	.72	.79	.68	.76	.98	1.0	.62	.77	.80	.75
PGM	n	**19**	**31**	**45**	**9**	**25**	**49**	**21**	**22**	**28**	**24**	**28**	**22**
	a	—	—	.01	—	—	—	—	—	—	—	—	—
	b	—	—	.11	.11	.02	.01	—	—	.18	.04	.02	—
	c	—	.97	.83	.83	.82	.84	.02	.98	.79	.73	.89	.91
	d	.37	.03	.05	.06	.02	.03	.29	—	.015	.02	—	.02
	e	.63	—	—	—	.14	.12	.69	.02	.015	.21	.09	.05
	f	—	—	—	—	—	—	—	—	—	—	—	.02

Table 2.4. Results of chi-square contingency table tests for heterogeneity in allele frequencies among host-associated populations and among species of *Arctonoe*. For comparisons within symbiont species, see Table 2.3 for the hosts from which collections were made. In the among-symbiont species comparisons, allele frequencies are those of populations on the hosts *Diodora aspera* (*A. vittata*), *Evasterias troschelii* (*A. fragilis*), and *Parastichopus californicus* (*A. pulchra*). Chi-square values are reported. "—" means that the locus was effectively monomorphic and a comparison could not be carried out. * = significant at the table-wide significance value of $p<0.05$.

Among host-associated populations, within symbiont species

	CD	*G3PDH*	*GPI*	*PGM*
1996				
A. pulchra (df=1)	0.02	80*	22.38*	6.68*
A. vittata (df=2)	4.43	—	5.48	0.07
1997				
A. pulchra (df=1)	1.57	100*	20.05*	7.99*
A. vittata (df=2)	16.53*	—	0.20	7.26*

Among symbiont species (df=1 in all comparisons)

	CD	*G3PDH*	*GPI*	*PGM*
1996				
A. fragilis x *A. pulchra*	5.87*	—	16.71*	76.48*
A. fragilis x *A. vittata*	76.10*	—	18.36*	58.34*
A. pulchra x *A. vittata*	35.17*	—	0.20	0.04
1997				
A. fragilis x *A. pulchra*	8.35*	—	17.00*	56.10*
A. fragilis x *A. vittata*	98*	—	6.99*	35.57*
A. pulchra x *A. vittata*	78.06*	—	3.90*	2.38

Table 2.5. Composition of fecal pellets of field-collected *Arctonoe* spp. (in parentheses, the hosts they were collected from). All fecal pellets produced for the three days following collection were examined; after this time period pellets contained only "excretory" granules ~20 μm in diameter, and no remaining prey items.

	A. fragilis (*Evasterias*)	*A. pulchra* (*Parastichopus*)	*A. vittata* (*Diodora*)
Number of individuals examined	28	29	26
Percent containing			
crustacean parts	7	69	85
polychaete setae	0	31	58
echinoderm ossicles	79	0	0
dipteran larvae	0	0	65
unidentified tissue	43	31	12
sediment	0	66	100
no prey items; "excretory" granules only	21	14	0

Figure 2.1. Survival of symbionts in transplants of the three described *Arctonoe* spp. among their three most common hosts in the San Juan Islands. The number of surviving symbionts (and in bold, the starting sample size) is reported in for each symbiont-host combination. Two experiments (a, in May 1997, and b, in June 1997) were carried out because experimental tanks were not large enough to accomodate adequate sample sizes of hosts in one experiment.

HOST

SYMBIONT	*Diodora aspera*	*Evasterias troschelii*	*Parastichopus californicus*
A. fragilis	a) 4 (**4**) b) 9 (**9**) Total: 13 (**13**)	a) 3 (**3**) b) 9 (**9**) Total: 12 (**12**)	a) 4 (**4**) b) 9 (**9**) Total: 13 (**13**)
A. pulchra	a) 5 (**5**) b) 8 (**8**) Total: 13 (**13**)	a) 0 (**5**) b) 0 (**8**) Total: 0 (**13**)	a) 5 (**5**) b) 8 (**8**) Total: 13 (**13**)
A. vittata	a) 5 (**5**) b) 8 (**8**) Total: 13 (**13**)	a) 0 (**5**) b) 0 (**8**) Total: 0 (**13**)	a) 5 (**5**) b) 8 (**8**) Total: 13 (**13**)

Chapter Three

GAMETE COMPATIBILITY AMONG THREE SYMPATRIC POLYCHAETES: IMPLICATIONS FOR THE EVOLUTION OF GAMETE INTERACTIONS

Abstract.—The evolution of gamete incompatibility between free-spawning marine invertebrate species has been explained by three hypotheses: (1) divergence at gamete recognition loci as a by-product of reproductive isolation, (2) selection against hybrids, and (3) a process of sexual selection involving polymorphic gamete recognition loci (Metz and Palumbi 1996). The first two hypotheses predict that gamete incompatibility appears only after reproductive isolation has arisen for other reasons, and the third that gamete incompatibility appears simultanously with reproductive isolation. Here I show that gametes of three sympatric polychaetes in the genus *Arctonoe* are compatible in all possible crosses, over a range of gamete concentrations and contact times. Allozyme and mitochondrial DNA sequence data indicate that the three species are indeed reproductively isolated lineages. These data are consistent with predictions of the first two hypotheses for the evolution of gamete incompatibility, but allow rejection of the third hypothesis. Gametes of the three species are compatible despite estimated divergence times of 1-3 million years before present; in several other marine invertebrates, divergence times of the same magnitude are associated with complete or asymmetric gamete incompatibility. It appears likely that segregation of symbiotic adults on their respective host species restricts mating opportunities, and thus gene flow, among *Arctonoe* spp.

INTRODUCTION

Speciation requires the evolution of some degree of intrinsic reproductive isolation. Hence, biologists often study speciation by examining processes leading to reproductive isolation among closely-related taxa (*e.g.*, Mayr 1942; Otte 1991; Coyne *et al.* 1994; Craig *et al.* 1997), in the hopes that these are directly related to speciation events. In marine

invertebrates, a variety of such processes have recently been examined in some detail. For example, the spatial distributions and flow environments of free-spawning invertebrates strongly influence fertilization success through gamete dilution effects (Pennington 1985; Denny and Shibata 1989), such that even slight habitat segregation among sympatric species may prevent hybridization. Even when gametes are present in high concentrations, interactions of gamete recognition molecules on the surfaces of eggs and sperm control fertilization success in particular crosses (Metz *et al.* 1994; Vacquier *et al.* 1997). Finally, even if gametes successfully fuse, fitness of the first or later generations of hybrids may be low due to hybrid inviability or breakdown (Burton 1990a,b).

Gamete interactions are among the most important of these processes in free-spawning marine invertebrates, which release gametes into the sea where fertilization occurs. Here, mating interactions between relatively sedentary reproductive adults are often limited to a few environmental or chemical cues, and simultaneous spawning by multiple species may be relatively common (*e.g.*, Babcock *et al.* 1986; Pearse *et al.* 1988). Hence, gamete interactions may be important mediators of mate choice. They are also amenable to study, because the gametes of free-spawning invertebrates are often easy to obtain and because the results of fertilization experiments are apparent after only a few minutes or hours. Fertilization success has been examined in crosses among closely-related species in diverse taxa (see Palumbi 1994 for review). These experiments show three types of results. Most commonly, gametes are incompatible -- fertilization success in reciprocal crosses of heterospecific gametes is significantly, and sometimes drastically, lower than in crosses of conspecific gametes (*e.g.*, Palumbi and Metz 1991; Marsden 1992). Also common is asymmetric incompatibility, with eggs of one species compatible with sperm of another but the reciprocal cross failing (*e.g.*, Strathmann 1981; Lessios and Cunningham 1991). The least common result is complete gamete compatibility -- that is, equivalent fertilization success in crosses of both conspecific and heterospecific gametes.

Complete or near-complete gamete compatibility has previously been reported among species in only two taxa, echinoderms (Lessios and Cunningham 1991; Byrne and Anderson 1994) and corals (Wallace and Willis 1994; Miller and Babcock 1997).

That the gametes of closely-related species are usually incompatible to some degree suggests a link between the evolution of gamete incompatibility and speciation. Three general hypotheses have been proposed to explain how this association might arise. First, gamete recognition molecules in reproductively isolated populations are expected to diverge over time in independently evolving populations. Divergence may occur as a result of drift (Lessios and Cunningham 1991), the accumulation of selectively neutral mutations, or sexual selection within each population (Palumbi 1994). This hypothesis postulates that gamete incompatibility evolves as an incidental consequence of change at gamete recognition loci in reproductively isolated populations.

A second hypothesis is that selection against hybrids promotes divergence at gamete recognition loci (*i.e.*, reinforcement: Butlin 1991; Lessios and Cunningham 1991; Vacquier *et al.* 1997). In contrast with the first hypothesis, reinforcement requires the reproductive interaction of two genetically differentiated populations. Both of these hypotheses lead to the prediction that gamete incompatibility evolves only after reproductive isolation, or in the second case, a certain amount of genetic differentiation, has arisen for other reasons.

A third hypothesis based on the population genetic models of Wu (1985) has been suggested by Metz and Palumbi (1996). Wu's model shows that in large populations with polymorphic mate recognition loci and relaxed selection on the female locus, stochastic changes in allele frequency combined with sexual selection can lead to the formation of sympatric reproductively isolated groups. These groups may eventually form permanently reproductively isolated species. Metz and Palumbi (1996) suggest that this scenario may explain the origin of gamete incompatibility, and speciation, among some members of the sea urchin genus *Echinometra*. These species are characterized by large population sizes,

polymorphism at the bindin locus (which encodes a sperm protein involved in species-specific fertilization), low genetic divergences (suggesting recent origin), and strong gamete incompatibility (Palumbi and Metz 1991). This hypothesis suggests that reproductive isolation can arise simultaneously with, and even as a result of, the evolution of gamete incompatibility.

Differentiating among these hypotheses in particular cases requires, at a minimum, reliable data on fertilization patterns and reproductive isolation among the taxa in question. Such data are not always easy to obtain. In some cases it may be difficult to detect gamete incompatibility. For example, fertilization success in crosses of both conspecific and heterospecific gametes is strongly dependent on sperm concentration (Levitan 1995; Leighton and Lewis 1982). In some cases, crosses of heterospecific gametes may result in high fertilization success only at extremely high sperm concentrations rarely seen in the field (*e.g.*, Leighton and Lewis 1982; Strathmann 1987). Thus it is important to carefully control sperm concentrations in fertilization experiments, and to use a broad range of sperm concentrations in order to ensure that such effects are not obscuring real incompatibilities.

Another more subtle problem has not been addressed in previous studies of interspecific gamete interactions. In the field, diffusion may rapidly dilute sperm, reducing the length of time that eggs and sperm interact (contact time). No field measurements of gamete contact time have been made. The model of Denny and Shibata (1989) suggests that in a fully turbulent environment (*e.g.*, the wave-swept intertidal zone) sperm may be diluted to ineffective concentrations within about 20 seconds after release. These conditions may not be experienced by most free-spawning marine invertebrates, but field experiments suggest that even under less extreme flow conditions gamete contact times may be short, on the order of one to several minutes (Pennington 1985; Levitan *et al.* 1991, 1992). In laboratory fertilization experiments, though, gametes are usually allowed to interact for periods of tens of minutes to hours (*e.g.*, Lessios and Cunningham 1990;

Palumbi and Metz 1991; Byrne and Anderson 1994). Do these long contact times obscure ecologically relevant patterns of incompatibility?

The answer to this question depends on the form of the relationship between fertilization success and contact time. Fertilization success in crosses of heterospecific gametes may increase more slowly with contact time than in crosses of conspecific gametes; in such cases experiments might show gamete incompatibility at ecologically relevant short contact times, but complete compatibility at longer contact times. As an illustration of such effects, consider the results of an experiment designed to compare fertilization success as a function of contact time in crosses within and among two closely-related species of sea urchins (Hagström and Lønning 1961; Fig. 3.1). In a cross of conspecific gametes, fertilization success was nearly 100% after only 1 minute of contact time. In contrast, in a cross of heterospecific gametes fertilization success was only 35% after 1 minute. However, after allowing the gametes "infinite" contact time, fertilization success in both crosses had reached 100% (Hagström and Lønning do not define "infinity", but its upper limit is set by the longevity of diluted sperm, ~1 hr). In this case allowing gametes to interact for longer than a few minutes might obscure a potentially important incompatibility. Gamete contact time is thus another important variable that should be examined in experiments designed to identify gamete incompatibilities. Of course, comparing fertilization success over a range of contact times is relevant only when more obvious gamete incompatibilities are not evident. Previous reports of gamete compatibility among closely-related species have not been designed to detect this effect (Lessios and Cunningham 1991; Byrne and Anderson 1994; Wallace and Willis 1994; Miller and Babcock 1997).

In this paper I describe gamete interactions among three species of sympatric, free-spawning polychaetes in the genus *Arctonoe*. I examined fertilization success in crosses of conspecific and heterospecific gametes over broad ranges of sperm concentrations and

gamete contact times; in all cases, crosses of heterospecific gametes were almost or completely as successful as crosses of conspecific gametes. In addition, patterns of genetic variation at multiple loci suggest strongly that the three species are reproductively isolated. These data show that speciation in the genus has occurred without the evolution of gamete incompatibility; hence, in this case, the third hypothesis for the evolution of gamete incompatiblity can be rejected. In the three *Arctonoe* spp., gamete incompatibility has not evolved despite estimated divergence times of from 1-3 M.Y.B.P. At present, reproductive isolation in the genus is likely maintained by spatial segregation of these symbiotic polychaetes on their respective host species. Because both theory and empirical generalizations suggest that closely-related, reproductively isolated lineages should eventually evolve gamete incompatibility, the three *Arctonoe* spp. provide a rare system in which to explore the early stages of divergence in gamete interactions.

NATURAL HISTORY OF *ARCTONOE*

The three described species in the polychaete genus *Arctonoe* are sympatric over most of their ranges in the northern Pacific, including around San Juan Island, Washington, where this study was carried out (Pettibone 1953; Hanley 1989). The species are distinguishable by the morphology of setae and elytrae (Table 2.1). Color patterns of the dorsum and elytrae are also usually diagnostic, though the extensive intraspecific variation in these traits has not been subject to rigorous analysis in northeastern Pacific populations.

All three species are obligate ectosymbionts of other marine invertebrates (Pettibone 1953). Around San Juan Island, *Arctonoe vittata* is found on a diverse array of hosts including seastars, keyhole limpets and polychaetes; the other two species, *A. fragilis* and *A. pulchra*, use fewer species of hosts (Table 2.1). Though hosts for all three species often coexist in the same subtidal habitats, overlap in host use is extremely rare (pers.

obs.). Isolated adult worms can locate new hosts using waterborne chemical signals, and such worms usually show strict preferences for their original host species (Davenport 1950; Gerber and Stout 1968; Dimock and Davenport 1971; Britayev 1991). Dimock and Davenport (1971) showed that host preferences in adult *A. pulchra* are influenced by the history of host use by individual worms.

Arctonoe pulchra and *A. vittata* feed primarily on small crustaceans and polychaetes (Chapter Two), and probably rarely if ever consume host tissues; they may, of course, influence host fitness in other ways (*e.g.*, Dimock and Dimock 1969; Wagner *et al.* 1979). *A. fragilis*, in contrast, frequently consumes tissue of host seastars, though it also feeds on crustaceans and polychaetes (Chapter Two). Its effects on host fitness are unknown.

The reproductive modes of the three species are similar. Around San Juan Island, adult worms free-spawn gametes in spring and summer. Fecundity is high, with each female spawning about 10^4 to 10^6 80-µm diameter eggs (Britayev 1991). In the laboratory, a complete gametogenic cycle can occur in one month (Chapter Two); hence, it seems possible that in nature females spawn several times during the reproductive season. Fertilized eggs develop into larvae that spend at least 6 wk feeding in the plankton (Britayev 1991; Phillips and Pernet 1996; Chapter Two). Selection of a host presumably occurs by the juvenile soon after metamorphosis (Palmer 1968), as *Arctonoe* spp. are never found free-living except as larvae.

MATERIALS AND METHODS

Collections.—I obtained worms for fertilization experiments and surveys of genetic variation in 1995 and 1996 from the San Juan Islands and Puget Sound, Washington, USA. *Arctonoe fragilis* was collected from the seastar *Evasterias troschelii* in the intertidal zone at Alki Point, Seattle, and in the subtidal zone (by divers) on the east side of San Juan Island. *A. pulchra* was also collected from the subtidal zone on the east side of San Juan

Island, from the sea cucumber *Parastichopus californicus*. Finally, *A. vittata* was collected from the intertidal zone at Deadman Cove, on the west side of San Juan Island, from its host keyhole limpet *Diodora aspera*.

Effects of sperm concentration on gamete interactions.—These experiments were designed to examine the effects of sperm concentration on fertilization success in crosses of conspecific and heterospecific gametes. They involved assessing fertilization success in eggs of a single female exposed to sperm of each of the three species at each of three concentrations, as well as a "no sperm" control for contamination or parthenogenesis. Each treatment was replicated five to ten times for eggs of each of the three species.

Worms relaxed in a 1:1 mixture of seawater and 7.5% $MgCl_2$ were sexed by removing a parapodium and examining the released gametes. Females were isolated in bowls of fresh seawater at 10-13 °C (ambient seawater temperature) until they spawned, and males were reunited with hosts to prevent them from spawning prematurely. Isolated females often spawned 12-24 hrs after isolation from their hosts, though in some cases worms were held for several weeks before spawning. Bowls were checked for spawned eggs at least four times a day in order to minimize the effects of egg aging on results. As soon as possible after spawning eggs were rinsed in 0.45 µm (mesh size) filtered seawater and used in experiments. Each worm was used only once in an experiment.

Eggs of a single female were divided into 10 treatments: fertilization by *Arctonoe fragilis* sperm (three concentrations), *A. pulchra* sperm (three concentrations), *A. vittata* sperm (three concentrations), or no sperm. Rinsed eggs were suspended in clean seawater, and 5 ml aliquots were placed into each of ten tissue culture plate wells. While egg concentration was not quantified, it was controlled within experiments -- that is, within an experiment, all treatments received the same egg concentration. Egg concentration almost certainly varied among experiments. Tissue culture plates were then floated in aquaria with running seawater, keeping treatments at 10-13 °C.

A male worm to be used as a sperm source was haphazardly selected from stocks. Sperm was surgically removed from a posterior body segment and used to make a dense sperm suspension. The concentration of sperm in this suspension was measured in a hemocytometer (four replicates), and the mean of these measurements was used to calculate the proper amount (usually ~50 μl) to add to bring the final sperm concentration to 10^5 sperm·ml^{-1} in one well. Dilutions of the suspension were then made so that the same amount could be added to bring final sperm concentrations to 10^4 sperm·ml^{-1} and 10^3 sperm·ml^{-1} in two other wells. Sperm were then added to the appropriate three wells and the contents of each well briefly stirred. This process was repeated for males of the remaining two species. Nothing was added to the control treatment. Because measuring sperm concentrations took 10-20 minutes for each species, sperm of the first species were added to eggs up to 40 minutes before sperm of the third species. Effects of the resulting variation among treatments in egg age were minimal, for two reasons. First, the order of addition of sperm was deliberately varied -- that is, over the five to ten replicate experiments for eggs of each species, sometimes conspecific sperm was added first, and sometimes heterospecific sperm. Second, egg aging does not appear to significantly reduce fertilization success until about 24 hr after spawning (pers. obs.); in all of these experiments eggs were used between one and twelve hrs after spawning.

Two hours after addition of sperm, samples of eggs were taken from each treatment and observed at 200x with a compound microscope. The first 100 eggs encountered on a microscope slide were scored as fertilized (if at the two or four-cell stage) or unfertilized (if still uncleaved). At 10-13 °C first cleavage occurs ~1.5 hr after fertilization (Chapter Two). Fertilization success was estimated as the percentage of eggs in each treatment which had undergone cleavage. Using cleavage as a marker of fertilization confounds two processes, fertilization and subsequent early development. However, in *Arctonoe* there are no obvious morphological signs of fertilization until polar bodies are given off (Chapter Two).

Because polar bodies are small and their presence difficult to score rapidly, I instead used first cleavage as a marker of fertilization. The similarity of fertilization success in crosses of conspecific and heterospecific gametes reported here suggests that this was not an important source of error.

Fertilization success was compared using ANOVA of arcsine-transformed percent fertilization data. Because each replicate used the eggs of a single female in all treatments, I used a randomized block design (Zar 1996, p.257), treating each maternal worm as a (random) block and sperm identity as a fixed factor. This allows partitioning the variance due to among-female differences in egg quality or egg concentration. Separate analyses were performed for each of the three sperm concentrations. When ANOVA indicated significant effects of sperm treatment, Tukey HSD multiple comparisons were used to identify their sources.

Effects of contact time on gamete interactions.—Here I examined the effects of gamete contact time on fertilization success in crosses of conspecific and heterospecific gametes. Because gamete contact times in the field may routinely be low (on the order of seconds to minutes: Denny and Shibata 1989; Levitan *et al.* 1991), fertilization success at short contact times may be an important indicator of gamete incompatibility.

Two primary methods have been used to manipulate gamete contact times. Spermicides can be conveniently used to rapidly incapacitate sperm at known times (*e.g.*, Rothschild and Swann 1951; Presley and Baker 1970). However, preliminary experiments with gametes of *Arctonoe* spp. revealed that tested spermicides (dilute seawater, sodium lauryl sulfate in seawater, and EDTA or potassium chloride in artificial seawater: Presley and Baker, 1970) not only incapacitated sperm, but also killed eggs. Therefore I manipulated gamete contact times using a second method -- rapidly diluting gamete mixtures in large volumes of clean seawater to bring sperm concentrations below levels effective in fertilization (*e.g.*, Levitan *et al.* 1991).

Gametes of a single female worm were obtained, washed, and suspended in clean seawater as described above. Egg concentration was estimated in three 50 µl samples, and the mean of these measurements used to adjust egg concentration to 2000 eggs·ml^{-1}. Ten ml of this egg suspension were placed into each of three 20 ml glass vials and held at 10-13 °C.

A sperm suspension from one of the three species was obtained and its concentration measured as described above. At the moment that a timer was started, a measured aliquot of this suspension was added to one of the vials of eggs to bring the final sperm concentration to 10^5 sperm·ml^{-1}. The vial was inverted once to mix gametes, and 1 ml subsamples were removed with a pipet at intervals of 10, 30, 60, 120, and 300 sec after fertilization. Immediately after removal, subsamples were expelled into a 100 ml plastic beaker whose bottom had been replaced with 30 µm (mesh size) Nytex® screen (Tetko Inc., Elmsford, N.Y.). This beaker was suspended in an 800 ml beaker of filtered seawater. The 100 ml beaker containing gametes was briefly agitated to dilute sperm, then transferred through two more 800 ml beakers of filtered seawater. By the end of the first wash, sperm concentration should have been reduced to about 10^3 sperm·ml^{-1}, a concentration shown to be ineffective in fertilization in the first series of experiments (see Results). The complete washing procedure took only 10-15 seconds. This cycle of preparing a sperm suspension, fertilizing and washing eggs was repeated with sperm of the remaining two species. As in the first set of experiments, systematic errors resulting from variation among treatments in egg age were minimal, because the order of addition of sperm was deliberately varied, and because eggs were used soon after spawning.

Two hours after insemination, fertilization success was assessed as described above. Fertilization success in eggs left in the original vial with sperm was also assessed; here, gamete contact time was 2 hrs. For eggs of each female worm, then, fertilization success was assessed for sperm of each of the three species at each of six contact times:

10, 30, 60, 120, and 300 secs, and 2 hrs. This experiment was replicated five times for eggs of *Arctonoe vittata*, and three times for eggs of *A. fragilis*. I was not able to obtain eggs of *A. pulchra* in sufficient quantities for these experiments, though sperm were available. I used ANOVA (on arcsine-transformed percent fertilization data) to test for effects of sperm identity and gamete contact time on fertilization success. Fisher's PLSD tests were used for post-hoc comparisons of means.

Culture of hybrid larvae and juveniles.—In an attempt to determine whether or not hybrids were viable, I attempted to raise them through the larval stage to sexual maturity. Hybrid larvae were raised in 600 ml beakers at densities of ~1 larva•ml^{-1}, at temperatures of 9-11° C. Every third day, the larvae were fed a mixture of *Rhodomonas* sp., *Isochrysis galbana*, and *Coscinodiscus radiatus*; early veliger larvae of bivalves (*Mytilus californianus* or *Crassostrea gigas*) or gastropods (*Melibe leonina*) were also added as food occasionally. Beakers were stirred once daily to resuspend settled food particles. Cultures were cleaned weekly by siphoning most of the seawater out of the beaker through a 50-µm mesh, and transferring the larvae to fresh seawater in a clean beaker. After metamorphosis (6-12 wks after fertilization), juveniles were removed from larval cultures and isolated in 5 ml seawater in small dishes. Juveniles were fed newly hatched *Artemia* nauplii daily, and small pieces of polychaete (*Platynereis bicanaliculata*) and unidentified amphipods weekly. They were transferred to clean dishes containing fresh seawater every few days.

Allozyme electrophoresis.—To test the hypothesis that the three species are reproductively isolated lineages, I surveyed enzyme variation using allozyme electrophoresis. Worm tissues were used either fresh or after freezing at -80 °C for up to 1 yr. Samples were prepared for electrophoresis by homogenizing a ~2 mm^3 piece of tissue in an approximately equal volume of grinding buffer (0.05 M Tris-HCl, pH 7.5). Paper wicks were soaked in the homogenate and inserted into 13% (amine-citrate morpholine

buffer) or 11% (Tris-borate-EDTA II buffer) starch gels, which were run for 4 hours at 300-350V. Procedures, buffer and stain recipes are described in Murphy *et al.* (1996).

After a preliminary survey of 17 enzyme systems, I selected five that I could consistently score and interpret. Glucose-6-phosphate isomerase [*GPI*, EC 5.3.1.9], isocitrate dehydrogenase [*IDH*, EC 1.1.1.42], and malate dehydrogenase [*MDH*, EC 1.1.1.37] resolved well in the amine-citrate morpholine buffer, and phosphoglucomutase [*PGM*, EC 5.4.2.2] and superoxide dismutase [*SOD*, EC 1.15.1.1], in the Tris-borate-EDTA II buffer. Each of the five enzyme systems encoded a single scorable locus. Alleles were named alphabetically in order of decreasing electrophoretic mobility. I used chi-square tests of independence (Zar 1996) to identify significant differences in allele frequencies at each locus among the three species. Rare alleles were pooled so that expected allele frequencies were at least five in each category. Because data were used in multiple comparisons, significance levels were adjusted by the procedures of Hochberg (1988; see Lessios 1992).

DNA sequences.—I used sequence data from the mitochondrial 16S ribosomal RNA gene to further test the hypothesis that the three *Arctonoe* spp. are reproductively isolated lineages. I extracted DNA from fresh tissues of four individuals of each species using the divalent cation-binding resin Chelex-100 (BioRad, CA). Small pieces of fresh tissue (single parapodia) were added to 200 μl of a 5% Chelex suspension, incubated at 95° C for 20 minutes, and briefly centrifuged. One μl of the supernatant was used as template in 50 μl PCR reactions, using the 16Sar and 16Sbr primers and protocols described by Palumbi (1996). PCR products were cleaned with a Qiagen PCR Purification kit, quantified spectrophotometrically, and sequenced with an Applied Biosystems Taq FS kit and automated sequencer. Products were sequenced in only one direction, starting with the 16Sbr primer.

RESULTS

Effects of sperm concentration on gamete interactions.—Fertilization success in all crosses was strongly dependent on sperm concentration (Fig. 3.2). At the lowest sperm concentrations, 10^3 sperm·ml^{-1}, essentially no eggs were fertilized; at the highest sperm concentrations, 10^5 sperm·ml^{-1}, however, nearly all eggs were fertilized. No eggs in the control treatments were fertilized, so neither sperm contamination nor parthenogenesis complicate these results.

ANOVA and post-hoc tests revealed significant effects of sperm identity on fertilization in only two crosses, both involving the eggs of *Arctonoe vittata*. At sperm concentrations of 10^4 sperm·ml^{-1}, *A. vittata* eggs showed significantly higher fertilization success when fertilized with *A. vittata* sperm (mean 5.7%, range 1-25%) than when fertilized with *A. fragilis* sperm (2.7%, 0-10%) (Tukey HSD test, $p<0.05$). At the highest sperm concentration, 10^5 sperm·ml^{-1}, *A. vittata* eggs showed significantly higher fertilization success when fertilized with *A. vittata* sperm (97.8%, 92-99%) than when fertilized with either *A. fragilis* sperm (84.2%, 51-100%) or *A. pulchra* sperm (88.0%, 62-98%) (Tukey HSD test, $p<0.05$). In all other comparisons, fertilization success was not significantly different in crosses of heterspecific gametes and conspecific gametes.

Effects of contact time on gamete interactions.—For all combinations of gametes examined, the curves relating fertilization success to contact time are similar in shape (Fig. 3.3). ANOVA showed that both gamete contact time and sperm type significantly influenced fertilization success in both types of eggs ($p<0.001$). For eggs of *A. fragilis*, sperm of *A. pulchra* and *A. vittata* produced significantly higher fertilization success than did conspecific sperm (Fishers PLSD, $p<0.01$). For eggs of *A. vittata*, conspecific sperm produced higher fertilization success than did sperm of the other two species (Fisher's PLSD, $p<0.05$).

Culture of hybrid larvae and juveniles.—Juveniles were obtained from cultures of all of the six possible hybrid crosses. Normal and hybrid juveniles grew rapidly in culture, reaching 6-10 mm in length three months after metamorphosis. After five months individuals of three hybrid genotypes -- *A. pulchra* egg x *A. fragilis* sperm, *A. pulchra* egg x *A. vittata* sperm, and *A. vittata* egg x *A. pulchra* sperm -- were sexually mature. Backcrosses of their gametes (both female and male) to parental gametes produced morphologically normal larvae; these were not cultured through metamorphosis. Individuals of the other three hybrid genotypes did not produce gametes in my cultures, but this may be a consequence of small sample sizes (n=1-4 individuals of each genotype).

Allozyme electrophoresis.—Staining patterns observed for the five loci surveyed were consistent with standard descriptions of enzyme subunit structure (Murphy *et al.* 1996). Allele frequencies at each of the loci for each of the three species are shown in Table 3.1. I found significant differences in allele frequencies for 2-3 loci in pairwise comparisons among all three species (Table 3.2). *Arctonoe fragilis* carried a unique allele at high frequency at the *PGM* locus, and *A. vittata* was nearly fixed for a unique allele at the *MDH* locus.

DNA sequences.—Amplifications with the 16Sar and 16Sbr primers yielded a single DNA fragment about 470 bp long. Because I used only the 16Sbr primer in sequencing reactions, only 400 bp of this fragment were sequenced, from four individuals of each of the three species. Sequences are shown in Table 3.3. There was no nucleotide variation in the 400 bp 16S mtDNA sequences among individuals within any of the three species. In contrast, there was marked variation among the three species (Table 3.4). Transitions outnumbered transversions in all pairwise comparisons (Table 4). Total sequence divergence in the three pairwise comparisons ranged from 1.25% to 2.75%.

DISCUSSION

These data show that the gametes of the three *Arctonoe* spp. are nearly completely compatible in all reciprocal crosses over a wide range of sperm concentrations and gamete contact times. The few detected gamete incompatibilities are small. In experiments with the eggs of *A. vittata*, for example, addition of conspecific sperm at high concentrations and long contact times resulted in 97.8% fertilization; heterospecific sperm performed significantly worse, but still fertilized 84-88% of eggs (Fig. 3.2). These small but consistent incompatibilities were also found in experimental manipulations of gamete contact time with *A. vittata* eggs. Here again conspecific sperm performed significantly, but only slightly, better than heterospecific sperm (Fig. 3.3). Further, the form of the curves relating fertilization success to contact time were similar in crosses of conspecific and heterospecific gametes, suggesting that there are no important differences in rate of fertilization in the two types of crosses.

Results of experiments with eggs of *A. fragilis* are more complex. In the sperm concentration experiments, there were no significant differences between the performance of conspecific and heterospecific sperm (Fig. 3.2). However, in experiments designed to identify effects of gamete contact time on fertilization success, conspecific sperm performed significantly worse than heterospecific sperm (Fig. 3.3). I view the results from the first series of experiments (replicated eight times) as more convincing than the second (replicated three times), and suspect that eggs of *A. fragilis* do not distinguish among sperm of the three species.

The lack of differentiation among the three species in gamete interactions contrasts with the results of surveys of genetic variation. Both allozyme data (Tables 3.1, 3.2) and mtDNA sequence data (Table 3.4) reveal significant genetic differentiation among the three species. Such data do not permit definitive conclusions about reproductive isolation; the three species may, in fact, exchange genes at some loci while selection curbs exchange at

other loci (*e.g.*, Porter *et al.* 1997). However, the concordance of morphological, ecological, and genetic data (Pettibone 1953; Hanley 1989; this study) indicates that these three species are reproductively isolated lineages.

These results show that speciation has occurred in this genus without the evolution of gamete incompatibility. In turn, this means that the hypothesis of Metz and Palumbi (1996) concerning the association of gamete incompatibility and speciation can be rejected for *Arctonoe*. I expect gamete incompatibility to evolve in the genus over time as a result of either independent divergence or reinforcement. Indeed, the small but significant reductions in fertilization success in crosses of *A. vittata* eggs and heterospecific sperm may represent an early stage in the divergence of gamete recognition molecules in the genus.

There are only a few other published reports of complete or near-complete gamete compatiblity among closely-related species of marine invertebrates (echinoderms: Lessios and Cunningham 1991; Byrne and Anderson 1994; corals: Wallace and Willis 1994; Miller and Babcock 1997). All report fertilization success of crosses performed at only a single high sperm concentration and with long (minutes to hours) contact times. [Miller and Babcock (1997, their Fig. 3) did one reciprocal cross over a range of sperm concentrations; results of that cross are suggestive of asymmetric incompatibility, but this was not confirmed statistically, perhaps because of low replication (n=2).] With these parameter choices, some ecologically relevant gamete incompatiblities may have been obscured. Performing experiments over a range of sperm concentrations and gamete contact times is essential in testing the hypothesis of gamete compatibility, because we do not have good estimates of natural values for these parameters. Another complicating factor in reports of gamete compatibility among coral species is that, despite attempts, no genetic evidence of reproductive isolation has been found among the species studied (Wallace and Willis 1994; Miller and Babcock 1997). Hence, it is possible that morphologically distinct coral

"species" regularly exchange genes, and should not evolve gamete incompatibility under current hypotheses. Without positive evidence on the taxonomic status of the organisms involved, interpretation of these studies is difficult.

In general, existing data do not permit generalizations about how gamete incompatibility evolves in marine invertebrates. The simplest hypothesis, that gamete incompatibility evolves as a result of divergence between reproductively isolated populations, has little empirical support. One type of data that would lead to the rejection of alternative hypotheses would be the detection of gamete incompatibilities among pairs of species that had speciated as a result of the appearance of geographic barriers, and had not hybridized since speciation (for example, geminate species on either side of the Isthmus of Panama: Lessios and Cunningham 1991). However, even if two species in allopatry evolve independently from each other, each may be interacting with other sympatric species. If these sympatric interactions lead to reinforcement, one might expect that gametes of the allopatric species might also become incompatible as a result. A similar case of "indirect reinforcement" has been described in *Drosophila mojavensis* (Zouros and d'Entremont 1980). Thus, careful selection of taxa for tests of the hypothesis of independent divergence is critical.

Recent molecular evidence on patterns of diversification at loci that code for gamete recognition proteins are suggestive of reinforcement. First, sequences of several gamete recognition loci in several taxa (bindin in sea urchins: Metz and Palumbi 1996; lysin and another protein in abalone: Vacquier *et al.* 1997) show signs of positive selection for divergence in amino acid sequence. There are several possible explanations for this pattern, including reinforcing selection (Vacquier *et al.* 1997). Second, Lee and Vacquier (1992) show that abalone species pairs that have more similar sequences of lysin, a gamete recognition protein, also have higher rates of non-synonymous substitutions in the lysin gene. If close relatives are more likely to be sympatric, and thus able to hybridize, and if

sequences of the lysin gene accurately reflect abalone phylogeny, this is strongly suggestive of reinforcement. Independent data on abalone phylogeny and biogeography are needed to test this hypothesis.

The third hypothesis, that reproductive isolation and gamete incompatibility are simultaneous results of drift and sexual selection in particular kinds of populations (Metz and Palumbi 1996), may apply to some sea urchins in the genus *Echinometra*. These may meet its assumptions, especially that of functional polymorphism at gamete recognition loci. Further, the hypothesis may adequately explain the apparently rapid evolution of strong, reciprocal barriers to fertilization in some members of the genus (Palumbi and Metz 1991). An alternative scenario is that *Echinometra* spp. diverged initially through isolation by distance, albeit over very large spatial scales (Palumbi *et al.* 1997); hybridization could then lead to reinforcement and the evolution of the strong gamete incompatibility seen in modern species. The results of this study clearly show that speciation has occurred without the evolution of gamete incompatibility in *Arctonoe* spp.; here, at least, this hypothesis can be rejected.

The mtDNA sequence data reported here bear on the issue of the timing of evolution of gamete incompatibility relative to speciation. While the rate of divergence at this locus has not been studied in polychaetes, estimates of divergence rates (calibrated with biogeographic or fossil data) of the sequence amplified by the 16Sar and 16Sbr primers are available from several other invertebrate taxa. These show divergence rates ranging from a minimum of 0.05-0.09%/M.Y. in corals (Romano and Palumbi 1996) to a maximum of 1%/M.Y. in mussels (Rawson and Hilbish 1995), with rates for fiddler crabs (Sturmbauer *et al.* 1996), hermit crabs (Cunningham *et al.* 1992), littorinid snails (Reid *et al.* 1996), and conid snails (T. Duda, pers. comm.) intermediate at 0.28-0.9%/M.Y. Taking 1%/M.Y. as an upper limit on divergence rate at this locus, and assuming that the three *Arctonoe* spp. diverged from a common ancestor carrying a single ancestral 16S mtDNA sequence, we

can estimate minimal divergence times for the three species of 1.25-2.75 M.Y.B.P. If these divergence rate estimates are reasonable, then despite 1-3 M.Y. of isolation, little or no gamete incompatibility has evolved among *Arctonoe* spp. These results can be compared to those found for sea urchins in the genus *Echinometra* by Lessios and Cunningham (1991). One pair of these species shows near-complete gamete compatibility despite over 3.5 M.Y. of biogeographic isolation. In other cases, though, gamete incompatibility may evolve more rapidly. Lessios and Cunningham (1991) describe another species pair with an estimated divergence time of 3.5 M.Y.B.P. that show asymmetric gamete incompatibility. More striking is the finding of Palumbi and Metz (1991) of strong reciprocal gamete incompatibility among several sea urchins with estimated divergence times of 0.5-2 M.Y.B.P. Hence, the rate of evolution of gamete incompatibility among species may be quite variable. These differences may be a consequence of variation among taxa in (i) mutation rates at gamete recognition loci, (ii) selective constraints operating on gamete recognition loci (*e.g.*, stabilizing selection), or (iii) the mode of evolution of gamete incompatibility.

If gamete incompatibility does not restrict gene flow among the three sympatric *Arctonoe* spp., what does? One possibility is that spatial segregation of spawning adults prohibits hybridization because of gamete dilution effects. Overlap in host use among the three species is rare, and alternate host species use different microhabitats, though there is a great deal of overlap in their distributions (pers. obs). This degree of spatial segregation may be enough to minimize hybridization among the three species of *Arctonoe*. Gamete dilution drastically reduces fertilization success in echinoids over distances of a few meters (Pennington 1985; Levitan 1995), and the decline of fertilization success with distance might be even more drastic in *Arctonoe* spp., simply because the animals are small and produce relatively few gametes. Hybridization would be virtually impossible if *Arctonoe* spp. mated in pairs on their respective host species, but there is no convincing evidence that

this occurs. On the contrary, at least in *A. pulchra* and *A. vittata*, only one individual is usually found per host, a pattern that is generated by the vigorous defense of host "territories" by resident worms (Palmer 1968; Dimock 1974; Britayev 1991).

Spawning asynchronies may also reduce hybridization. The reproductive seasons of the three species are similar (Chapter Two), and currently there is no reason to suspect that spawning is asynchronous on shorter time scales; however, natural spawnings have never been observed. Eggs are fertilizable with high success for 24 hours after spawning (pers. obs.), further minimizing constraints of any small-scale spawning asynchronies.

A final possibility is that hybrids may be inviable, restricting gene flow after fertilization. I was able to rear all six genotypes of hybrid larvae through metamorphosis in the laboratory, showing that hybrids are viable in early stages, and three of these F1 hybrids reached sexual maturity and produced viable gametes in the laboratory. That the other three hybrid genotypes did not reach sexual maturity may be a consequence of small sample size rather than hybrid inviability. These results suggest that hybrid inviability is not an important impediment to gene flow among *Arctonoe* spp. (especially among *A. pulchra* and *A. vittata*). At present, it seems likely that segregation of symbiotic adults on their respective host species, possibly in concert with small-scale spawning asynchronies, restricts mating opportunities and thus gene flow among the three species. Other cases where reproductive isolation among sympatric symbiotic or phytophagous species is likely mediated by disjunct host use patterns have been described in several other aquatic and terrestrial taxa (*e.g.*, Katakura *et al.* 1989, Waring *et al.* 1990, Duffy 1996, Mosco *et al.* 1997).

Table 3.1. Allele frequencies at five enzyme loci among the three *Arctonoe* spp. Sample size is given in bold at the top of each column.

		SPECIES		
		A. fragilis	*A. pulchra*	*A. vittata*
GPI	**n**	**36**	**52**	**65**
	A	--	.01	--
	B	1	.63	.78
	C	--	.02	--
	D	--	.33	.21
	E	--	.01	.01
IDH	**n**	**30**	**40**	**44**
	A	--	--	.01
	B	.03	--	--
	C	.97	1	.98
	D	--	--	.01
MDH	**n**	**32**	**43**	**58**
	A	1	.97	--
	B	--	--	.98
	C	--	.015	.01
	D	--	--	.01
	E	--	.015	00
PGM	**n**	**37**	**59**	**66**
	A	--	--	.01
	B	.51	--	.12
	C	--	.03	.02
	D	.49	--	--
	E	--	.88	.82
	F	--	.01	--
	G	--	.08	.03
SOD	**n**	**37**	**56**	**66**
	A	1	1	1

Table 3.2. Results of chi-square contingency table tests for allele frequency differences among pairs of *Arctonoe* species. Chi-square values are reported. "--" means that the locus was effectively monomorphic and a comparison could not be carried out. * = significant at $p<0.05$. Significance values are adjusted for multiple comparisons by the standard Bonferroni technique (Lessios 1992).

	A. fragilis/A. pulchra	*A. fragilis/A. vittata*	*A. pulchra/A. vittata*
GPI	34.30*	18.75*	6.22*
IDH	--	--	--
MDH	--	182*	202*
PGM	192*	142*	17.34*
SOD	--	--	--

Table 3.3. 16S mitochondrial DNA sequences from *Arctonoe* spp. Sequences start at the 5' end, ~40 bps from the 16Sar primer, and end ~30 bps from the 16Sbr primer.

	1	10	20	30
A. fragilis	AATGTTCAAC	GGCCGCGGTA	TCCTGACCGT	GCAAAGGTAG
A. pulchra	----------	----------	----------	----------
A. vittata	----------	----------	----------	----------
	40	50	60	70
A. fragilis	CATAATCATT	TGCCTTTTAA	TTGAAGGCTT	GTATGAATGG
A. pulchra	----------	----------	----------	----------
A. vittata	----------	----------	----------	----------
	80	90	100	110
A. fragilis	ATTAACGAGG	TTTTAGCTGT	CTCCCTTATA	ACCCTTTAAT
A. pulchra	----------	----------	----------	--T------C
A. vittata	----------	----------	----------	--T-------
	120	130	140	150
A. fragilis	TTAAATTTTA	GGTGAAGATG	CCTAAATCTA	GTAGAAAGAC
A. pulchra	----------	----------	---------T	---------A
A. vittata	----------	----------	-------T--	---------A
	160	170	180	190
A. fragilis	AAGAAGACCC	TATAGAGTTT	CAATAAAAAG	AAATTTCTTT
A. pulchra	----------	----------	-----G----	----------
A. vittata	----------	----------	----------	----------
	200	210	220	230
A. fragilis	TTTCTTTATT	ATTTTGGTTG	GGGCGACCAG	GGATTATAAA
A. pulchra	--A-----C-	----------	----------	-------G--
A. vittata	----------	----------	----------	----------
	240	250	260	270
A. fragilis	AACCATCCCT	AAACAATTAG	GCTACAAGCC	GTTTTATTGA
A. pulchra	----------	---T---C--	---G------	----------
A. vittata	----------	---T------	----------	A---------
	280	290	300	310
A. fragilis	CCCTCCTGGA	TTATCTGAAT	CAACTACCTT	AGGGATAACA
A. pulchra	----------	----------	----------	----------
A. vittata	----------	----------	----------	----------
	320	330	340	350
A. fragilis	GGCTTATCTT	CCTAGAGAGC	CCAAATCGAT	AGGATGGATT
A. pulchra	----------	----------	----------	----------
A. vittata	----------	----------	----------	----------
	360	370	380	390
A. fragilis	GGCACCTCGA	TGTTGGCTTA	GAGAAACTTA	TTGTCGCAGC
A. pulchra	----------	----------	----------	----------
A. vittata	----------	----------	----------	----------

Table 3.4. Divergence in nucleotide sequence of a 400 bp fragment of 16S mitochondrial DNA in pairwise comparisons of the three *Arctonoe* spp. Reported are the number of varying nucleotide sites, and, in parentheses, percent sequence divergence.

	Transitions	Transversions	Total
A. fragilis/A. pulchra	9 (2.25%)	2 (0.5%)	11 (2.75%)
A. fragilis/A. vittata	5 (1.25%)	0	5 (1.25%)
A. pulchra/A. vittata	8 (2%)	2 (0.5%)	10 (2.5%)

Figure 3.1. Fertilization success as a function of gamete contact time in crosses of *Echinus acutus* eggs with *E. acutus* sperm (circles) and *E. esculentus* sperm (squares). These results are unreplicated. Data from Hagstrom and Lonning (1961: their Table 5). "Infinity" is not defined, but an upper limit is set by sperm longevity, probably ~1 hr.

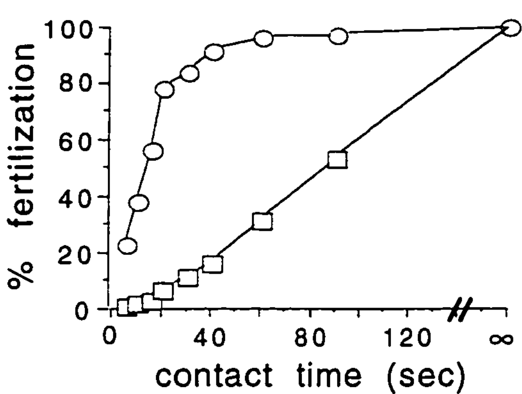
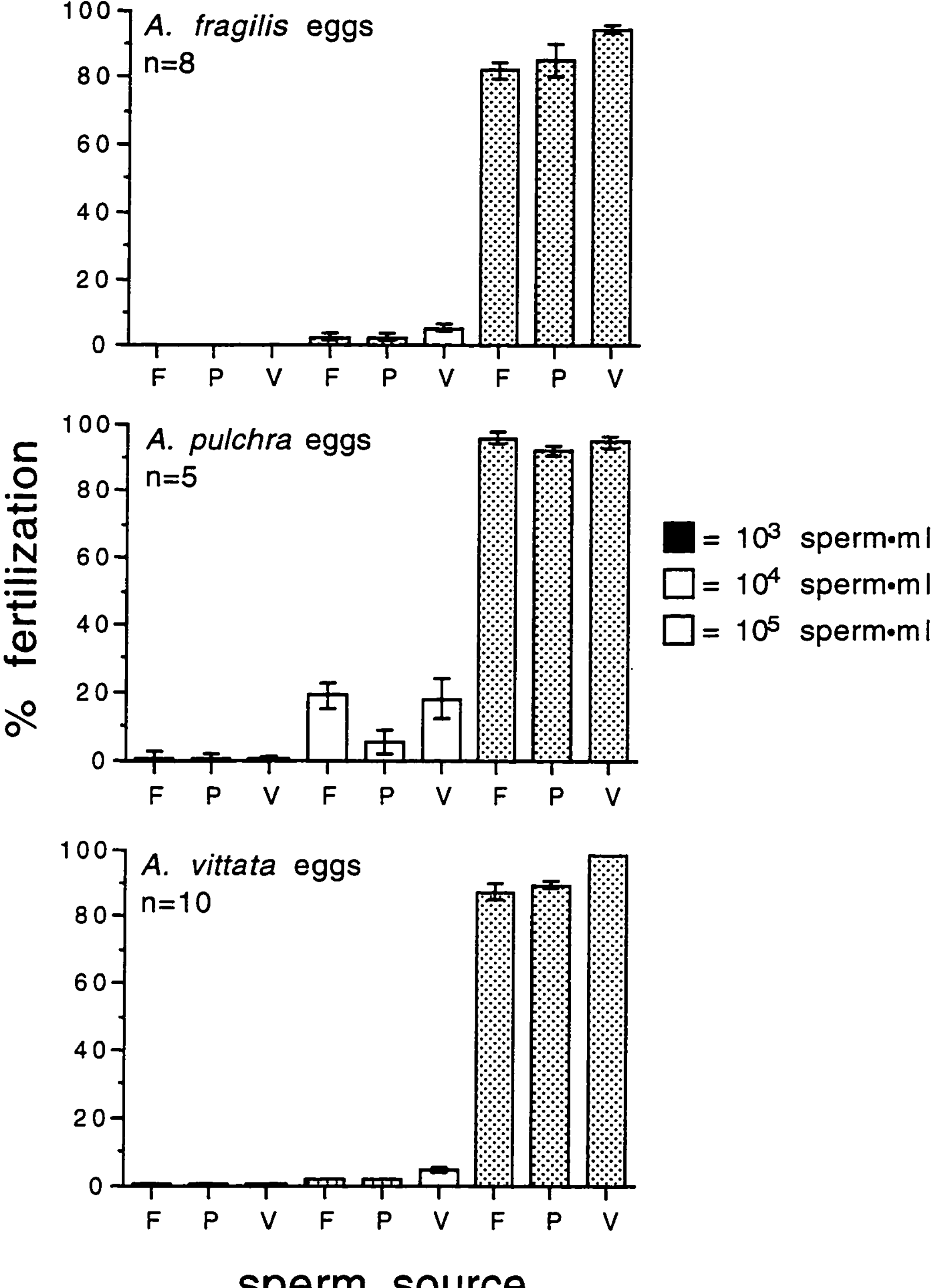
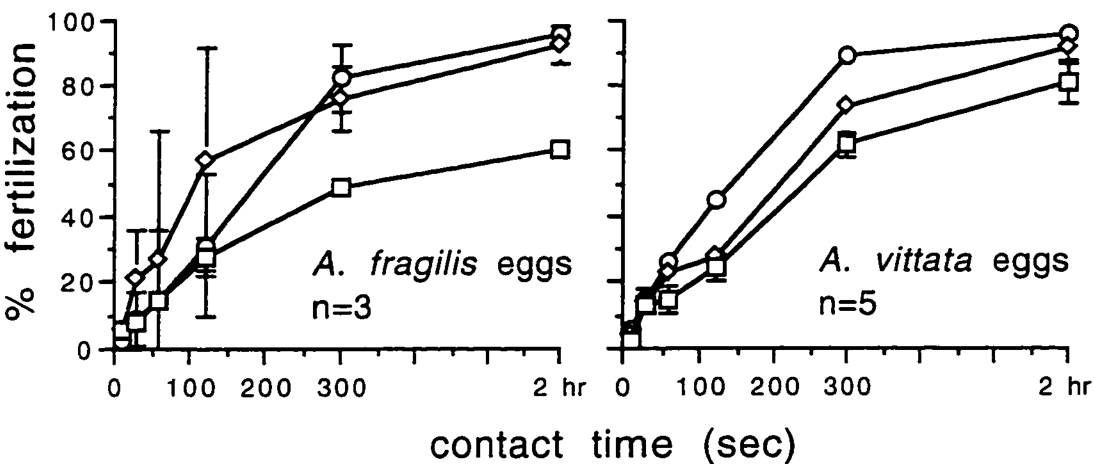

% fertilization
100
80
60
40
20
0
0
40
80
120
∞
contact time (sec)

Figure 3.2. Fertilization success over a range of sperm concentrations in crosses of conspecific and heterospecific gametes of *Arctonoe* spp. One hundred eggs were surveyed for cleavage in each replicate of each treatment. Percent data were arcsine transformed, and means and 95% confidence intervals plotted after back transformation. "n" refers to the number of replicate experiments, each using eggs of a single female.



A. fragilis eggs
n=8
A. pulchra eggs
n=5
A. vittata eggs
n=10
% fertilization
0
20
40
60
80
100
F
P
V
F
P
V
F
P
V
= 10³ sperm•ml
= 10⁴ sperm•ml
= 10⁵ sperm•ml
sperm source

Figure 3.3. Fertilization success over a range of gamete contact times in crosses of conspecific and heterospecific gametes of *Arctonoe fragilis* and *A. pulchra*. One hundred eggs were surveyed for cleavage in each replicate of each treatment. Percent data were arcsine transformed, and means and 95% confidence intervals plotted after back transformation. Symbols represent: *A. fragilis* sperm (squares), *A. pulchra* sperm (diamonds), and *A. vittata* sperm (circles). "n" refers to the number of replicate experiments, each using eggs of a single female. Crosses using eggs of *A. pulchra* were not done.



% fertilization
100
80
60
40
20
0
0
100
200
300
2 hr
A. fragilis eggs
n=3
A. vittata eggs
n=5
contact time (sec)

Chapter Four

HOW DO FERTILIZATION ASYMMETRIES AMONG CLOSELY-RELATED SPECIES ARISE?

Asymmetry in sexual isolation -- when crosses between males of one taxon and females of another taxon are successful, but the reciprocal cross fails or is less successful -- is common among animals (*e.g.*, Snell and Hawkinson 1983; Coyne and Orr 1989; Palumbi 1994; Arnold *et al.* 1996). The evolutionary origins of such asymmetries are controversial (Kaneshiro 1976, 1980; Watanabe and Kawanishi 1979; Barton and Charlesworth 1984; DeSalle and Templeton 1987; Fraser and Boake, 1997). In some taxa, they result from complex mate choice behaviors that are likely controlled by many genes (Coyne 1992). In lieu of detailed mechanistic knowledge of these behaviors and their genetic bases, the origins and dynamics of asymmetry in sexual isolation may best be understood in terms of quantitative genetic models (Arnold *et al.* 1996).

However, in other cases asymmetry in sexual isolation is likely controlled by only a few genes, and simpler models can explain its origin and fate (*e.g.*, Coyne *et al.* 1994). Many free-spawning marine invertebrates may fall into this category. Here, gametes are simply shed into the sea, and mate choice is mediated by the interactions of a few sets of molecules associated with sperm and eggs (termed gamete recognition molecules; Garbers 1989). In sea urchins, for example, the interaction of the sperm acrosomal protein bindin with an egg surface receptor appears to be a major determinant of fertilization species-specificity (Vacquier *et al.* 1995). A different system, involving interactions between the sperm protein lysin and the egg vitelline envelope, mediates fertilization species-specificity in abalone and some other molluscs (Shaw *et al.* 1994). Fertilization between closely-related species of marine invertebrates is often markedly asymmetric, with eggs of one species being compatible with the sperm of a second, but the reciprocal cross failing (Palumbi 1994). Such fertilization asymmetries, which have been noted most frequently in

echinoderms (*e.g.*, Strathmann 1981; Lessios and Cunningham 1990; Uehara *et al.* 1990), but also in cnidarians (Buss and Yund 1989; Miller and Babcock 1997) and molluscs (Leighton and Lewis 1982; Grant *et al.* 1998), are presumably mediated by interactions of these same gamete recognition molecules. Despite the relative simplicity of these systems, the origins of fertilization asymmetries in marine invertebrates are not understood (Strathmann 1981; Lessios and Cunningham 1991; Palumbi 1994). Their consequences may include asymmetric introgression of mitochondrial DNA, and nonconcordant patterns of introgression of mitochondrial and nuclear genomes, in hybridizing taxa (Arnold 1997; Rawson and Hilbish 1998).

Here I develop the idea that the evolution of fertilization asymmetries in marine invertebrates may be explained by an extension of the Dobzhansky-Muller model for the evolution of post-zygotic inviability (Dobzhansky 1937; Muller 1942; Orr 1995). Markow (1997) made a similar suggestion to explain mating asymmetries in *Drosophila*. My version of the model, explicitly applied to the loci that control gamete recognition molecules, is illustrated in Figure 1. Consider a population of animals in which only two gamete recognition molecules are important in mediating fertilization -- a molecule associated with sperm, encoded by a single gene, and a molecule associated with eggs, encoded by another gene. This initial population is homozygous at both gamete recognition loci (aabb). Individuals in the population produce sperm with the a phenotype, and eggs with the b phenotype; these eggs and sperm are fully compatible, and produce high yields of zygotes when combined.

Let this population be divided into two reproductively isolated populations. In one, a new allele (A) appears at the sperm locus and is fixed. This population is now comprised of AAbb individuals, which produce sperm with the A phenotype and eggs with the b phenotype. These gametes must be compatible, or the A allele would not have become fixed. At this point the two allopatric populations still have reciprocally compatible

gametes: the only possible crosses, A-sperm x b-eggs and a-sperm x b-eggs, produce zygotes efficiently.

As the populations continue to diverge, a new allele (B) arises at the egg locus in the second population and is fixed. This population is now composed of aaBB individuals, which produce sperm with the a phenotype and eggs with the B phenotype. Again, these gametes must be compatible, or the B allele would not have become fixed. Crosses of a-sperm (from the second population) and b-eggs (from the first population) are successful, as these are the ancestral gamete recognition alleles. However, the reciprocal cross (A-sperm from the first population x B-eggs from the second) involves gamete recognition molecules that have never been tested together, and these molecules may not be compatible with each other. If not, fertilization in crosses between these two populations will be asymmetric. If the new molecules are compatible with one another, then the process of substitution at gamete recognition loci outlined above simply continues until an incompatibility appears between gametes of the two populations. The actual pattern of substitutions at the two loci is not important; changes can occur in both populations, or in one. As long as changes occur at both loci, the first incompatibility that arises will result in asymmetric fertilization in the hybrid crosses.

This model relies on several assumptions about how gamete recognition systems work. First, I assume that only one pair of gamete recognition molecules is important in determining fertilization success. In reality, fertilization success is likely influenced by numerous sets of gamete interaction molecules. For example, in sea urchins fertilization may be blocked at a minimum of four steps, each of which likely involves interactions between a pair or more of sperm and egg molecules (Ohlendieck and Lennarz 1996). Violation of this assumption should not alter the predictions of the model; in fact, the more sets of gamete recognition molecules involved in sperm-egg interactions, the more

opportunities there are for the evolution of fertilization asymmetries, and the more rapidly it should arise as two taxa diverge.

Second, gamete interaction molecules of both sperm and eggs must tolerate some variation in their interaction partners. Small changes in the sperm molecule must be tolerated by the egg molecule, and vice versa; otherwise, new alleles at either locus would be selected against, and divergence at gamete recognition loci would not occur. This assumption is probably correct. In sea urchins in the genus *Echinometra*, for example, egg bindin receptors apparently interact successfully with a variety of sperm bindin molecules (Metz and Palumbi 1996).

Third, interactions between gamete recognition molecules must at least sometimes be intransitive. For example, I assume that the gamete recognition molecules A and B are not necessarily compatible, even though the combinations ab, Ab, and aB are compatible. An example of such interactions is found in the vetigastropod *Haliotis*. Here, the sperm protein lysin dissolves the egg vitelline envelope, allowing the sperm to pass through and contact the egg membrane. Purified lysin of *H. corrugata* is effective in dissolving vitelline envelopes of both *H. corrugata* and *H. rufescens*; however, *H. rufescens* lysin can only dissolve *H. rufescens* vitelline envelope (Vacquier *et al.* 1990).

Thus fertilization asymmetries among closely-related taxa may arise as a simple consequence of divergence at gamete recognition loci. Indeed, given sequential substitutions of new alleles at both recognition loci, asymmetry is an inevitable consequence of divergence. The model described here makes no predictions about the magnitude of the initial asymmetry; this is determined by how novel alleles at gamete recognition loci interact with alleles of the other population, and will depend on the actual identities of the novel alleles.

Other considerations suggest that fertilization asymmetry is probably a temporary condition. In the absence of hybridization between two isolated populations, additional

substitutions will likely accumulate at gamete recognition loci until eventually fertilization fails in both hybrid crosses. This process may occur quickly if gamete recognition loci coevolve within each population, as seems likely. If the two populations hybridize while fertilization is asymmetric, several outcomes are possible. Interactions among other complementary loci (Dobzhansky 1937; Orr 1995) may result in reinforcing selection against hybrids, which might act to make fertilization incompatibility symmetric. Alternatively, gene flow might eventually erase the among-population differences at gamete recognition loci.

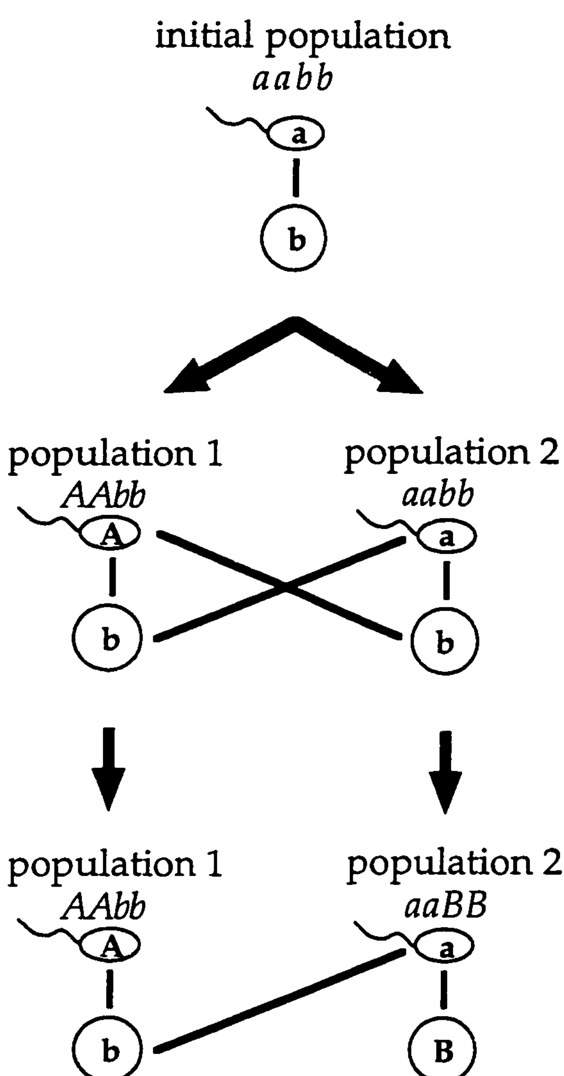
If fertilization asymmetry is only a temporary condition, with further divergence at gamete recognition loci leading to symmetric incompatibility, the frequency of asymmetric fertilization should be higher among pairs of recently-diverged species than it is among pairs of species that diverged a long time ago. This prediction can be tested in two ways. First, one might plot a calculated index of asymmetry (*e.g.*, Arnold *et al.* 1996; Fraser and Boake 1997) versus an estimate of divergence time for numerous species pairs. The resulting distribution should be curvilinear, with low asymmetry (symmetric compatibility) between recently-diverged species, maximal asymmetry in species with intermediate divergence times, and low asymmetry (symmetric incompatibility) among species that diverged a long time ago. (Note that this prediction is identical to that of Arnold *et al.* [1996], who derive it from a quantitative genetic model.) Alternatively, one could map the results of fertilization experiments on to phylogenies of the taxa in question. In this case, only closely-related species pairs would be expected to show asymmetric fertilization.

These tests are not yet possible in any taxon of marine invertebrates. Data on fertilization success in hybrid crosses are relatively rare (especially in large and well-sampled clades), and when available are often of relatively low quality because important parameters (especially gamete concentration) have not been controlled or specified by investigators, or because of lack of replication. Hence, it is difficult to accumulate

meaningful comparative data from the literature. In the few taxa where adequate fertilization data are available phylogenies or estimates of divergence times are not available.

In summary, applying the Dobzhansky-Muller model for the origin of hybrid inviability specifically to the genes that control gamete recognition yields important insights into how asymmetries may evolve in simple molecular recognition systems. The model introduced here suggests that fertilization asymmetries arise as an inevitable consequence of divergence at gamete recognition loci. Further, it makes the testable prediction that fertilization asymmetries are likely temporary, as additional changes at gamete recognition loci will eventually lead to symmetric incompatibility in hybrid crosses. These insights are especially relevant as the role of gamete recognition interactions in the speciation of marine invertebrates begins to receive increased scrutiny (Palumbi 1994; Lee and Vacquier 1995).

Figure 4.1. The origin of fertilization asymmetries between reproductively isolated populations. An initial population is homozygous at two recognition loci (one of which controls sperm phenotype, and the other egg phenotype). It is divided into two isolated populations by some prezygotic barrier (*e.g.*, vicariance). A mutation arises and is fixed at the sperm locus in the first population; then a mutation arises and is fixed at the egg locus in the second population. While these new alleles are compatible with ancestral alleles, they have never been tested together and may not interact properly, leading to fertilization asymmetry. Lines joining gametes indicate compatibility.


initial population
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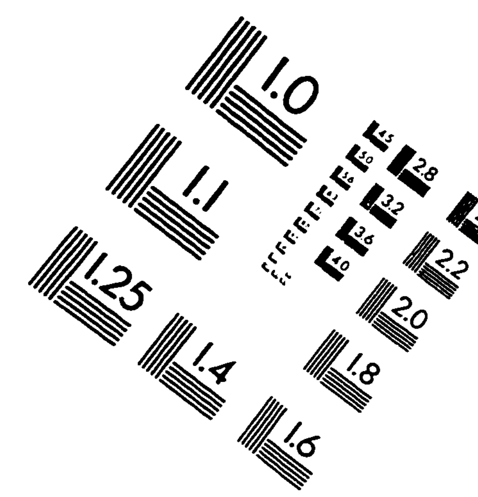

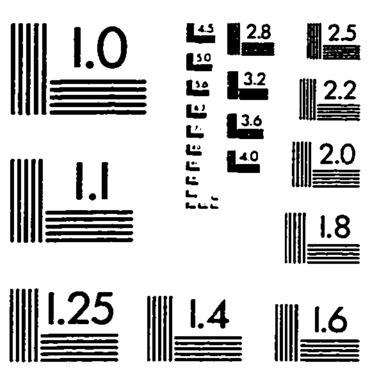

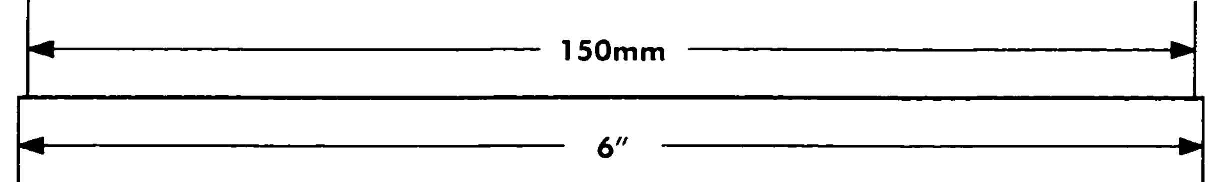

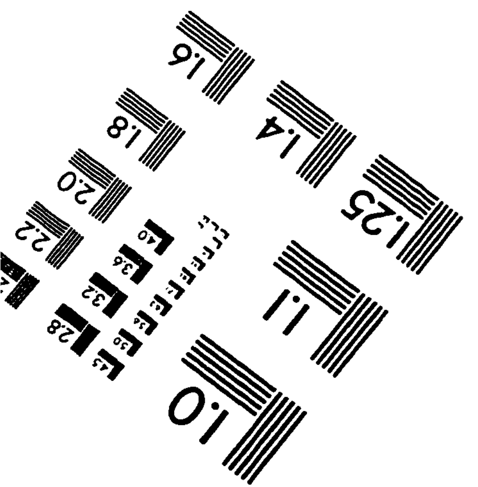

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