

# FINAL REPORT

## *Manayunkia speciosa*: Life History, Rearing, and Associated Development of *Ceratomyxa shasta* (2005-FP-10)

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## ABSTRACT

*Ceratomyxa shasta* is a serious pathogen of anadromous salmonids in the Klamath River Basin. It has a complicated life history using salmonids as the vertebrate host and the freshwater polychaete *Manayunkia speciosa* as the invertebrate (definitive) host. The objectives of this study were to: (1) rear *Manayunkia speciosa* in the laboratory so that its life history could be described, (2) examine uninfected and infected polychaetes histologically so that histopathology of infection could be described, and (3) describe the sequential development of *C. shasta* in the polychaete. This final report is broken into three parts which correspond both to these objectives and to three papers submitted or to be submitted to peer-reviewed journals. Part 1 includes rearing and life history of the polychaete. Part 2 includes internal anatomy and histology of the polychaete. Part 3 includes sequential development of *C. shasta* in *M. speciosa* with notes on the histopathology.

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Life History of a Freshwater Polychaete Host for Salmon Diseases

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Running title: Culture and Life History of *Manayunkia speciosa*

**Abstract.** Infection of juvenile salmon with the myxosporean parasites *Ceratomyxa shasta* and *Parvicapsula minibicornis* is adversely affecting their survival and freshwater production in the Klamath River and elsewhere in the Pacific Northwest USA. The freshwater polychaete *Manayunkia speciosa* serves as the obligate intermediate host for both parasites. Scant information on the life history of the polychaete and inability to maintain individuals in culture has hindered development of management strategies to control disease outbreaks in Klamath River salmon. Through trial-and error- manipulations of food and water sources, culture environments and water velocities, we established conditions that allowed us to maintain polychaetes in culture over a 10-month period. In lab culture, eggs and young were brooded within the maternal tube for a 6-8 week period during which 5 developmental stages were distinguished. Peak egg production occurred in late spring-early summer, with each female brooding up to 35 offspring. Juveniles left the maternal tube at a length of approximately 1 mm, and become reproductively mature in late fall-early winter at a length of approximately 2 mm. The absence of adult polychaetes in preserved river samples that were collected in mid-summer suggests a generation time of 1 year. Establishment of juveniles in close proximity to the maternal parent suggests that widespread dispersal within a river may be primarily due to mechanical dislodgement rather than active drifting.

*Manayunkia speciosa* LEIDY 1858 (Polychaeta: Fabriciidae) is a tube-dwelling freshwater polychaete distributed in coastal rivers of eastern and western North America, as well as in the Great Lakes region (Hiltunen 1965, Mackie & Qadri 1971, Rolan 1975, Spencer 1976). Until recently, it was placed in the family Sabellidae (Kupriyanov & Rouse 2008). Within the Pacific Northwest, *M. speciosa* has been identified as the obligate intermediate host for *Ceratomyxa shasta* NOBLE 1950 and *Parvicapsula minibicornis* KENT et al 2001, two myxozoan parasites of salmonid fishes (Bartholomew et al. 1997; Bartholomew et al. 2006). These parasites have been implicated as a cause of recent extensive losses in juvenile Chinook salmon (*Oncorhynchus tshawytscha* WALBAUM 1792) in the Klamath River, a 423 km river flowing through northern California and southern Oregon (USA), (Foott et al. 1999; Foott et al 2002). For example, an estimated 45 percent of juvenile Chinook salmon captured in 2004 in the lower Klamath River were infected with *C. shasta* (Nichols & Foott 2006), which invades the intestine and is usually fatal. Infection prevalence by *P. minibicornis*, which infects the kidney, has been found to be greater than 90 percent. Dual infection with both parasites is common.

Management objectives of reducing salmonid mortality from these parasitic diseases in the Klamath River and elsewhere has generated interest in life cycle details of *M. speciosa*. Reduction of polychaete populations in affected river reaches represents a potential means by which disease levels could be reduced; however, implementation of this approach is hampered by poor understanding of the polychaete life history.

Knowledge of the reproduction and development of *M. speciosa* is largely limited to histological observations (Leidy 1883, Pettibone 1953, Holmquist 1973, Rouse 1995). In *M. speciosa* the sexes are separate (Pettibone 1953, Holmquist 1973), and non-feeding larvae are brooded in the tube of an adult (Leidy 1883). Female *Manayunkia* have extraovarian oogenesis,

in which oocytes develop independently in the coelom, rather than developing in clusters (Eckelbarger 2005). Males have a dorsal sperm duct (Holmquist 1973), and females have a dorsal sperm storage structure located in the radiolar crown (Rouse 1995). This suggests that sperm is released into the water by the males, and then captured and stored by the females, likely making physical contact between the male and female unnecessary.

Detailed life history information is available only for approximately three percent of polychaete species worldwide (Eckelbarger 2005), and studies of related polychaete species likely provide the best basis for inference about life cycle timing in *M. speciosa*. The timing of development in congeneric *Manayunkia aestuarina* BOURNE 1983 was described by Bick (1996). In the Baltic Sea population of *M. aesturina* that Bick studied, gametogenesis began in January, and eggs were first brooded in mid- March. Independent juveniles appeared in mid- May, suggesting a brooding time of approximately 8 weeks. Juveniles made up half of the population by mid- July, and by October most adults had died. Reproduction of *M. speciosa* may be less synchronous. In the Klamath River, Stocking (2006) found brooding larvae in tubes of *M. speciosa* that were collected in March, July, August, and November of 2005, with greatest abundance in March and November.

A more complete understanding of the life history of *M. speciosa* and its role in disease dynamics have been limited by an inability to maintain *M. speciosa* in culture (Stocking 2006). In this paper, we describe culture conditions we developed through trial-and-error manipulations of varying levels of water sources, food types, culture environments, and water velocities that allowed us to maintain a captive, breeding population of *M. speciosa* over a 10-month period, and we present life history information obtained from observation of the laboratory population and from preserved field samples.

## Methods

### *Field collection*

*M. speciosa* were collected live in September 2006 from the mouth of the Williamson River, in Chiloquin, Oregon (N42° 28' 03.6", W121° 57' 25.2"), in May 2007 from the Klamath River at Keno, Oregon (N42 08' 59.5", W122 01' 10.2") , and in July 2007 from the Cayuse River Access (N41° 51' 11.6", W122° 41' 41.1") near Gottsville, California. At the collection site on the Williamson River, the substrate from which the animals were collected consisted of gravel and sand- sized volcanic particles, overlain with a layer of silt and mud. The highest density of polychaetes at the location was found in and around sparse macrophyte beds. Polychaetes here were collected with a D-frame 250 µm mesh net bounced lightly over the river substrate. Timed samples of 30 seconds at this location often yielded approximately 100 polychaetes. At the Keno collection site, *M. speciosa* were collected from an eddy adjacent to a riffle tailout. Substrate consisted of large, periphyton-covered boulders, with silt trapped in the periphyton. Polychaetes were collected by scraping a D-frame net over the surface of the boulders. At the Cayuse River Access, polychaetes were collected in and around beds of mussels and freshwater sponges. At all sites, samples were sieved through a 250 µm sieve to remove ultrafine particles, placed in plastic zip top bags, and transported to the laboratory in coolers. Additional samples were collected in August and October of 2006 at the mouths of Beaver Creek (N41° 51' 51.5" ,W122° 49' 8.9") and Horse Creek (N41° 49' 24.2" ,W123° 00' 17.1") in Siskiyou County, California, to compare the timing of life history stages between lab and field conditions. These samples were preserved in 70% ethanol.

### *Culture Conditions*

Polychaete samples were maintained in covered outdoor circular tanks at the Humboldt State University hatchery, in Arcata, California. Each tank had a diameter of 62 cm and was filled to a depth of 19 cm. A bank of two tanks shared a common sump, which consisted of a 55 liter box into which each circular tank drained. An external pump returned water to the tanks. Water entered each tank through a spray bar. A valve could be opened or closed to control the amount of water entering each tank. The velocity in each tank was controlled by a combination of positioning the spray bars and opening or closing valves until the desired velocity range was reached ( $\pm 2$  cm/s), but precise control of velocity was not possible.

Trays constructed to house single polychaetes, which allowed us to track growth and reproductive status of individuals, were placed within each circular tank (Fig. 1). Trays were made of two 12mm thick sheets of plexiglass that were cut to 250 x 105 mm and glued together. Four rows of seven evenly spaced holes each were drilled into each tray. Each hole was sized to exactly fit one size 00 Beem® capsule (8 mm inner diameter x 20 mm high). Lids were removed from Beem® capsules before the capsules were placed in the trays. Approximately half of the depth of each capsule was filled with glue from a hot glue gun to reduce the amount of sediment that had to be sorted to retrieve each polychaete at the end of the experiment. Remaining space with filled with substrate from the Klamath River. Substrate was collected near the boat launch at the U.S. Forest Service Tree of Heaven campground on the Klamath River (N41° 49' 35.6" W122° 39' 37.2") in September 2006. We have observed polychaetes to be abundant at this location. Substrate that passed through a 250  $\mu$ m sieve was retained, allowed to settle in a graduated cylinder, and excess water was decanted off the top. Remaining material was frozen to kill contaminant organisms, and thawed before use.

An alternative rearing environment using plastic live boxes placed within a Living Stream™ unit was also evaluated. Live boxes measured 28 cm by 18 cm x 6 cm, with side, top, and bottom panels of 25-250 µm mesh Nyltex to allow flow through each box. The Living Stream environment allowed temperature control and uniform flow conditions, and live boxes kept samples separate, but fouling of the boxes and reduced flow through mesh panels resulted in reduced polychaete survival relative to survival in trays within the circular tanks.

Tanks were originally filled with water from the salmonid hatchery system at Humboldt State University, which originates from a small natural stream near the campus. The presence of predatory flatworms in the water supply, which were observed to consume polychaetes, made it necessary to switch to using de-chlorinated city water for the remainder of the study. In a few instances, we observed that a polychaete was able to regenerate its crown following a non-lethal attack. Water temperature was not controlled, but was recorded on a weekly basis with a YSI® unit from October 2006 until January 2007, and continuously from January 2007 through July 2007 with a Hobo® temperature recorder. Temperature ranged between 3 and 19° C. Tanks received ambient lighting, but were covered with chicken wire netting to keep out raccoons. Velocity was maintained at approximately 3-4 cm/s, based on preliminary trials which indicated that individual growth in length of polychaetes was greater at this velocity than at 1-2 cm/s, and that velocities of 5-6 cm/s led to substantial displacement of polychaetes. Velocity was measured with a Marsh-McBirney, Inc. Model 2000 Portable Flowmeter. Velocity was checked weekly and adjusted as needed. Circular tanks were filled with water and water velocity was set before adding polychaete trays. Trays were gently submerged to avoid dislodging polychaetes from capsules. All trays were placed so that they were not directly under the inflow.

Each of the 4 circular tanks used in this study received 1 ml of Kent Marine, Inc. Micro-Vert Invertebrate Food®, 3 times per week. This food source supported polychaete growth. We estimated specific growth rates (SGR) in units of animal length, which was measured as:

$$\text{SGR} = \frac{\ln L_t - \ln L_o}{t} \times 100$$

where  $L_t$  = final length,  $L_o$  = initial length, and  $t$  = duration of the experiment in days (Ricker 1975). We compared SGR between polychaetes fed with this food source and polychaetes without added food in preliminary trials of approximately 1-month duration during December-January. Under conditions of similar velocity (3-4 cm/s), the specific growth rate of polychaetes receiving the invertebrate food averaged 0.89 percent per day ( $n = 24$ ,  $SD = 0.21$ ), while the specific growth rate of polychaetes without added food averaged 0.54 percent per day ( $n = 18$ ,  $SD = 0.20$ ). We also explored the use of powdered nutritional yeast as a potential food source for the polychaetes. This food source was rejected because it promoted rapid bacterial growth within the rearing tanks.

### *Polychaete Censuses*

Individual polychaetes were removed from field samples with the aid of a dissecting microscope. If a live polychaete was inside a tube, it was removed by gently probing one end of the tube until the worm emerged. The worms were transferred with fine flexible forceps (Bioquip™) to glass Petri dishes for measurement of length ( $\pm 0.1$  mm). A ruled plastic strip was placed on the stage of the dissecting scope, so that it was visible through the bottom of the Petri dishes. Measurements were made without use of an anesthetic to avoid stress to the animal. The worms usually relaxed after being removed from the lighted microscope and placed on a dark countertop for several minutes. They were then placed back under the microscope and quickly measured before they started moving again.

After being measured, each polychaete was placed in an individual, substrate-filled Beem® capsule in a tray. Once each tray was filled, a light sprinkling of substrate was added to top off the capsules. Based on previous observations, polychaetes were allowed at least ten minutes to begin tube reconstruction prior to being placed in a rearing tank.

Polychaetes were censused on a monthly basis by carefully removing a tray from the tank, and pipetting the contents of each Beem® capsule into a Petri dish. The material was examined under a dissecting scope for the presence or absence of polychaetes. If a polychaete was found, it was measured as described earlier, and sexed if possible, before being returned to its capsule. In some instances, adult males and females were paired within one capsule to encourage reproduction. Numbers of eggs or brooding larvae were also counted during censuses, and their developmental state was described. Usually only 1 tray (of 28 individuals or up to 56 adults once males and females were paired up, plus whatever young were produced in the time period) could be censused per day due to time constraints.

#### *Egg incubation/rearing Study*

Eggs and brooded young were removed from tubes of female polychaetes following a census to determine if these could be reared independently of its parent and to avoid double-counting offspring. Eggs were usually expelled as the female left her tube upon being disturbed. In cases where not all eggs were expelled, fine flexible forceps were used to gently push the eggs out of the tube. Eggs were gently picked up with forceps and moved into labeled well slides containing de-chlorinated tap water at room-temperature. Egg length was measured and eggs were observed using a dissecting microscope. After observation, a glass cover slip was placed over each slide to keep eggs from being flushed out as slides were transferred to an incubation

container. Once slides were transferred, coverslips were removed by slowly sliding them off of the well slides.

The incubation container consisted of a rectangular Tupperware™ container (16.5 x 21.5 cm), which was filled to a depth of approximately 4 cm with de-chlorinated tap water. Gentle aeration was provided, and water was maintained at room temperature of approximately 21° C. Salinity of the tap water was 0.3ppt. Water was replaced and the entire incubation set-up cleaned on a weekly basis to control fungal growth. In addition, developing eggs were moved to clean well slides as needed based on fungal growth, generally every other day. Eggs and developing juveniles were observed and measured daily. Juveniles were observed to have endogenous nutrition supplies, so no supplementary food was added.

## **Results**

### *Lab rearing units*

Few polychaetes abandoned the rearing trays. After leaving the maternal tube, free-living juvenile polychaetes established tubes in close proximity to the maternal tube within the same capsule, and did not enter the water column to relocate.

Gender of individual polychaetes could usually be determined by examination at 100x magnification when polychaetes became reproductively mature at approximately 1.8 – 2 mm. In the lab population, gender was apparent during late winter (February and March 2007) (Fig. 2). In males, developing sperm gave a cloudy appearance to the posterior half of the body. Females were easier to sex because of the relatively large size of eggs, which developed in the anterior half of the body.

In lab rearing units, eggs first appeared in the tubes of a very few females in February 2007. During February and March, only 3-4% of 75 observed females were brooding eggs. Early brooding females were all dead by May, and none produced more than a total of four eggs each. The first substantial egg production occurred in early to mid April 2007, with 22% of 41 observed females brooding young. This increased to 55% of 58 observed females in May, and 76% of 41 observed females in July (Fig. 3), at which time the artificial rearing study was terminated.

Juvenile polychaetes remained in the tube of the female until reaching a length of approximately 1.0-1.2mm. Juveniles began to reach this size in lab rearing units during the last week of May, suggesting a brooding time of around 6-8 weeks. The greatest number of eggs produced by any female polychaete in lab rearing units by the end of May was 11 (Fig. 4). By mid July, most females had brooded between 1-10 eggs, and the most produced by a single female was 36 eggs (Fig. 5).

#### *Artificial Incubation of Eggs*

When disturbed, most polychaetes abandoned their tubes, usually dislodging any brooding offspring in the process. However, brooding females were slower to leave their tubes than were males or other females without brooding offspring. Once out of the maternal tube, developing juveniles that had not reached a length of approximately 1 mm prior to tube abandonment invariably died. We attempted to artificially rear abandoned eggs to better understand the development of the young within the maternal tube. The greatest difficulty in rearing eggs was in controlling fungal growth. Very few eggs survived the process, and none were reared entirely from newly fertilized egg to independent survival. However, many survived for a week or more, and enough individuals of different developmental stages were collected to

give some indication of the stages and timing of development. Individuals that were closer to the end of the brooding process when they were removed from the maternal tube had a better survival rate, and many of these survived through the onset of independent feeding.

Early development of *M. speciosa* within the maternal tube was broken down into five stages, which are described in Table 1 and illustrated in Figs 6-10. At stage VI, animals left the maternal tube to begin independent existence. Because of the variety of egg sizes present in the tube of a single female, egg production was likely continuous, with an egg or two laid every few days. For example, in the tube of one female on one censusing date, 10 eggs were at stage 1, 6 were at stage 2, 2 were at stage 3, 7 were at stage 4, and 2 were at stage 5.

#### *Preserved River Samples*

In May 2007, the sex ratio of preserved adult polychaetes was approximately 50:50. In the same sample, 71% of 17 in-river females were brooding versus 55% of 58 females in the rearing units (Fig. 4). Wild females also had more offspring brooding at that time than females in the lab population. The highest number of offspring brooded by a single wild-caught female by May was 35, whereas the most produced by a captive female was 11 (Fig. 4).

In preserved river samples, brooding was evident in May 2007 samples, but larvae had not yet left the tubes, so only adults were measured. At that point the majority of the population measured between 2.0-3.9mm (Fig. 12). By July 2007, when the young had been released, offspring outnumbered adults, and the proportion of young continued to increase. Very few worms longer than 1.9mm were found in August 2006, suggesting that the adults had largely died off by that time. In September and October 2006, most were in the 1.0-1.9 mm range, but some juveniles had grown in to the 2.0-2.9mm category. A few had reached the 3.0-3.9mm category.

## Discussion

Comparison of the timing of the reproductive season of *M. speciosa* between Stocking (2006) and this study must be considered in light of annual variation in water flow and temperature in the Klamath River. In 2005, Stocking found tubes with 2-3 brooding offspring in March, July, August, and November, with peaks occurring in March and November. This study suggested a shorter reproductive season, with a peak in brooding numbers occurring in May, and juveniles being released during June and July. The adults died off by October, leaving only immature offspring which did not begin to mature until the following January-February. Differences in length and timing of the reproductive season are likely due to annual temperature variation, which Bick (1996) suggested was the case for *M. aestuarina*.

Sampling techniques used in field collections may lead to under-estimation of the numbers of offspring produced by *M. speciosa*. Disturbance created by sampling usually causes polychaetes to leave their tubes, causing any brooded offspring to be dislodged and destroyed, or lost through the sieve. Our experimental use of artificial substrates alleviated this problem, by allowing the polychaetes to be collected and preserved quickly, with minimal disturbance to the brooded offspring.

In the original description of *M. speciosa*, Leidy mentioned finding "...about a half a dozen..." developing young in a tube (1883), and Stocking reported tubes with 2-3 larvae (2006). The highest number of eggs and embryos reported developing in a single tube of *M. aestuarina* was 16 (Bick 1996). In contrast, we found a single female *M. speciosa* in May brooding 35 offspring at once. In addition, 36% of the 17 sampled females had more than 20

developing young per female. Since these were found relatively early in the breeding season, it is quite possible that a single female could produce 40-50 offspring in a single reproductive season, although further investigation is needed to confirm this.

Females collected from the river began brooding earlier and produced greater numbers of offspring than captive females. This difference in timing is likely due to temperature. The difference in numbers of offspring produced is likely primarily due to disturbance. Captive females were removed from their tubes and measured on several occasions, causing the loss of any brooded offspring, and presumably causing a fair amount of stress to the adults. However, it is also possible that temperature or dietary variation may have contributed to the difference. Further experimentation with controlled amounts of disturbance would be useful, as this could provide insight into the effects of river flow management on polychaete populations.

Though straying rates of captive polychaetes were not quantified in this experiment, they appeared to be very low. On two occasions loose organic detritus was removed from the tanks in which the polychaete rearing trays were located, and was examined for the presence of stray polychaetes. No more than 1-2 polychaetes were found per tank, and the rate remained low even after the young left the maternal tube. The establishment of juvenile polychaetes in close proximity to the maternal parent suggests that widespread dispersal in the river may be primarily due to mechanical dislodgement rather than active drifting. These findings would be of significance in planning any program to limit polychaete populations through manipulation of river flows, and may explain why polychaetes are found in such high densities at preferred sites.

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## Figure Captions

**1.1 FIGURE 1. TRAYS USED FOR REARING OF INDIVIDUAL *M. SPECIOSA*. TRAY SIZE WAS 250 MM X 105 MM. INSIDE DIAMETER OF EACH WELL WAS 8 MM. WELLS WERE FITTED WITH BEEM CAPSULES THAT WERE FILLED WITH FINE SEDIMENTS FROM THE KLAMATH RIVER. AN INDIVIDUAL POLYCHAETE WAS PLACED WITHIN EACH CAPSULE.**

Figure 2. A. Mature female *M. speciosa*, 3.3 mm long, with eggs visible in the coelom midway along the length of the animal. B. Mature male *M. speciosa*, 3.3. mm long. The posterior half of the body becomes cloudy as sperm develop.

Figure 3. Percentage of captive *M. speciosa* females brooding eggs during Dec 2006 - July 2007. Numbers in parentheses near each datapoint represent sample sizes.

Figure 4. Percentage of captive and wild female *M. speciosa* with differing numbers of brooded offspring. Brooded offspring of captive females (n = 41) were counted in monthly censuses during Dec 2006-May 2007 and represent the cumulative total through May. Brooded offspring of wild *M. speciosa* (n = 17) were counted in Klamath River samples collected May 13, 2007.

Figure 5. Percentage of captive female *M. speciosa* with differing numbers of brooded offspring from Dec 2006 – July 2007.

Figure 6. Stage I egg of *Manayunkia speciosa*. Egg length is approximately 0.15 mm.

Figure 7. Early (top) and late (bottom) stage II eggs of *Manayunkia speciosa*. The anterior end of the egg becomes rectangular in shape, while the posterior end remains ovular. Egg length is approximately 0.2 mm.

Figure 8: Stage III eggs of *Manayunkia speciosa*, showing early crown formation that appears as individuals approach 0.3mm in length.

Figure 9: Stage IV development of *Manayunkia speciosa*, showing early eyespot formation at 0.6mm body length. Note setal development.

Figure 10: Stage V development of *Manayunkia speciosa*, after which individuals leave the maternal tube and take up independent existence. This occurs at approximately 1.0mm in body length. Larva shown outside of its tube.

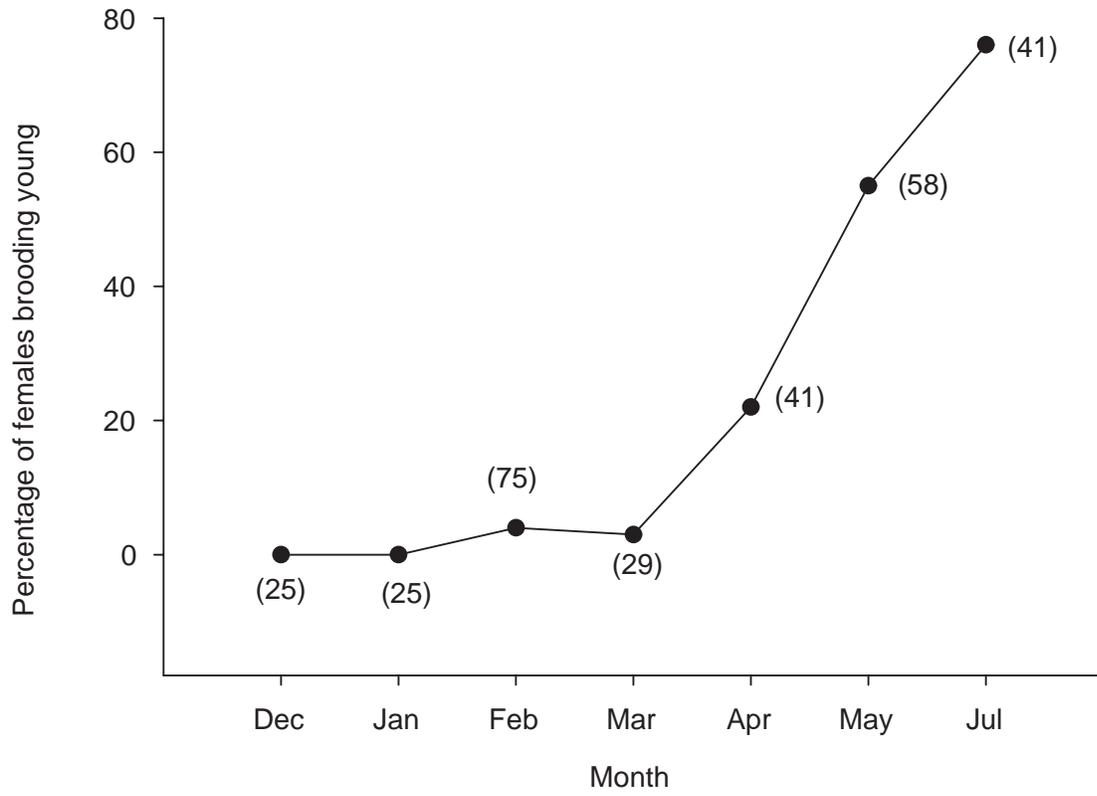
Fig. 11. Size frequency distribution of wild *Manayunkia speciosa* collected from the Klamath River in August, September, and October 2006, and in May and July 2007.



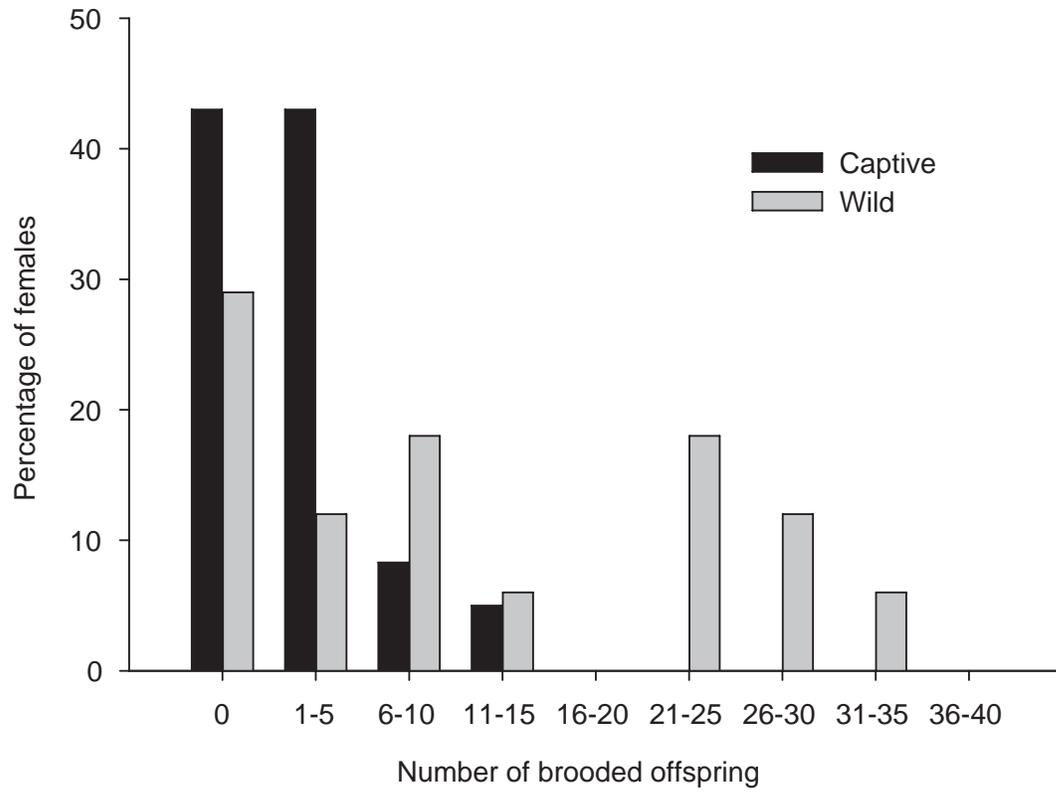
**Figure 1**



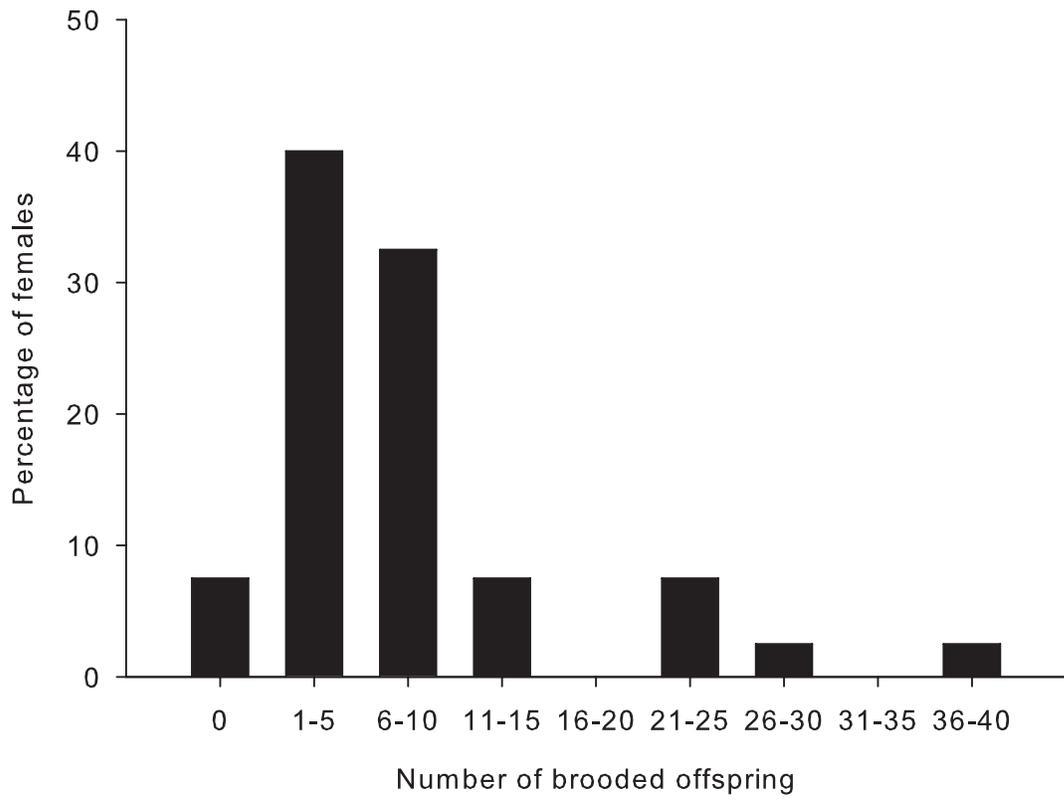
**Figure 2**



**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**



**Figure 7**



**Figure 8**



**Figure 9**



**Figure 10**

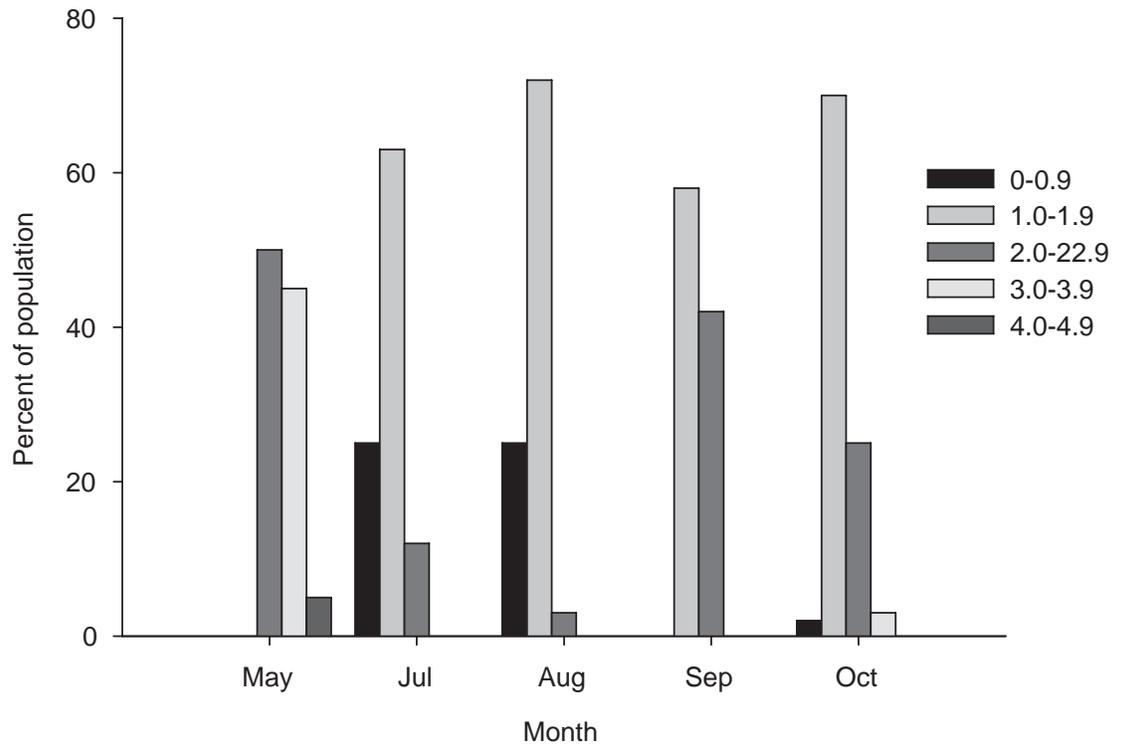


Figure 11

Table 1. Duration, ranges of individual length, and significant features of early developmental stages of *M. speciosa* grown in laboratory culture at 21° C. Stages 1-V occur within the maternal tube.

Stage	Duration (d)	Polychaete Length (mm)	Description
I	undetermined	0.15	Fertilization, early division of oval-shaped egg
II	2-3	0.2	Elongation of egg prior to crown formation; at end of stage, anterior end of egg flattens, while posterior end remains oval
III	3-5	0.2-0.3	Crown formation begins with formation of two anteriorly directed projections; at end of stage, first movement arises, characterized by slight sideways bending with both ends of worm moving simultaneously
IV	14	0.3-0.6	Eyespots appear; setae appear at animal length of 0.5 mm; rudimentary tube construction
V	3	0.6-0.7	Increasing movement, characterized by separate movement of anterior and posterior ends of body, but without lengthwise movement; setae lengthen; digestive tract empties as endogenous nutrition is depleted
VI		0.7-1.0	Lengthwise movement on flat surface, tube construction; exogenous feeding; independent survival

**INTERNAL ANATOMY, STRUCTURE, AND FUNCTION IN RELATION TO LIFE  
HISTORY CHARACTERISTICS OF THE FRESHWATER POLYCHAETE,  
*MANAYUNKIA SPECIOSA***

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Running title: Internal anatomy of *Manayunkia speciosa*

**ABSTRACT.** *Manayunkia speciosa* (Fabriciidae) is a basal organism, representing a relict form of a marine incursion. The internal anatomy of *M. speciosa* is specialized for freshwater environments. Longitudinal and oblique musculature allows mobility, which in combination with manipulation of chaetal structures provides stability within riverine habitats. Furthermore, arrangement of chaetal bundles and uncini supports an additional level of maneuvering capability. Feeding habits are diverse, which allows for survival under a variety of environmental conditions and nutrient availabilities within freshwater environments. Waste processing is efficient and simplistic. The excretory system is a metanephridium paired with a simple ciliated gut within each segment. Organization of circulatory and nervous systems of *M. speciosa* provides evidence that it is a basal organism. The circulatory system is closed with two main vessels that line the dorsal and ventral portions of the gut. The nervous system includes a ventral nerve cord that is in an intraepithelial position lined with basal cells. In addition, posterior segments are innervated with chaetal nerves that maintain coordination between musculature and chaetae. The sensory system is composed of nuchal organs, statocysts, and simple ocelli. Together these structures provide information on chemical composition, gravity, and light. Female *M. speciosa* have spermathecae or sperm storage organs. This is a fairly unusual characteristic, which is not consistently found throughout Fabriciidae. Conversely, spermiogenesis and associated organs in males are similar to that described for other fabriciids. Similarities and differences found between *M. speciosa* and other fabriciids demonstrate that it is a basal organism with marine ancestry that has adapted to the freshwater environment.

Foster (1972) reported that there were three families of polychaetes (Nereidae, Sabellidae, and Serpulidae) with freshwater representatives in North America. Since the time of that publication, polychaetes within the subfamily Fabriciinae have been separated from Sabellidae into Fabriciidae (Kupriyanova & Rouse 2008), making *Manayunkia speciosa* LEIDY 1858 the only freshwater representative in North America of a predominantly marine polychaete family (Table 1). During the late Quaternary, marine populations of Fabriciidae established themselves in freshwater environments through a marine incursion (Croskery 1978). The current patchy distribution of *M. speciosa* within limited areas of freshwater lakes and rivers of North America is evidence of this marine incursion.

Fabriciidae, more commonly known as feather-duster worms, are generally characterized by the presence of body segmentation, prostomium branchial crown with paired radioles, biramous parapodia with numerous chaetae, fused prostomium and peristomium, and an epibenthic mode of life (Rouse & Pleijel 2001b). *Manayunkia speciosa* is characterized externally by grooved V-shaped radioles with ciliated paths, short total length (3 to 4 mm), and 12 total body segments in adults (Leidy 1883).

*Manayunkia speciosa* is the invertebrate host of two serious myxozoan parasites (*Ceratomyxa shasta* NOBLE 1950 and *Parvicapsula minibicornis* KENT 1997) of salmonids found in Pacific Northwest drainages. Myxozoans are spore-forming parasites with two morphologically different spore phases of their life cycle: myxospores and actinospores (Lom & Dyková 2006). The majority of myxozoan parasite life cycles involve both an invertebrate (definitive) host and vertebrate host (typically teleost fish). Both *C. shasta* and *P. minibicornis* use *M. speciosa* as the invertebrate host and a salmonid as the vertebrate host. Infection by the

two myxozoan parasites in the salmonid host results in the diseases ceratomyxosis and glomerulonephritis, respectively.

Evidence presented by Siddall et al. (1995) established myxozoans as a clade of highly derived parasitic cnidarians. Although simplification of morphology has occurred from its diversion into Myxozoa, phylogenetic data substantiate the connection to Cnidaria. What is interesting about the two myxozoan parasites in the Klamath River basin is that they share a similar evolutionary pattern to both their hosts. Both *C. shasta* and *P. minibicornis* are lone freshwater representatives of marine genera (Bartholomew et al. 2006, Lom & Dyková 2006). Additionally, freshwater colonization came secondary to the marine existence.

The goal of this study was to histologically describe internal anatomy of the invertebrate host, *Manayunkia speciosa*. Internal anatomy was then related to life history characteristics of the polychaete. It is important to fully understand internal structure and function of uninfected tissue before being able to report on how parasites may alter both of these parameters.

According to Hoffmann (1999), the first steps in studying parasites are to (1) identify the parasite, (2) obtain a thorough knowledge of its life history, and (3) understand ecological requirements of the parasite. Because so much of what we need to understand about myxozoan parasites is directly related to the host(s), this study is designed to improve our basic information on the invertebrate host of both *C. shasta* and *P. minibicornis*. The following is a general description of the internal structure and function of *M. speciosa* based on samples acquired from the upper Klamath River, Oregon.

## **METHODS**

*Manayunkia speciosa* was collected at multiple sites within the upper Klamath River, Oregon. The majority of samples used in this study were collected from the Williamson River

upstream of Upper Klamath Lake, and Keno Reservoir just south of Klamath Falls. Samples were collected with a long handled, D-frame 250  $\mu\text{m}$  mesh dipnet, targeting (1) sand-silt embedded with fine benthic organic matter, (2) large substrate with *Cladophora* spp., or (3) sediment with large beds of vascular macrophytes (Stocking & Bartholomew 2007). Samples were then washed through a 250  $\mu\text{m}$  mesh Tyler<sup>®</sup> sieve, placed in Zip-Lock<sup>®</sup> bags, and transported to Humboldt State University in Arcata, California, where they were sorted under a dissecting microscope for adult polychaetes.

Adult polychaetes were fixed in alcohol-formalin-acetic acid (AFA) in a 5 mL glass scintillation vial with at least 10 times the volume of tissue being fixed for at least 24 hours (Strange 1996, Bartholomew et al. 2006). Samples were then transferred to a solution of 70% ethanol (EtOH) for another 24 hours prior to processing to ensure that all fixative was removed from the tissue. Samples were then placed in a series of tertiary butyl alcohol (TBA, ascending) and EtOH (descending) solutions to dehydrate the tissue and infiltrate it with a medium that is miscible with paraffin (Humason 1979). After infiltration with TBA, the alcohol was slowly replaced with paraffin. Tissue was then embedded into 100% paraffin and cut with a Reichert Histostat<sup>®</sup> rotary microtome at 4 to 6  $\mu\text{m}$ . Sections were affixed onto standard microscope slides using a thin layer of Haupt's adhesive, 4% formalin, and heat (~50°C).

Histological sections were stained with either a Hemotoxylin and Eosin (Humason 1979) or May-Grünwald Giemsa protocol (Yasutake & Wales 1983). Histological sections were examined using a standard light microscope under phase and bright field with at least 400x magnification. Photographs were taken with a Nikon<sup>®</sup> ED and Martin<sup>®</sup> adapter for light microscopes.

## RESULTS

Rouse & Pleijel (2001a) described three main regions to the polychaete body that develop from the larval form: (1) presegmental region, (2) metameric repetition or segmentation, and (3) pygidium or non-segmented posterior end (Fig. 1). There is still some debate as to exactly where the prostomium and peristomium originate in the larval form, because they are fused in adult Fabriciidae. However, it is hypothesized that development of the prostomium expands from the episphere and the peristomium expands from the prototroch and buccal region (Rouse & Pleijel 2001b). The location where the prostomium and peristomium separate is the dorsal lip of the peristomial collar, which is located along the dorsal surface of the presegmental region. Below this is the fecal groove, which is a ciliated path that leads from the peristomial collar along the dorsal side of the thorax. At the thorax-abdomen junction, the fecal groove curves around the left side of the body and then runs along the ventral side in the abdomen, where it terminates at the anus in the ventral portion of the pygidium (Lewis 1968a; Rouse & Pleijel 2001b).

Segments are added along the anterior edge of the pygidium in the larval form. Each segment contains a muscular, digestive, vascular, and nervous system that are united to previous segments through transverse septa (Gardiner 1992). Based on a 12-segmented body morphology, the following labels will be used for the remainder of this work: (1) prostomium/peristomium is segment 1, (2) metameric repetition is within segments 2 through 12 (i.e., thoracic chaetigers 1-8 and abdominal chaetigers 9-11), and (3) pygidium at the posterior end of segment 12.

During fixation and washing in 70% EtOH, polychaetes shrunk an average 34% in length. This was followed by an additional 15% average loss of length during tissue processing,

sectioning, and staining for histology. Note that all measurements (e.g., epidermal width, cross sectional width, etc.) that are provided in this study have a similar loss due to processing.

### **Integument**

Integument of polychaetes is generally composed of: (1) supporting cells (epidermal cells), (2) basal cells (associated with neural cells), (3) sensory cells, (4) secretory cells, and (5) an overlying cuticular layer (Gardiner 1992). *Manayunkia speciosa* epidermis consists primarily of simple columnar epithelial cells. However, along the length of the body, there are variations in thickness and cell organization (Figs. 2-4). For example, each segment between the peristomium and pygidium contains parapodia with chaetae. Areas where parapodia begin to form coincide with narrowing of the epidermis. Additionally, thickness of the epidermis varies by segment, with a thicker epidermis anteriorly (up to 48  $\mu\text{m}$  in a 2.4 mm worm), decreasing within mid-segments (down to 2.4  $\mu\text{m}$ ), and increasing again posteriorly. Overall, there is a wide range of epidermal thickness in each segment of the polychaete (Table 2).

There is a predominance of secretory cells throughout the length of the polychaete. Secretory cells that contain mucus stain as dark objects with an amorphous shape (Fig. 5). Secretory cells in *M. speciosa* are concentrated around the periphery of the pygidium and radioles. This agrees with the branchial structure that Lewis (1968a) reported in *Manayunkia aestuarina* BOURNE 1883 from brackish lentic habitat. *Manayunkia aestuarina* has mucous cells in the radioles but not at the top edges of the V-shaped grooves. In the mid-region of *M. speciosa*, secretory cells are concentrated along the ventral surface. However, the integument is also thicker ventrally compared to dorsally, so this may be more a factor of available surface area. Aside from secretory cells, standard columnar epithelial cells are found anteriorly, giving way to basal cells posteriorly. Basal cells are especially concentrated around the ventral nerve

cord and chaetal structures, but are also interspersed throughout the integument. There is a limited cuticular layer in *M. speciosa*, which is a common trait of tube-dwelling organisms (Gardiner 1992).

### **Tube-Building**

Under laboratory conditions, *Manayunkia speciosa* constructed tubes out of a variety of materials of various sizes and shapes. These materials ranged from fine particulate organic matter to 210  $\mu\text{m}$  glass beads. Based on studies of natural *M. speciosa* populations, Poe & Stefan (1974) reported that the preferred size range for tube material was less than 2.5  $\mu\text{m}$  (fine particulate matter). Preferred microhabitat was later substantiated by Stocking & Bartholomew (2007) with Klamath River polychaete populations. This study identified populations primarily within sand-silt embedded with fine benthic organic matter and *Cladophora* attached to larger substrate material. The method of tube construction has been described in detail by Lewis (1968a) for *Manayunkia aestuarina*, and only a few comments will be added to this thorough account in relation to *M. speciosa*.

Rouse & Pleijel (2001b) indicated that the family Fabriciidae has the ability to size-select particles with their radioles. Each radiole has a grooved path that is V-shaped toward its base, and as particles are collected, larger particles lodge at the top and smaller particles fall to the base (Lewis 1968a). Radioles emerge from the junction of the prostomium and peristomium and are ciliated along longitudinal grooved paths. Specifically, grooved radioles of *M. speciosa* form a prostomial branchial crown in a symmetrical pattern with two main clusters or lophophores, each with one radiole connecting internally (Fig. 6). The number of radioles varies by age of the worm; a mature worm will typically have 10 to 15 per lophophore. This number agrees with both Leidy (1883) and Holmquist (1966).

Proteineaceous mucus, within the secretory cells along the body wall and within radioles, is used in holding tube materials together. Particles used for tube-construction are coated with mucus and transported by cilia to the dorsal lip of the peristomial collar, where they are added to the top of the tube (Lewis 1968a). In addition, while in its tube, *M. speciosa* reinforces construction by moving in a circular motion and simultaneously secreting mucus. In this way, *M. speciosa* is able to construct a stable tube that can be many times longer than the polychaete. During construction, the tube is typically attached to larger substrate or an epilithic alga (*Cladophora* spp.) within transitional zones between riffle and pool habitat (Stocking & Bartholomew 2007).

Polychaetes are soft-bodied organisms. Therefore, the tube provides a hard layer of protection against predation. Typically, the outer surface of the epidermis is a thin collagenous cuticle (Gardiner 1992). However, the cuticular layer of *Manayunkia speciosa* is much reduced in structure and function. Both transverse and longitudinal sections of *M. speciosa* still within a tube contain a continuous dark-staining layer (Figs. 7-8). This layer is a product of numerous mucous layers laid down during tube construction.

### **Locomotion**

Although *Manayunkia speciosa* has poor swimming capabilities compared to other polychaetes (Croskery 1978), it is relatively mobile for a tube-dwelling organism. When observed under a dissecting microscope, *M. speciosa* typically moved through substrate in either a circular or undulating pattern with the pygidium in front. This behavior was also observed in *Fabricia sabella* EHRENBERG 1836 by Lewis (1968b), who theorized that this orientation protects the branchial crown. As previously mentioned, *Manayunkia speciosa* also uses circular locomotion to exit and enter its tube.

Both longitudinal and oblique striated musculature is used in locomotion (Rouse & Pleijel 2001a). In segments 2 through 12, there are four longitudinal muscle bands around the perimeter of the coelom (Fig. 9). Due to segmentation of the body, the polychaete can contract its longitudinal muscles to elongate or widen its fluid-filled coelom and move in an undulating pattern (Gardiner 1992). It is also worth noting that the coelom of *M. speciosa* is typically much reduced in size, which is common for smaller polychaetes. However, in each sex, the coelom is larger at the site of gamete development.

Oblique muscle fibers are the primary source of locomotor control (Gardiner 1992). In general, these fibers manipulate chaetae, which in turn provide traction and maneuver the body. Chaetae (setae) are basically  $\beta$ -chitin bound with protein (Rouse & Pleijel 2001a). Chaetal shape in *M. speciosa* is the most basic type of tapering cylinder (flexible capillary chaetae). Chaetae emanate from parapodia and are surrounded by oblique muscle fibers (Fig. 10).

The last external chaetal-type of structures that are important to *M. speciosa* locomotion are uncini. These are hook-like structures that are individual projections in thoracic segments 3 to 9 and dense rows in abdominal segments 10 to 12 (Fig. 11). The number and shape of abdominal uncini can be used in both aging and species identification (Croskery 1978). In *M. speciosa*, thoracic uncini are in two rows and typically range from 4 to 6 per segment. They are simple recurved hooks with small denticles that can point in both an anterior or posterior direction. In contrast, abdominal uncini are found in one dense row with typically 9 to 25 hooks per segment. Number of uncini within the last three segments decreases along with the tapering of the polychaete body. Abdominal uncini are broader in shape with larger denticles that typically point posteriorly. Both types of uncini are primarily used for maintaining position within the tube (Leidy 1883; Rouse & Pleijel 2001a). In this way, *M. speciosa* can anchor itself

within the tube while feeding. Simultaneously, it can maintain space for water exchange and fecal evacuation. This same anchoring strategy was reported for other species within Sabellidae and Fabriciidae (Knight-Jones 1981).

A general feature of fabriciid, sabellid and serpulid polychaetes is biramous parapodia, which means that lateral appendages consist of an upper notopodium and lower neuropodium. Segmental inversion is where capillary chaetae are located in notopodia and uncini in neuropodia in anterior segments, and then inverted in posterior segments (Kupriyanova et al. 2006). The location where this inversion occurs is the thorax-abdomen junction. In *Manayunkia speciosa*, the thorax is segments 2 through 9, while the abdomen is segments 10 through 12. Although this segmental inversion is considered pleisomorphic for polychaetes in Fabriciidae, Sabellidae and Serpulidae, the specific organization of uncini and chaetal bundles can also be considered an adaptation to freshwater environments. For example, dense rows of notopodial uncini in the posterior three segments can be used for hooking into algae or other substrates to feed or stabilize within the current while constructing a tube. Additionally, capillary chaetal bundles in the anterior segments can be used to direct the location of feeding within riverine habitat. This theory is supported in part by Gardiner (1992), who commented that chaetal organization allowed for detection of surrounding objects, current, and particles. Additional support is provided by Woodin & Merz (1987), who reported that uncini were oriented to resist removal from the tube. Notopodial uncini of *M. speciosa* are primarily oriented posteriorly (away from the tube mouth), which would protect them more efficiently against water currents rather than predators. Additionally, neuropodial uncini are oriented both anteriorly and posteriorly, which would allow for shifts in current direction and protect against predation.

## Feeding

There are three types of feeding in *Manayunkia* spp. (Lewis 1968a): (1) deposit feeding, (2) suspension feeding, and (3) secondary suspension feeding. Grooved ciliated radioles of *M. speciosa* that are used for tube construction are also used for feeding. Radioles and cilia are used in tandem to create a current within the surrounding water (Lewis 1968a; Rouse & Pleijel 2001b; Meaders, pers. obs.). Cilia and chaetae may also act as sensory receptors for muscles and direct the specific location of feeding (Gardiner 1992).

Directly following collection, food particles are transferred to the base of the branchial region where they are sorted. Smaller particles are pushed into the upward current of the fecal groove (Lewis 1968a). Mucous glands present along the sides of the buccal opening, fecal groove, and esophagus can also aid in capture and transport of food particles to the gut (Lewis 1968a; Gardiner 1992). The buccal cavity in *M. speciosa* lacks differentiation and is seen as a simple opening of the esophagus into the gut.

The gut itself is a simple structure that follows the segmentation of the polychaete. In general, a polychaete gut consists of an inner wall, intestinal epithelium, splanchnic musculature, and an outer layer of noncontractile peritoneal cells (Gardiner 1992). Additionally, the gut cavity is lined with cilia that aid in transport of nutrients through each segment down to the anus. Gut morphology, in *M. speciosa*, varies between anterior and posterior sections. Within segment 1 (prostomium/peristomium), the buccal opening is ventral to the nephridium and fecal groove (Figs. 12-13). By segment 2, the gut is in a more dorsal position with paired nephridia lining the thoracic region (Fig. 14). By segment 3, the gut expands laterally and the nephridia taper down to single openings in the coelom (Fig. 15). In segments 4 to 7, the gut is the dominant feature and possesses a thicker wall and glandular epithelium (Fig. 16). Posteriorly (segments 8 to 11),

the gut is again located dorsally (Fig. 17) and tapers down to the anus, which is located along the ventral surface of the pygidium.

The fabriciid excretory system is a metanephridium. The excretory organ opens into the coelomic cavity through a ciliated funnel (Gardiner 1992; Rouse & Pleijel 2001a). *Manayunkia speciosa* has one pair of functional nephridia, or ducts, that act as excretory organs. Paired nephridia are located in segment 1, segment 2, and the anterior portion of segment 3. Nephridia fuse to a single pore in segment 1, which then opens outside of the body wall adjacent to the fecal groove (Fig. 12).

In general, processing waste involves filtration units (podocytes) that increase the pressure between blood vessels and the coelom. Peritoneal cells have also been reported to provide diffusion channels between coelomic fluid and musculature (Gardiner 1992). The filtered vascular fluid, which contains waste products, enters the coelom and exits through the fused nephridial opening. In addition, fecal matter leaves through the anus along the ventral side of the pygidium (Lewis 1968a). Feces are ovoid pellets that are pushed vertically up the ventral abdomen by cilia, around the thorax to the dorsally-located fecal groove, and moved out of the tube by the branchial crown.

### **Circulatory System**

The circulatory system of *Manayunkia speciosa*, like most polychaetes, is a closed system with two main longitudinal vessels: dorsal and ventral (Rouse & Pleijel 2001a). In general, blood flows anteriorly toward the branchial crown through the dorsal vessel and posteriorly toward the pygidium through the ventral vessel. The larger ventral vessel, which is located directly below the gut, supplies blood to the body wall muscles and epidermis (Fig. 18). There are also median vessels that link the two main vessels within each segment. These median

vessels supply blood to capillary beds in the body wall and gut lacunae (Barnes & Harrison 1992). Blood is pumped by specialized organs typically associated with tube-dwelling organisms (Rouse & Pleijel 2001b). In *M. speciosa*, these organs are basically muscularized vessels.

Oxygen is acquired via two different methods: (1) direct respiration through the branchial crown in the water column and (2) auxiliary respiration by generating water currents through the tube or case (Rouse & Pleijel 2001b). Respiratory pigments of *M. speciosa* are chlorocruorins, which give the blood a green color (Leidy 1883; Rouse & Pleijel 2001b). Chlorocruorins are extracellular and transport oxygen to tissues throughout the body (Barnes & Harrison 1992).

### **Nervous System**

*Manayunkia speciosa* has an accumulation of cerebral ganglia that are fused to nerve cells, which include the ventral nerve cord, septal nerves, and chaetal nerves (Fig. 19). The ventral nerve cord in *M. speciosa* is connected to transverse septal nerves. Chaetal nerves are also connected to septal nerves and innervate the epidermis and musculature. Basal cells, which are associated with nerve function, line the perimeter of the ventral nerve cord and chaetal nerves. The ventral nerve cord is considered a more basal organization because it lies between the outer epithelium and extracellular matrix preceding the coelom (intraepithelial; Barnes & Harrison 1992).

### **Sensory System**

The sensory system of Fabriciidae is comprised of four main structures: ciliated radioles, statocysts, nuchal organs, and eye spots (Rouse & Pleijel 2001a). Radioles are assumed to have a certain amount of tactile control. Statocysts, which are gravity receptors, are simple open pits in the epidermis. Nuchal organs are paired ciliated structures within the fused

prostomium/peristomium, which function as chemoreceptors (Rouse & Pleijel 2001a; Gardiner 1992). In *Manayunkia speciosa*, nuchal organs have been internalized and are centrally located just above the posterior end of the peristomial collar in segment 1 (Fig. 20). Eye spots in *M. speciosa* are simple ocelli with sensory cells and pigmented support cells (Fig. 21). Eye spots of *M. speciosa* most likely only provide information about light intensity and direction. The combination of eye spots and statocysts result in a response similar to that reported by Lewis (1968b) with *Fabricia sabella*, in which the polychaetes exhibited negative phototaxis and positive orthokinesis out of the tube. This means that they crawl away from light and toward inclined surfaces. Although, given the opportunity, *M. speciosa* would preferentially burrow into sediment.

## **Reproduction**

Both sexes of *Manayunkia speciosa* have a reproductive organ immediately behind the radiolar crown, dorso-lateral to the buccal opening. Male *M. speciosa* have a sperm duct that runs along the dorsal side of the body from segment 9 to the radiolar crown (Fig. 22). Female *M. speciosa* have paired spermathecae or sperm receptacles (Fig. 23). Rouse (1995b,1996) described spermathecae in *M. aestuarina* as two openings, anterior to the peristomial collar, which led into a duct that extended laterally and dorsally into ellipsoidal sacs (Fig. 24). In *M. speciosa*, the ducts that start at the peristomial collar were  $48.3 \pm 8.2 \mu\text{m}$  long (n=15). The ducts then led into a similar 'atrium' as described by Rouse (1995b,1996) in *M. aestuarina*, with the exception that it extended anteriorly into the chamber preceding the ellipsoidal sacs. This anterior 'connecting piece' to the sperm-storage area in *M. speciosa* was  $13.4 \pm 5.7 \mu\text{m}$  (n=15). The ellipsoidal sacs of spermathecae measured  $18.2 \pm 3.2$  by  $15.2 \pm 2.3 \mu\text{m}$  (n=15). There is much

confusion surrounding spermathecae in Fabriciidae, because they are consistently misidentified as a second pair of eye spots (G. W. Rouse, pers. comm.).

Gamete development occurs in different locations of the coelom within the two sexes of *Manayunkia speciosa*. Laboratory observations of female *M. speciosa* confirm that they are egg-laying with larval care. This agrees with Bick (1996), who looked at larval development in *M. aestuarina*. Development of oocytes in *M. speciosa* occurs within thoracic chaetigers of the coelom within segments 4 to 6, in conjunction with funnels and short canals or gonoducts (Rouse 1995a). Oocytes are released through gonoducts once they are approximately 0.1 mm long (Fig. 25). Gonoducts are small openings in the body wall that develop only when oocytes are ready for release into the maternal tube (G. W. Rouse, pers. comm.). There is also some evidence that gametes may be released through rupturing of the body wall (Barnes & Harrison 1992; Rouse 1995a), although this has not been observed under laboratory conditions for *M. speciosa*.

Larvae are continuously developed every 2 to 4 weeks between January and July (Bick 1996; Willson et al., in press). Throughout the reproductive season, oocytes and larvae occur in varying stages of development. Bick (1996) reported that the youngest developmental stages of *M. aestuarina* were located in the front of the maternal tube, and more advanced stages occurred in more posterior locations, but all larvae ‘faced’ the mouth of the tube. The same type of orientation was observed for *M. speciosa*. After release of oocytes, females fertilize eggs from sperm stored in their spermathecae. Direct larval development then occurs within the maternal tube until larvae are between 1.5 to 2 mm in length (Leidy 1883; Willson et al., in press). Larvae establish tubes in close proximity to their maternal tube, which Lewis (1968a) argued will consolidate a population once it colonizes an area. This also suggests that dispersal occurs only through adult transport or invertebrate drift. This is where higher velocities displace substrate

and organisms downstream (catastrophic drift) or organisms inadvertently enter the water column and are transported downstream (behavioral drift; Brittain & Eikeland 1988).

Sperm in male *M. speciosa* are continuously developed within the coelom of thoracic trunk segments 7 to 9. Rouse (1995a) thoroughly described spermiogenesis for *M. aestuarina*, which agrees with histological observations of *M. speciosa* sperm cells. According to Rouse (1995a), development of sperm cells occurred in large clusters attached to a cytophore in elongate and conical groups (Fig. 26). At the posterior end of each sperm-bearing segment, there are paired ducts that lead to a dorsal sperm duct.

According to Rouse (1995a,1999), the genus *Manayunkia* released sperm through the dorsal sperm duct as spermatophores, or bundles of sperm that were surrounded by a mitochondrial sheath or capsule, that protected them from deterioration in the surrounding environment. Spermatophores are then collected by females from the water column and stored in spermathecae. This is yet another good adaptation to a dynamic riverine environment, where reproduction can occur without leaving the tube that is secured onto larger substrate. However, this type of reproduction is only successful in large, closely associated communities, which has been consistently reported for *M. speciosa* (Hiltunen 1965; Poe & Stefan 1974; Stocking & Bartholomew 2007).

## **DISCUSSION**

*Manayunkia speciosa* is one of only a few polychaetes in the freshwater environment. Evidence was provided by Croskery (1978) that North American populations of *M. speciosa* were a result of a marine incursion. Various aspects of its internal anatomy support this theory

of marine ancestry. The best examples of these freshwater adaptations presented in this study, included: (1) uncini orientation, (2) reproduction, and (3) feeding.

Although *M. speciosa* found in the Klamath River prefers a velocity range of 0.02 to 0.05 m/s (Stocking & Bartholomew 2007), it has been found in currents up to 0.5 m/s (Poe & Stefan 1974). Uncini are the structures that maintain stability within these currents. Abdominal uncini are positioned primarily in a posterior orientation, while thoracic uncini are found in either an anterior or posterior orientation. According to Woodin & Merz (1987), this type of dual orientation directly relates to the type of external forces that can displace the polychaete from the tube (e.g., water velocity fluctuations in riverine systems and predation).

Presence of spermathecae in female *M. speciosa* allow for fertilization to occur within the tube without contact from the male (Rouse 1995b). This type of reproduction takes advantage of currents in freshwater environments in order to distribute spermatophores produced by male polychaetes. However, an important caveat is that populations must occur in large communities for this type of reproduction to succeed. This is typically the case for *M. speciosa*.

Stocking & Bartholomew (2007) reported *M. speciosa* population abundances up to 40,607/m<sup>2</sup> in the Klamath River. Additionally, Hiltunen (1965) reported population abundances of 45,292/m<sup>2</sup> at the mouth of the Detroit River as it entered Lake Erie. Although these populations are patchy in distribution, there are some commonalities in habitat that can predict overall success. The most favorable habitats are: (1) those directly downstream from the mouth of a river, (2) those within transition zones between lentic and lotic environments, and (3) those with a continuous supply of nutrients (Hiltunen 1965; Croskery 1978; Bick 1996; Stocking & Bartholomew 2007).

According to Croskery (1978) and Bick (1996), organic loading into a system leads to a direct increase in polychaete abundance. The diversity of feeding habits of *M. speciosa* is a good adaptation for the dynamics of a freshwater system, but is especially useful in areas of nutrient loading. In accordance with this pattern, dams on the Klamath River contribute to nutrient loading downstream, and the highest concentrations of *M. speciosa* populations are located directly downstream of a river mouth or dam spillway (Hiltunen 1965; Stocking & Bartholomew 2007).

Ecological conditions supporting an increased abundance of *M. speciosa* in the Klamath River may also be supporting increased production of two myxozoan parasites, *Ceratomyxa shasta* and *Parvicapsula minibicornis* (Bartholomew et al. 2006; Stocking & Bartholomew 2007). Data gaps left to resolve include what ecological requirements are specifically related to the polychaete host and what are necessary for the survival of the myxozoan parasite. For example, it is understood that the parasite develops in the epidermis of the polychaete (Bartholomew et al. 1997), but what are the conditions that trigger its release from the host into the water column? There are substantial intricacies to cellular organization of *M. speciosa* that may have future significance in discerning why it is a good host for these two parasites.

**ACKNOWLEDGEMENTS.** We would like to thank Klamath River Restoration Program of US Fish & Wildlife Service for their partial financial support. A big thanks goes to Nick Ingram for the original drawings of *Manayunkia speciosa*. Thanks to Greg Rouse at Scripps Institute of Oceanography for multiple reviews of this work and for help with polychaete internal anatomy. Special thanks to Peggy Wilzbach, Ken Cummins, Sarah Willson, and David Malakauskas for help in collecting and rearing polychaetes. Thanks to Jerri Bartholomew, Sarah Bjork, and Rick

Stocking for help throughout this project and providing us with some initial specimens. Thanks also to George Meaders and Mark Yost for help in collecting samples for this study. Lastly, we would like to thank Marty Reed for advice and guidance in equipment maintenance and design.

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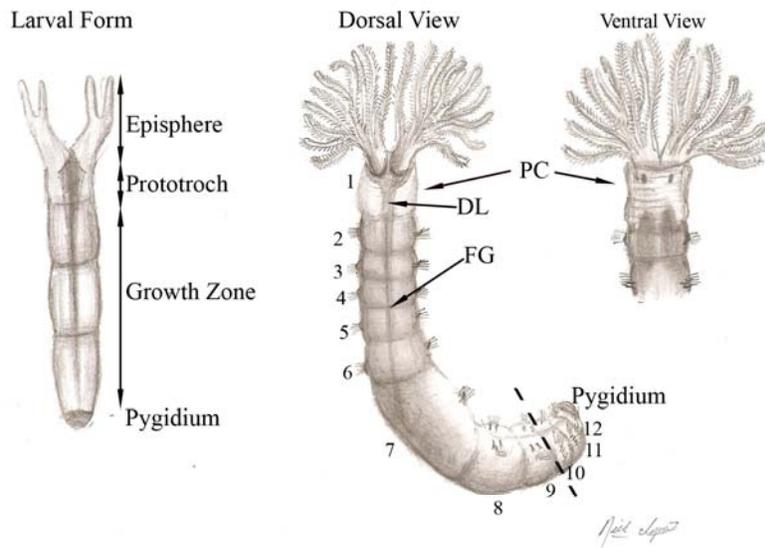
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**Table 1.** Systematics of *Manayunkia speciosa*; based on cladistics presented by Rouse & Pleijel (2001a) and amended by Kupriyanova & Rouse (2008).

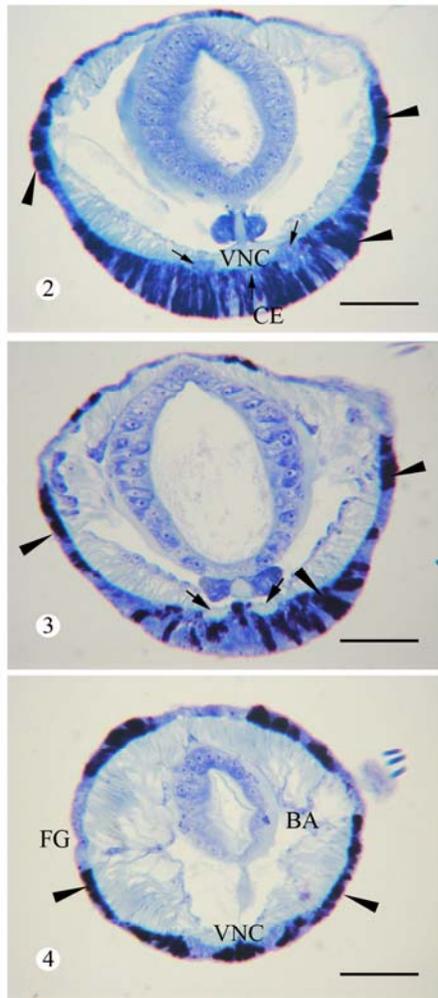
<b>Kingdom</b>	Animalia
<b>Phylum</b>	Annelida
<b>Class</b>	Polychaeta
<b>Order</b>	Sabellida
<b>Family</b>	Fabriciidae
<b>Subfamily</b>	Fabriciinae
<b>Genus</b>	<i>Manayunkia</i>
<b>Species</b>	<i>speciosa</i>

**Table 2.** Average epidermal width of *Manayunkia speciosa* (n = 10). Measurements start just posterior to presegmental region (at segment 2) and continue through segment 11. Epidermal widths of segment 12 and the pygidium are not reported here.

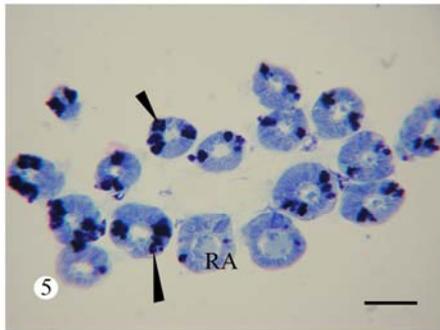
<b>Segment #</b>	<b>Average Epidermis Width (mm)</b>	<b>Standard Deviation (mm)</b>
2	15.4	3.6
3	14.3	2.7
4	14.0	2.5
5	11.9	2.0
6	11.5	1.9
7	7.8	1.6
8	7.6	1.7
9	8.0	1.5
10	10.9	3.1
11	12.1	3.0
<b>AVERAGE</b>	<b>11.3</b>	<b>2.3</b>



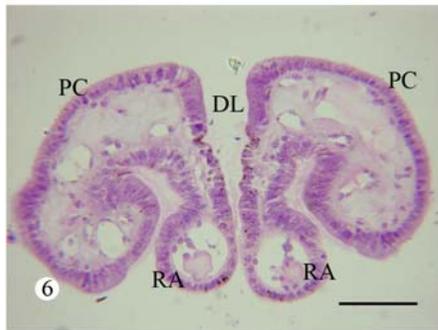
**Fig. 1.** Zones of polychaete growth and development. Diagram illustrates major external features of adult *Manayunkia speciosa* and how each zone of growth develops from the polychaete larvae. Entire animal is shown in ventral view and anterior end is shown in dorsal view. Each segment is numbered; dashed line indicates thorax-abdomen junction. Drawings by Nick Ingram. DL, dorsal lip; FG, fecal groove; PE, peristomial collar.



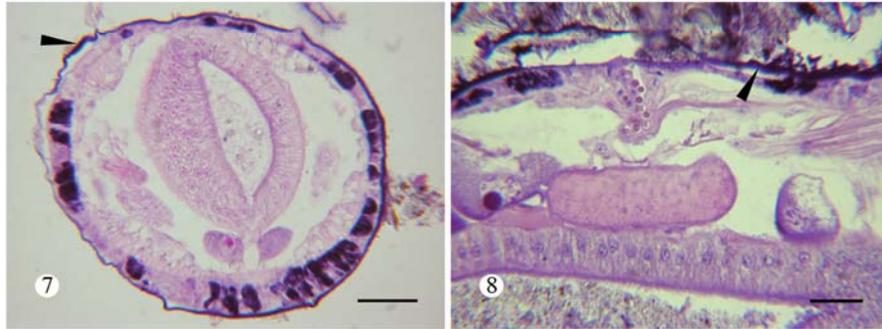
**Figs. 2-4.** Transverse histological sections through a 1.4 mm long *Manayunkia speciosa*, illustrating the difference in thickness of epidermis and cellular organization from anterior to posterior segments. **Fig. 2.** Section at 0.46 mm (segment 3). The epidermal layer in anterior segments is relatively thick and consists primarily of columnar epithelial (CE) cells, with randomly distributed secretory cells (arrowheads). A few basal cells (arrows) are present surrounding the ventral nerve cord (VNC). Scale bar, 40  $\mu$ m. **Fig. 3.** Section at 0.74 mm (segment 6). The ventral portion of the epidermal layer starts to narrow in segment 6 and is nearly the same width as the dorsal portion by segment 7. Secretory cells (arrowheads) are present in limited volume within the epidermis compared to anterior segments. However, basal cells can be found surrounding the ventral nerve cord (arrows), along musculature, and within the epidermis. Scale bar, 40  $\mu$ m. **Fig. 4.** Section at 1.12 mm (segment 9). Epidermal width is more continuous in posterior segments. The number of basal cells (BA) increases both along musculature and along the ventral nerve cord (VNC). Secretory cells (arrowheads) are present again in higher number. The fecal groove (FG) migrates along the left margin from an anterior to posterior location. Scale bar, 40  $\mu$ m.



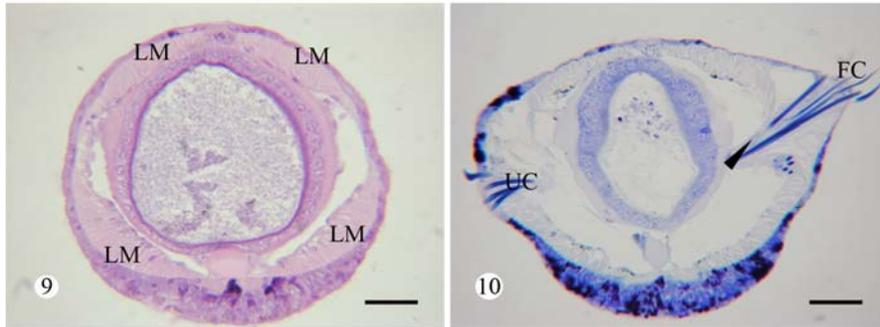
**Fig. 5.** Transverse histological section of radioles (RA) and associated secretory cells of *Manayunkia speciosa*. Each radiole contains a ring of secretory cells (arrowheads) in the anterior portion but not in more posterior locations. Scale bar, 40  $\mu$ m.



**Fig. 6.** Transverse histological section of *Manayunkia speciosa* within segment 1 (0.25 mm into a 1.8 mm worm), showing internal connections of each lophophore (RA). Anterior to each lophophore connection is the start of the peristomial collar (PC). The location where the peristomial collar separates is the dorsal lip (DL). Scale bar, 40  $\mu$ m.



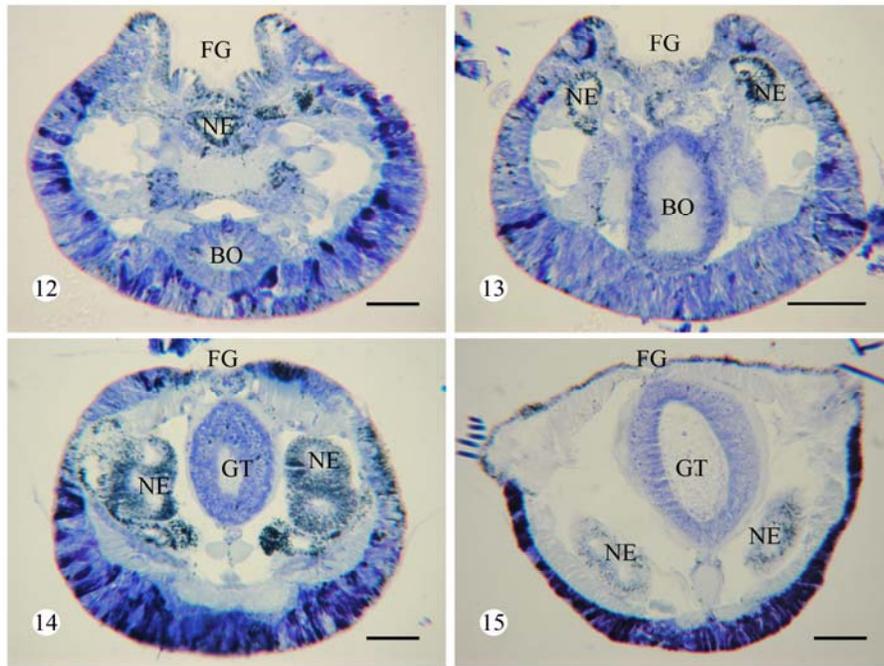
**Figs. 7-8.** Histological sections of *Manayunkia speciosa* inside its tube. **Fig. 7.** Transverse view. Note the continuous dark staining mucous layer (arrowhead), which is not present when the polychaete is outside of its tube. Scale bar, 40  $\mu$ m. **Fig. 8.** Longitudinal view. The material above the dark-staining mucous layer (arrowhead) is fine particulate matter, or tube material. Scale bar, 40  $\mu$ m.



**Figs. 9-10.** Orientation of musculature in *Manayunkia speciosa*. **Fig. 9.** Transverse view of longitudinal muscle (LM) bands within the coelom of *M. speciosa*. Note the reduced coelom and lack of a circular muscle layer. Scale bar, 40  $\mu$ m. **Fig. 10.** Transverse view of oblique muscle (arrowhead) within parapodium of *M. speciosa*. Oblique muscle fibers manipulate flexible capillary chaetae (FC) to maneuver the body. Note that chaetal bundles (or rows) are arranged in notopodia in anterior segments and neuropodia in posterior segments, while the opposite is true of uncini (UC) or hooks. The transition of chaetal bundles from notopodia to neuropodia indicates the location of the thorax-abdomen junction. Scale bar, 40  $\mu$ m.



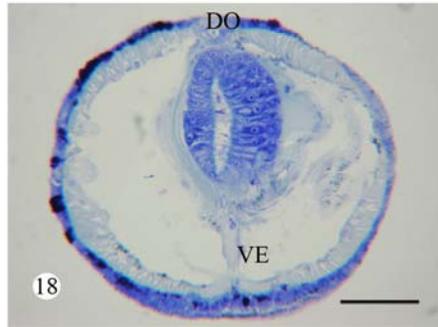
**Fig. 11.** Transverse histological section of uncini in *Manayunkia speciosa*. The section shown here is found only at the posterior end of the polychaete (segments 10-12), where dense rows of uncini are located in notopodia. Conversely, four to six hooked uncini are found in anterior segments (3-9) in neuropodia. Dense rows of uncini are also associated with an aggregation of basal cells (BA). Note that abdominal uncini are pointed toward the posterior end of the polychaete, which is how the polychaete is able to maintain position within its tube in higher water velocities. Scale bar, 40  $\mu\text{m}$ .



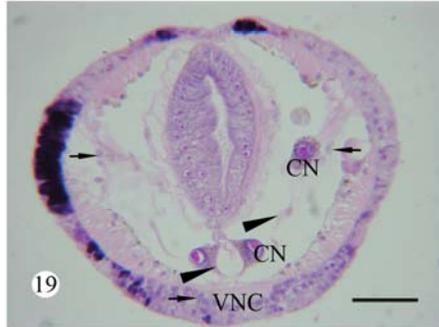
**Figs. 12-15.** Transverse histological sections of *Manayunkia speciosa* digestive system; anterior segments. **Fig. 12.** In segment 1, the buccal opening (BO) starts in a ventral position, posterior to both the fused opening of the nephridium (NE) and the fecal groove (FG). Scale bar, 40  $\mu$ m. **Fig. 13.** At the posterior end of segment 1, the buccal opening (BO) begins to expand toward the dorsal surface. Paired nephridia (NE), coiled organs adjacent to the fecal groove (FG), begin to surround the gut within the coelom. Scale bar, 40  $\mu$ m. **Fig. 14.** In segment 2, the gut (GT) is located dorsally with the paired nephridia (NE) in a more thoracic position. The fecal groove (FG) is little more than a shallow indentation at this point. Scale bar, 40  $\mu$ m. **Fig. 15.** In segment 3, the gut (GT) is the dominant feature in the coelom and the paired nephridia (NE) taper down to single openings. The fecal groove (FG) is still small and located dorsally. Scale bar, 40  $\mu$ m.



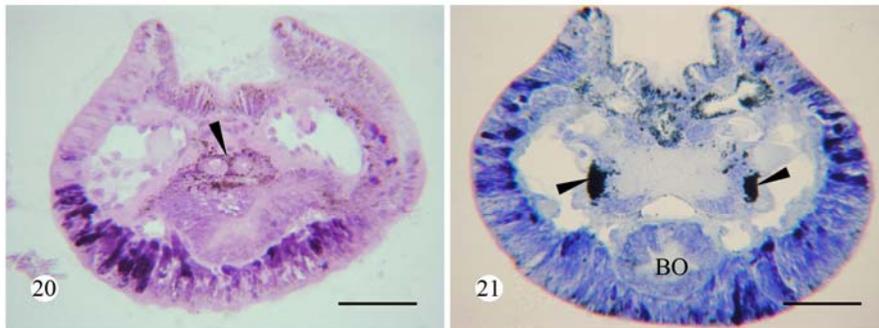
**Figs. 16-17.** Transverse histological sections of *Manayunkia speciosa* digestive system; mid- to posterior segments. **Fig. 16.** In segment 4, the gut is the predominant feature of the polychaete's body. Within segments 4-7, the gut encompasses the majority of the coelom. Splanchnic musculature (SM) and intestinal epithelial (IE) cells surround the perimeter of the gut. The fecal groove (FG) is still dorsally located. Scale bar, 40  $\mu$ m. **Fig. 17.** In segment 10, the gut (GT) is dorsally located and tapers down to the anus located in the pygidium. Anterior and posterior to these segments, the gut is reduced in size and located dorsally. In segment 9, the fecal groove (FG) moves from a dorsal to ventral position. The fecal groove terminates at the anus along the ventral portion of the polychaete. Scale bar, 40  $\mu$ m.



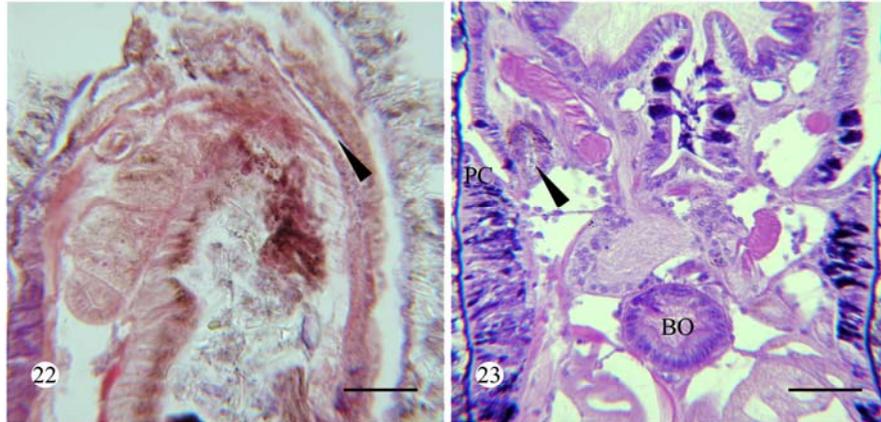
**Fig. 18.** Transverse histological section of the two main blood vessels in *Manayunkia speciosa*. Note that the dorsal vessel (DO) is much reduced compared to the ventral vessel (VE). The circulatory system in *M. speciosa* is closed. Blood flows anteriorly toward the branchial crown in the dorsal vessel and posteriorly in the ventral vessel. Scale bar, 40  $\mu\text{m}$ .



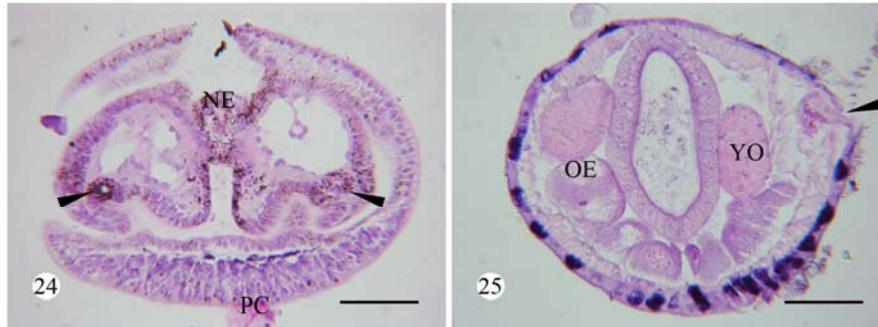
**Fig. 19.** Transverse histological section of the nervous system in *Manayunkia speciosa*. The ventral nerve cord (VNC) is intraepithelial in position and is connected to the septal nerve (arrowheads). Chaetal nerves (CN) are connected to the septal nerve. Basal cells (arrows) surround the ventral nerve cord, chaetal nerves, and musculature. Scale bar, 40  $\mu\text{m}$ .



**Figs. 20-21.** Histological sections of sensory organs in *Manayunkia speciosa*. **Fig. 20.** Transverse view of nuchal organs (arrowhead) in *M. speciosa*. Nuchal organs are internal paired structures located within segment 1. They are used for chemoreception. Scale bar, 40  $\mu$ m. **Fig. 21.** Transverse view of eye spots (arrowheads) in *M. speciosa*. Eye spots are simple ocelli with sensory cells and pigmented support cells. They are located posterior to the dorsal lip of the peristomial collar and dorsal to the buccal opening (BO). Note the accumulation of ganglia found between the eye spots. Scale bar, 40  $\mu$ m.



**Figs. 22-23.** Histological sections of dorsal reproductive organs in *Manayunkia speciosa*. **Fig. 22.** Longitudinal view of the dorsal sperm duct (arrowhead) in a male *M. speciosa*. The dorsal sperm duct runs along the dorsal surface of the gut and exits just posterior to the branchial crown. Scale bar, 40  $\mu\text{m}$ . **Fig. 23.** Longitudinal view of spermathecae (arrowhead) in a female *M. speciosa*. Spermathecae are located adjacent to the dorsal lip of the peristomial collar (PC) and anterior to the buccal opening (BO). Scale bar, 40  $\mu\text{m}$ .



**Figs. 24-25.** Histological sections of reproductive organs and gametes within female *Manayunkia speciosa*. **Fig. 24.** Transverse view of spermathecae (arrowheads). Note the duct starts just anterior to the peristomial collar (PC) and ends at the ellipsoidal sacs used in sperm storage. The buccal opening does not appear in this figure, but the fused opening of the nephridia (NE) is present along the dorsal surface of the polychaete. Scale bar, 40  $\mu$ m. **Fig. 25.** Transverse section of a gonoduct (arrowhead). Gonoducts only form in females that have well-developed oocytes (OE) with large associated yolk sacs (YO). Oocytes develop within segments 4-6, with the majority of development occurring within segment 5. Scale bar, 40  $\mu$ m.



**Fig. 26.** Transverse section of sperm (SP) cells in *M. speciosa* and the dorsal sperm duct (arrowhead). Sperm are generated on a continuous basis, and can be found in segments 7-9 in various stages of development. Scale bar, 40  $\mu$ m.

**CHRONOLOGICAL DEVELOPMENT OF *CERATOMYXA SHASTA* IN THE  
POLYCHAETE HOST, *MANAYUNKIA SPECIOSA***

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*Additional key words:* ceratomyxosis, Myxozoa, Annelida, actinospore production, myxozoan

Running title: Development of *C. shasta* actinosporeans

**ABSTRACT:** The life cycle from release of myxospore sporoplasm to mature actinospore is described for the myxozoan parasite *Ceratomyxa shasta* in the freshwater polychaete, *Manayunkia speciosa*. Infection was initiated through exposure to infected waters, from which the parasite was ingested by the polychaete. Mature myxospores were present in the gut of segments 4 to 7 by 2 hrs post-exposure (PE). Migration of early developmental stages occurred from gut epithelia, through the nervous system, and into the epidermis. Once the parasite reached the epidermis, development typically occurred in segments 6 to 10. Development of *C. shasta* from myxospore to actinospore appeared to mimic the life cycle of *Myxobolus cerebralis* in *Tubifex tubifex*. All life stages were present, including a proliferative stage (schizogony), sexual reproduction (gametogony), and spore formation (sporogony). At water temperatures averaging 17.3°C, schizogony began by 3 hrs PE, gametogony by 14 days PE, and sporogony by 35 days PE. Development to a mature actinospore took approximately 49 days PE. We estimated that up to 13,650 actinospores could be released from a single polychaete over a 14-day period. Actinospores appeared to be released directly through the epidermis into the water column.

The Klamath River drains approximately 12,100 mi<sup>2</sup> in northern California and southern Oregon. It was once the third most productive system for Chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*O. kisutch*), and steelhead (*O. mykiss*) on the West Coast of North America. Spring Chinook salmon alone have been reduced to 5% of their historic abundance in the Klamath River (Nehlsen et al. 1991). There are a variety of factors responsible for the decline in salmonid abundance within the system. However, myxozoan parasites are primary contributors to mortalities seen in juvenile Chinook salmon and outmigrant coho salmon in recent years (J. S. Foott, pers. comm.).

Myxozoans are spore-forming parasites with two morphologically different spore phases in their life cycle: myxospores and actinospores. The life cycle typically involves an alternation between vertebrate and invertebrate hosts (Markiw & Wolf 1983, Molnár et al. 1999). The two important myxozoan parasites that occur in the Klamath River, *Ceratomyxa shasta* and *Parvicapsula minibicornis*, alternate between a salmonid host (*Oncorhynchus* spp. and *Salmo* spp.) and an invertebrate polychaete host, *Manayunkia speciosa*.

Actinospores produced in the polychaete are the causative agents of the diseases ceratomyxosis (*C. shasta*) and glomerulonephritis (*P. minibicornis*), which target the posterior intestine and kidney in the salmonid host, respectively (Noble 1950, Kent et al. 1997). According to Hendrickson et al. (1989), occurrence of the infective stage of *C. shasta* in the Klamath River basin is seasonal. In accordance with this work, studies consistently report high levels of infection and mortality of outmigrant juvenile salmonids in the Klamath River from May through November (Palenzuela & Bartholomew 2002, Bartholomew et al. 2006, Stocking et al. 2006, Bartholomew et al. 2007). Susceptibility appears to vary by salmonid species and strain (Hendrickson et al. 1989, Nichols et al. 2003, Bartholomew et al. 2004). Species and

strains that have evolved in the presence of *C. shasta* are generally more resistant. However, even “resistant” strains can be overwhelmed by large numbers of myxozoan parasites. High infectious dose of both myxozoans, leading to dual infections, may be the reason that Klamath River fish show signs of immunosuppression (Stocking et al. 2006, Bartholomew et al. 2007, Foott et al. 2007).

El-Matbouli & Hoffmann (1998) studied development of the actinosporean of *Myxobolus cerebralis* in the oligochaete host, *Tubifex tubifex*. This study identified four main stages of actinosporean development: infection, schizogony, gametogony (including gametogamy), and sporogony. Myxospores of *M. cerebralis* were ingested by *T. tubifex*. Polar filaments extruded from the myxospore and attached to the gut epithelium. Directly following attachment, shell valves open along the suture line and release binucleate sporoplasm between gut epithelial cells. Schizogony was described as a stage of multiplication, resulting in infection of neighboring segments from the initial site of sporoplasm release. After multiplication of spore material, the parasite went into a stage of fusion and division (gametogony). Because gametogony involved sexual reproduction (gametogamy), the invertebrate is the definitive host of the parasite. Sporogony was the final stage of development from zygote to mature spore, in which development of each spore structure (polar capsules, polar filaments, sporoplasm, and valve cells) occurred within a pansporocyst.

Bartholomew et al. (1997) reported that *C. shasta* actinosporeans developed between the dermis and striated muscle layer (longitudinal musculature). Additionally, eight actinosporeans developed within a single pansporocyst in what appeared to be the remains of secretory cells. Although this work significantly advanced our understanding of the life cycle of *C. shasta* in the polychaete host, there are still unanswered questions regarding: (1) how actinospores are

released, (2) how the infectious cycle is initiated, and (3) the timeframe of actinosporean development.

The objective of this study was to expose *M. speciosa* to *C. shasta* myxospores to observe chronological development of actinosporeans in the polychaete. Observations were also made on associated host responses to a mature infection. This information may lead to management options for control of *C. shasta* mortality of salmonids in the Klamath River basin.

## **METHODS**

*Manayunkia speciosa* adults used in this study were initially collected from Keno Reservoir just south of Klamath Falls, Oregon. Samples were collected with a long handled, D-frame 250 µm mesh dipnet from boulder substrate (> 256 mm in diameter) that contained *Cladophora* spp. and an accumulation of fine sediments. The polychaete population was found just downstream from the mouth of the Klamath River as it entered Keno Reservoir in a transition zone between lotic and lentic habitat (Stocking & Bartholomew 2007). Collected samples were washed through a 250 µm mesh Tyler® sieve, placed in Zip-Lock® bags, and transported to Humboldt State University (HSU) in Arcata, California.

During transport, samples were held in river water and substrate from the area of collection, and then transferred to circular tanks at HSU within 48 hours. Circular tanks were 62 cm in diameter and held 57 L of water. The tanks recirculated filtered water pumped from Fern Lake, which is connected to the HSU hatchery water system.

Each tank was connected to a chiller, which set a maximum temperature of 15.5°C. The tanks were largely protected from the elements, but were located outside. Therefore, water temperature did not exceed 15.5°C, but were subjected to lower temperatures. However, the last

14 days of the experiment, one sample was held at room temperature. Water temperature was monitored on a daily basis between 9:00 and 10:00 with a laboratory grade reference thermometer. Flow was controlled through a vertical spray bar and velocities in each tank ranged from 0.03 to 0.04 m/s, which is within the preferred range of habitat conditions for *M. speciosa* (Poe and Stefan 1974, Stocking & Bartholomew 2007).

### **Lab rearing**

Following transfer to circular tanks at HSU, individual adult *Manayunkia speciosa* (2-4 mm in length) were sorted under a dissecting microscope and divided between five glass Petri dishes (11 mm diameter) and five Plexiglas containers (20.5 mm x 10.5 mm ) with 32 to 36 individual wells. Substrate used for infection experiments was collected from the Klamath River at Trees of Heaven campground in areas suitable for *M. speciosa*. Substrate was sorted through a 250  $\mu$ m mesh sieve and frozen for approximately one month prior to use. After freezing, substrate was autoclaved for one hour, cooled, pipetted into sterilized containers (i.e., Petri dishes or Plexiglas well plates). Petri dishes and well plates were then placed into two circular tanks with a common 55 L reservoir containing dechlorinated tap water. After one week of “aging” sterilized substrate, one adult polychaete was added to each well within a Plexiglas container and 10 adult polychaetes were added to each Petri dish. A total of 200 *Manayunkia speciosa* were placed in a combination of well plates and Petri dishes and then divided into the two circular tanks. Polychaetes were allowed to acclimate to the new environment for one to two weeks, which allowed for construction of new tubes with substrate provided prior to any experimentation. Each tank of polychaetes received 1 mL of micronutrients every two to three days (Micro-Vert<sup>®</sup> Invertebrate Food).

## **Exposure of polychaetes to aged myxospores of *Ceratomyxa shasta***

Approximately one month prior to experimentation, two juvenile rainbow trout (*Oncorhynchus mykiss*), provided by the HSU hatchery, were exposed to polychaetes infected with *C. shasta* from a separate tank experiment. The posterior intestine of each fish was removed and both the posterior intestine and remaining carcasses were placed into circular tanks with polychaetes. Additionally, posterior intestines of six juvenile Chinook salmon (*O. tshawytscha*), collected from the Kinsman weir on the Klamath River that were positive for *C. shasta*, were added to the tanks. All fish used in this experiment were tested for *C. shasta* by an intestinal scraping and viewed under 400x magnification.

A sample of 157 *M. speciosa* were collected and fixed for histological sectioning at various times from 1 hour to 49 days post-exposure (PE). Following simple random sampling protocol, polychaetes were sampled first from the Plexiglas well plates and then from Petri dishes. Collection alternated between the two infection tanks for each sampling.

### **Tissue processing**

Collected polychaetes from each sampling were fixed in alcohol-formalin-acetic acid (AFA) in a 5 mL glass scintillation vial with at least 10 times the volume of tissue being fixed for at least 24 hours (Strange 1996, Bartholomew et al. 2006). Samples were then transferred to 70% ethanol (EtOH) for another 24 hours prior to processing to ensure that all fixative was removed from the tissue. Samples were then placed in a series of tertiary butyl alcohol (TBA, ascending) and EtOH (descending) solutions to dehydrate the tissue and infiltrate it with a medium that is miscible with paraffin (Humason 1979). After infiltration with TBA, the TBA was slowly replaced with paraffin. Tissue was then embedded into 100% paraffin and cut with a

Reichert Histostat<sup>®</sup> rotary microtome at 4 to 6  $\mu\text{m}$ . Sections were affixed onto standard microscope slides using a thin layer of Haupt's adhesive, 4% formalin, and heat ( $\sim 50^{\circ}\text{C}$ ).

Histological sections were stained with either a Hemotoxylin and Eosin (Humason 1979) or May-Grünwald Giemsa protocol (Yasutake & Wales 1983). Histological sections were examined using a standard light microscope under phase and bright field with at least 400x magnification. Photographs were taken with a Nikon<sup>®</sup> ED and Martin<sup>®</sup> adapter for light microscopes.

### **Actinosporean development**

For each developmental stage of *C. shasta* actinosporeans, at least 10 measurements were taken (where applicable) for: (1) actinosporean length and width, (2) nuclei diameter, (3) pansporocyst length and width, and (4) epidermal width. Measurements for thickness of epidermis included both infected and uninfected tissue at four locations (dorsal, ventral, and two lateral) within each body segment between the peristomium collar and pygidium of the polychaete.

Potential spore production was based on the number of spores within late sporogony from both naturally- or laboratory-infected polychaetes. For the purposes of estimating spore production, late sporogony was defined as the presence of developing actinosporeans that contained polar capsules, valve cells, and sporoplasm material. Pre- and early sporogonic stages of the parasite were evident throughout each specimen, but were not included in these calculations. The following formula was used to calculate spore numbers:

$$\# \text{ spores} = \left( \frac{\# \text{ pansporocysts}}{\text{depth}(mm)} \right) * \left( \frac{1000 \mu\text{m}}{1mm} \right) * \left( \frac{8 \text{ spores}}{\text{pansporocyst}} \right) * (\text{tissue}(mm))$$

Where depth indicates the number of sections in which a pansporocyst develops and tissue refers to the length of infected tissue within each polychaete. It was assumed that every pansporocyst

contained a total of eight actinosporeans (Bartholomew et al. 1997). Pansporocysts were counted within 20 random sections. Mean ( $\pm$ SD) was taken to represent a total number of spores released from each infected polychaete.

## RESULTS

Based on a sample size of 157 individuals, *Manayunkia speciosa* shrunk an average of 34% in length during fixation in AFA and washing in 70% EtOH. This was followed by an additional 14% average loss of length during tissue processing and sectioning for histology. All measurements made of infected polychaetes ignored this shrinkage. All other measurements (e.g., spore measurements) that are provided in this study have an uncalculated, but probably similar, loss due to processing.

### **Development of actinosporeans in *Manayunkia speciosa***

With few exceptions, the actinosporean portion of the *C. shasta* life cycle in *M. speciosa* paralleled the life cycle of *Myxobolus cerebralis* actinospores in *Tubifex tubifex* reported by El-Matbouli & Hoffmann (1998; Fig. 1). In general, each stage of development was slightly larger than that reported for *M. cerebralis* actinosporeans (Table 1). However, morphology of each developmental stage for *C. shasta*, and the developmental process, was similar to that reported for *M. cerebralis*.

Infection was initiated through the water column, which contained *C. shasta* myxospores, which were then ingested by the polychaete through filter- or deposit-feeding. Mature myxospores were observed in the gut of *M. speciosa* of segments 4 to 7 at 2 hrs post-exposure (PE). Both intact myxospores and empty valves were observed in the gut until 24 hrs PE (Fig. 2). Within the first 3 hrs PE, myxospores attached to the gut lining and released sporoplasm

material between gut epithelial cells (Fig. 3). Sporoplasm material was compact, intensively stained, and consisted mostly of round aggregates of cells (Fig. 4). These aggregates were easily recognized due to a surrounding lytic zone, which is similar to what El-Matbouli et al. (1995) reported for development of *M. cerebralis* in *Oncorhynchus mykiss*. From 3 hrs to 7 days PE, sporoplasm aggregates were found in the gut epithelium, splanchnic (gut) musculature, and septal nerves. After 6 hrs PE, sporoplasm aggregates were observed in the epidermis of *M. speciosa* throughout the experiment.

Sporoplasm material multiplied intracellularly and produced the proliferative first stage of schizogony. This stage was identified as a multinucleate oval structure (Fig. 5). From 3 hrs to 7 days PE, this stage was observed in gut epithelium, splanchnic musculature, and septal nerves. After 8 hrs PE, this stage was observed in the epidermal layer of the integument throughout the experiment. By 24 hrs PE, the multinucleate stage of schizogony occurred throughout the epidermal layer of segments 4 through 7. Further expansion of this stage occurred by 36 hrs PE into segments 3 through 10. Through plasmotomy, multinucleate cells divided into uninucleate cells by 10 days PE; however, the occurrence of these cells was sporadic and only within segments 7 and 8. By 14 days PE, uninucleate cells were much more abundant within trunk thoracic segments 7 through 9. These cells were nearly round in cross-section, with a dark staining cytoplasm, centrally-located nucleus, and a distinct nucleolus (Fig. 6). El-Matbouli & Hoffmann (1998) described similar uninucleate cells, which were able to go back into a cycle of asexual reproduction to produce the multinucleate stage of schizogony. Additional evidence of this extension of schizogony, at later stages of the infectious cycle, was the simultaneous occurrence of developmental stages in the epidermal layer ranging from schizogony to late

sporogony. If uninucleate cells did not begin the cycle of schizogony again, then two cells fused and formed the first stage of gametogony.

By 14 days PE, the first stage of gametogony was observed within the epidermis. This stage was identified as a binucleate cell with dark-staining cytoplasm and a distinct nucleolus (Fig. 7). By 22 days PE, binucleated cells expanded dorsally and laterally (Fig. 8). According to El-Matbouli & Hoffmann (1998), the binucleate cell goes through karyogamy, wherein two nuclei fuse, thus becoming a uninucleate cell again. This uninucleate stage then undergoes plasmotomy (or mitotic division) to produce a four-celled structure (Fig. 9). This stage of gametogony appeared by 28 days PE. By 35 days PE, the four cells differentiated into somatic and generative cells (Fig. 10). Somatic cells (future pansporocyst) flattened out and started to wrap around generative cells (spore material). The first true pansporocyst also developed by 35 days PE, with two generative cells that were fully enveloped by two somatic cells (Fig. 11). According to El-Matbouli & Hoffmann (1998), there are two separate generative cells:  $\alpha$ - and  $\beta$ -cells. The two generative cells go through mitosis three separate times, producing eight  $\alpha$ - and eight  $\beta$ -gametocytes. This process was followed by a meiotic division, which produced an expulsion of small polar bodies and 32 total gametocytes. The initial stages of this process were observed at 35 days PE. However, the final stage of a pansporocyst that encapsulated 16  $\alpha$ - and 16  $\beta$ -gametocytes surrounded by polar bodies was not observed in the epidermal layer of *M. speciosa* until 49 days PE (Fig. 12).

Individual stages of sporogony were not seen independently of each other. All stages developed between 35 and 49 days PE. Description of each developmental stage is based on morphology characteristics of a mature spore and comparisons to the process reported by El-Matbouli & Hoffman (1998). The first stage of sporogony, was an  $\alpha$ - and  $\beta$ -gametocyte uniting

(i.e., sexual reproduction) to form a zygote. This stage was identified as eight fully developed zygotes with distinct nuclei encapsulated by a pansporocyst (Fig. 13). Zygotes divided mitotically to produce pansporocysts with the first stages of three morphologically-identifiable cell types: capsulogenic, sporoplasm, and valvogenic (Fig. 14). Each actinosporean contained three refractive cells (beginning of polar capsules with polar filaments) arranged in a pyramid around a dark-staining cell (beginning of sporoplasm). Early stages of valvogenic cells were observed encapsulating the other two cell types; these cells had an oblong dark-staining nucleus. El-Matbouli et al. (1995) reported that organelles of valvogenic cells eventually degenerate and become a narrow layer around the spore.

In advanced stages of spore maturation, each actinosporean went through a process of shrinkage and condensation (Fig. 15). At this stage, it was evident that sporogenesis occurred asynchronously within a pansporocyst. The next stage of spore development was actinosporeans that were more tetrahedral in shape (Fig. 16). The actinosporean sporoplasm material in advanced stages was relatively featureless but darkly stained. This is similar to what Yamamoto & Sanders (1979) reported for *C. shasta* myxosporeans developing in salmonids. The final stage of development was identified by presence of a suture line (septate junction) dividing the actinospore into three equal parts, each part containing the four cell structures of a mature spore: polar capsules, polar filaments, sporoplasm, and valve cells (Fig. 17). Polar filaments were not observed in histological preparations, but the fact that polar capsules were refractive denoted presence of polar filaments. Spores were approximately 37% smaller than that reported by Bartholomew et al. (1997), but were considered mature based on morphology. This value of shrinkage was consistent with comparisons of fresh myxospores reported by Yamamoto & Sanders (1979) and fixed myxospores found in the gut cavity of *M. speciosa* (32% loss).

## **Infection prevalence and trends**

Observations and measurements of mature *Ceratomyxa shasta* infections in *Manayunkia speciosa* were prepared from a sample of eight polychaetes. These samples were compared to an additional sample of 10 uninfected polychaetes of similar length. Based on infected samples, an average 41% of *M. speciosa* tissue was infected by *Ceratomyxa shasta* (Table 2). This represents 0.71 mm of tissue within the posterior half of the polychaete (segments 6-10). Correcting for a total loss of 48% during processing, a 2.5 mm live specimen would have approximately 1.1 mm of live infected tissue.

The sex of five individuals was definitively determined, and all were female. The remaining three were assumed to be male based on presence of a dorsal sperm duct and absence of spermathecae. However, gametes were so deteriorated that it was impossible to get a definitive determination on sex. In terms of infection percentage or segments affected, there were no obvious differences in prevalence between the two sexes. For all infected polychaetes, actinosporeans within late sporogony were more prevalent within segments 7 to 9 (thoracic chaetigers 6-8). There was a marked reduction in presence of pansporocysts within adjacent segments. However, these segments did contain early developmental stages of the parasite.

## **Location of parasite growth**

Bartholomew et al. (1997) first described *Ceratomyxa shasta* in *Manayunkia speciosa*. They found that development of actinosporeans occurred within the epidermis of *M. speciosa*. Furthermore, they found that development of pansporocysts occurred between columnar epithelial cells (Fig. 18). This study supports this finding. Additionally, within segments bearing pansporocysts, there was a significant reduction of *M. speciosa* secretory cells.

Development of pansporocysts typically occurred within the ventral surface of the host. However, in more advanced stages of parasite growth, pansporocysts were observed throughout the perimeter of the epidermis. Within each pansporocyst, there was asynchronous development of actinosporeans. Additionally, within a single host, there were multiple pansporocysts at different stages of the developmental cycle.

### **Host response**

Typical invertebrate responses to disease include cell-mediated responses and tissue alteration (Lackie 1980, Sheldon & Verhulst 1996). There was evidence of both responses in *Manayunkia speciosa* to actinosporean development. The primary cellular defense of *M. speciosa* is phagocytosis, which is an innate immune response of coelomocytes where foreign material is engulfed by a membrane bound phagosome and digested. In other invertebrates, epithelial cells mediate the immunological response (Bosch & David 1986). In cases where foreign objects are larger than 10  $\mu\text{m}$  in diameter (e.g., sporoplasm, later stages of gametogony), encapsulation rather than phagocytosis occurs (Lackie 1980). However, encapsulation may also be a strategy of the parasite to avoid detection by the host's immune system (Lackie 1980, Bartholomew 1998).

Tissue alteration was observed as displacement and hyperplasia. In other invertebrates, it has been shown that displacement is accompanied by phagocytosis (Bosch & David 1986). Epidermal cells of the polychaete were pushed aside as actinosporeans developed. Comparison of infected and uninfected tissue by histology showed a substantial increase in epidermal width in the presence of *Ceratomyxa shasta* (Fig. 19). Largest expansions of the epidermis occurred within segments 7 to 9 (thoracic chaetigers 6-8).

### **Release of parasite from polychaete host**

Release of mature actinospores from live *Manayunkia speciosa* has been observed along trunk segments through both the epidermis and gonoducts (Bartholomew et al. 2006, S. J. Willson, pers. comm.). Fully developed actinosporeans were found in histological sections being released directly through the epidermis through osmotic pressure (Fig. 20). There is a limited cuticular layer within *M. speciosa*, which leaves only a narrow layer of tissue to break through during release of the parasite.

### **Number of actinospores produced**

There was significant variation in the potential production of actinospores per infected polychaete (Table 3). If you take into account that the final stages of sporogenesis occur within a 14-day period, a 1.70 mm (i.e., 2.5 mm unprocessed) polychaete could produce an average of 4,759 ( $\pm 2,173$ ) actinospores over this timeframe. This does not take into account developmental potential within the polychaete. In other words, spores are continuously developed within *Manayunkia speciosa* and the values presented here represent only a single snapshot in time. The potential number of spores that could be produced over the lifetime of *M. speciosa* was not calculated in this study.

## **DISCUSSION**

According to Ratliff (1981), survival of a parasite is dependent on a long term association with its host. Based on current mortality estimates of outmigrating Chinook salmon in the Klamath River basin due to ceratomyxosis, the host/parasite balance has been disrupted. Many studies have reported that even resistant salmonid strains can be overwhelmed by *Ceratomyxa shasta* when given a high infectious dose or a long exposure time (Bartholomew et al. 2004,

Stocking et al. 2006, Foott et al. 2007). This suggests that actinospore production and release from *Manayunkia speciosa* is a key factor in the disease problems facing the Klamath River.

According to Foott et al. (2007), *C. shasta* actinospores in the Klamath River remain viable long enough to be distributed for tens of kilometers downstream of a polychaete population. Given that actinospores remain viable from 3 to 7 days at temperatures ranging from 11 to 18°C (Ratliff 1983, Foott et al. 2007), production must account for the likelihood of finding a salmonid host before deterioration of spore material. Potential production of *C. shasta* actinosporeans reported in this study closely resembles the value that Bartholomew et al. (2006) reported from one day of production. However, a maximum production of 13,650 (975 actinospores/day) seems rather small given the scale of the problem in the Klamath River basin. Given that the maximum reported infection prevalence in a Klamath River *M. speciosa* population is only 8% (Stocking & Bartholomew 2007), it does not seem that enough spore material would be generated to provide an infectious load capable of overwhelming salmonid immunity.

Some account for low production estimates may be provided by the fact that *M. speciosa* is able to function in the presence of *C. shasta*. Polychaetes are able to regenerate lost or broken tissue (Leidy 1883, Lewis 1968, Willson et al. *in press*). Therefore, it is unlikely that puncturing the narrow cuticular layer by *C. shasta* actinospores seriously affects the polychaete; at least during early stages of sporogenesis. Additionally, Bartholomew et al. (1997) proposed that development of pansporocysts within secretory cells may allow the parasite to be released from a secretory pore. Given the assumptions that release of actinospores do not kill the host, and that actinosporeans are produced on a continuous basis within *M. speciosa*, one polychaete may provide a significant number of actinospores throughout the course of the infectious season.

This is supported, in part, by what El-Matbouli et al. (1995) reported from *Myxobolus cerebralis* production in *Tubifex tubifex*. They found that infected tubificids released triactinomyxon spores for more than 12 months after the initial release of spore material. It was also established through work by El-Matbouli & Hoffmann (1998), and substantiated by this study, that through schizogony multiple life stages occur at the same time and in large volume. This would indicate that over the lifetime of a polychaete, a substantial amount of actinospores may be produced, which may lead to the high infectious loads seen in the Klamath River basin.

Additional support for how polychaetes may provide high infectious loads is from chronological development of actinosporeans. If indeed the only source of infection is through feeding, then it appears that only a few ingested myxospores produce hundreds of actinospores in only 49 days at temperatures averaging 17.3°C. Additionally, the majority of development (in terms of time) occurs in the early stages. Once the parasite reaches sporogony, development to a mature actinospore occurs relatively quickly. This information is confounded slightly by the fact that the last 14 days of the experiment occurred at temperatures that were 5°C higher. However, the faster rate of development may indicate that there is a mechanism for release that may coincide with salmonid presence in the basin. Many authors have hypothesized that temperature changes are the key trigger for parasite release (El-Matbouli et al. 1999, Stocking et al. 2006).

The primary goal of this experiment was to establish an infection of *C. shasta* in *M. speciosa*. Because lab rearing of polychaetes has not been successful until recently (Willson et al. *in press*), polychaetes used in this study were collected from the field two weeks prior to experimentation. This means that samples could have contained naturally infected individuals. Additionally, field collected individuals could have contained both *C. shasta* and *Parvicapsula minibicornis* myxospores. Thus, the information presented here may not be definitive.

However, understanding stages of development of the parasite will aid in narrowing future sampling protocol. Additionally, such tools as *in situ* hybridization may lead to a more definitive description of early schizogony stages and the migration route from the gut to the epidermis.

Understanding the development of *C. shasta* actinosporeans in *M. speciosa* will hopefully provide another tool useful in finding good management practices for control of ceratomyxosis in the Klamath River basin. For example, knowing that the infection is initiated through feeding may lead to viable solutions in controlling polychaete populations. There is also evidence that invertebrates may be able to build-up immunities to pathogens (Kurtz & Franz 2003, Steinbach 2003). If this is the case for *M. speciosa* and *C. shasta*, there could be a program to seed the Klamath River basin with parasite-resistant polychaete populations. The task that we are faced with is how to best prevent, or reduce, the occurrence of disease within the Klamath River basin. With a complete understanding of parasite and host life cycles, it may be possible to get at real solutions to support Klamath River fish.

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**Table 1.** Measurements ( $\pm$  standard deviation) of each life stage of *Ceratomyxa shasta* actinosporean development in *Manayunkia speciosa*. Life stage identification corresponds to the stages diagrammed in Fig. 1. All measurements in  $\mu\text{m}$ .

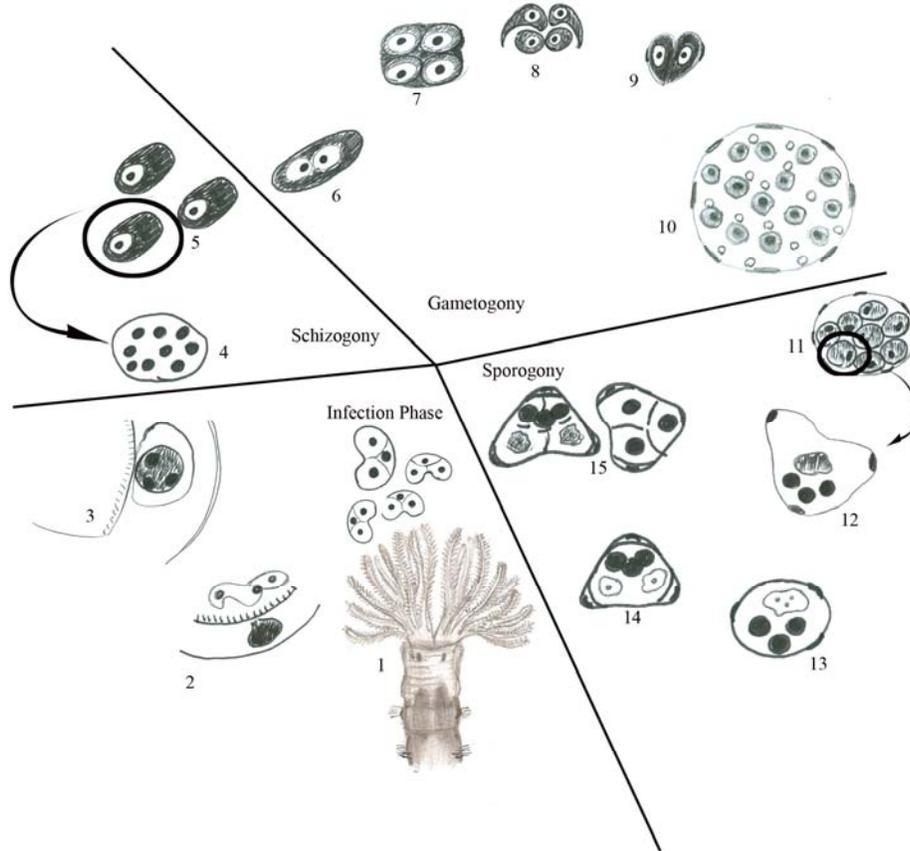
Life Stage	Pansporocyst		Actinosporean		Nucleus dia.
	Length	Width	Length	Width	
1			10.27 $\pm$ 1.2	6.64 $\pm$ 1.02	
3			5.62 $\pm$ 0.89	5.13 $\pm$ 0.94	
4			7.56 $\pm$ 0.72	5.28 $\pm$ 0.83	
5			4.31 $\pm$ 0.58	3.63 $\pm$ 0.52	2.54 $\pm$ 0.62
6a			8.19 $\pm$ 0.58	3.87 $\pm$ 0.51	2.18 $\pm$ 0.22
6b			10.47 $\pm$ 0.83	5.57 $\pm$ 1.17	2.25 $\pm$ 0.32
7			10.52 $\pm$ 1.51	6.93 $\pm$ 0.99	2 $\pm$ 0.18
8			9.01 $\pm$ 0.79	7.85 $\pm$ 1.24	2.24 $\pm$ 0.38
9			11.34 $\pm$ 1.29	7.27 $\pm$ 1.31	
10	15.22 $\pm$ 2.57	12.65 $\pm$ 2.31	3.99 $\pm$ 0.61	3.75 $\pm$ 0.24	2.18 $\pm$ 0.34
11	15.85 $\pm$ 1.25	11.44 $\pm$ 1.46	4.21 $\pm$ 0.32	3.92 $\pm$ 0.35	2.1 $\pm$ 0.72
12	24.91 $\pm$ 2.27	16.43 $\pm$ 2.3	9.16 $\pm$ 1.03	7.46 $\pm$ 0.52	
13			7.17 $\pm$ 0.3	6.3 $\pm$ 0.39	
14			7.99 $\pm$ 0.34	6.64 $\pm$ 0.32	
15			8.72 $\pm$ 0.6	7.07 $\pm$ 0.33	

**Table 2.** *Ceratomyxa shasta* infection in relation to sectioned length of *Manayunkia speciosa*. Dates refer to the date that the sample was fixed for processing. Note that the location of infection is reported by segment (out of 12), with the prostomium/peristomium representing segment 1. All measurements in mm.

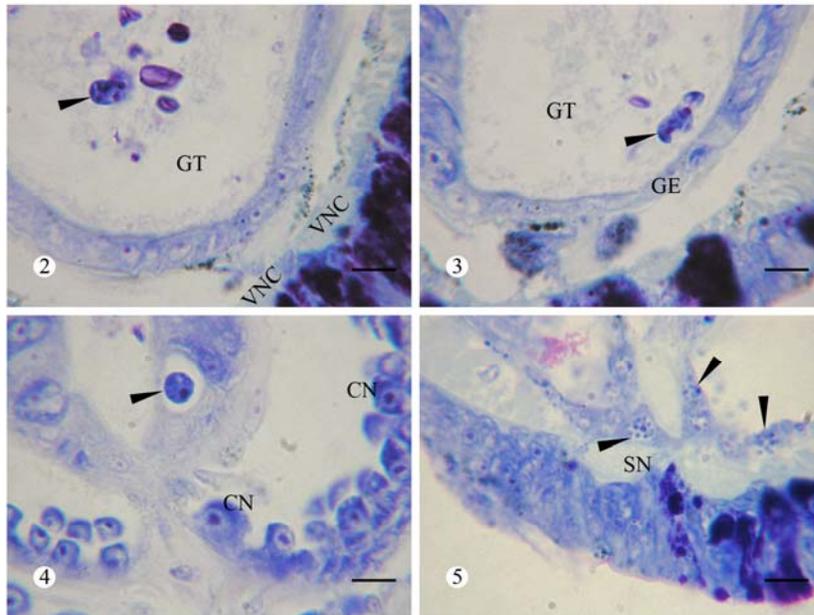
Specimen	Length of <i>M. speciosa</i>	Infection		
		Length	%	Location
25-Sep-06	1.97	0.59	30	6-10
25-Sep-06	1.73	0.85	49	6-10
28-Jun-07	1.43	0.53	37	6-10
11-Jul-07	2.18	1.14	52	4-10
11-Jul-07	1.49	0.53	36	6-9
11-Jul-07	1.18	0.59	50	5-10
14-Aug-07	1.92	0.72	38	6-10
14-Aug-07	1.76	0.71	40	6-11

**Table 3.** Number of *Ceratomyxa shasta* actinospores produced over a 14-day period from *Manayunkia speciosa*. Dates refer to the date that the sample was fixed for processing. Measurements for pansporocysts represent averages from 20 random sections. Depth of pansporocysts indicates the number of sections in which development occurred. Epidermal width represents average of four locations within a section from 20 random sections. All measurements in  $\mu\text{m}$ .

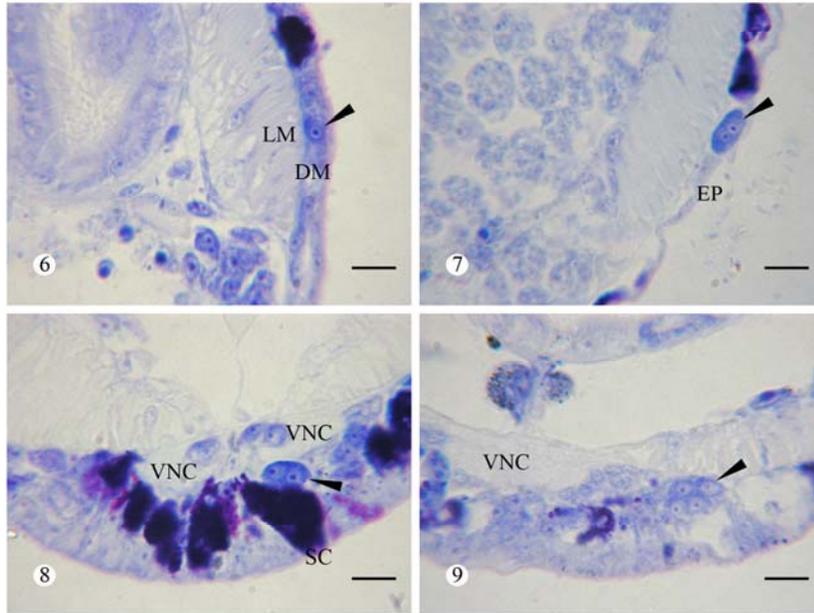
Specimen	Pansporocysts		Actinospores		Epidermal width
	#	Depth	#	SD	
25-Sep-06	6.60	12.00	2,605	1,269	13
25-Sep-06	6.90	12.75	3,661	937	19
28-Jun-07	3.20	12.50	1,124	878	13
11-Jul-07	17.95	12.75	13,650	7,807	22
11-Jul-07	14.95	13.00	5,017	2,195	18
11-Jul-07	12.30	12.75	4,602	1,702	20
14-Aug-07	6.10	12.25	3,005	1,320	18
14-Aug-07	9.80	13.00	4,411	1,275	21



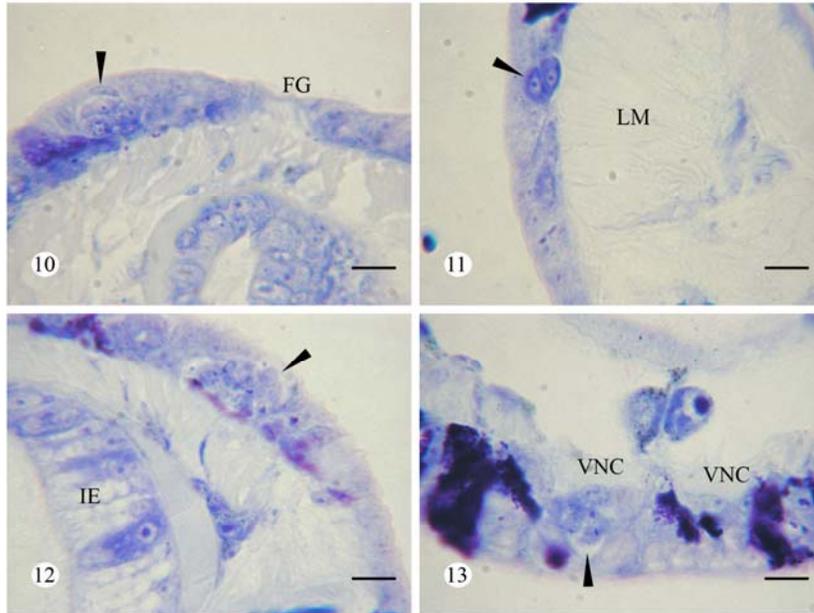
**Fig. 1.** Development of *Ceratomyxa shasta* actinosporeans in the freshwater invertebrate host, *Manayunkia speciosa* (schematic diagram). (1) Ingestion of *C. shasta* by *M. speciosa*. (2) Directly following ingestion, myxospores are present in gut cavity of segments 4 through 7. Shell valves open, releasing sporoplasm aggregate between gut epithelial cells. (3) Sporoplasm aggregates are found between gut epithelia after penetration of myxospores. (4) Proliferative (schizogonic) stage into a multinucleate cell-stage. (5) Uninucleate cell-stage that can either go back into schizogony or fuse. (6) Plasmogamy of two uninucleate cells to produce one binucleate cell-stage. (7) Fusion of nuclei, followed by mitotic division, to produce a four-celled stage. (8) Differentiation of four-celled stage into two somatic and two generative cells. (9) Formation of early pansporocyst, with somatic cells fully encapsulating generative cells. (10) Production of 16 gametocytes (8 $\alpha$  and 8 $\beta$ ) with polar bodies from two mitotic divisions and one meiotic division. (11) Combining of  $\alpha$ - and  $\beta$ -gametocytes to produce eight zygotes. (12) Initial stages of three main types of sporogonic cells: capsulogenic, valvogenic, and sporoplasm material. (13) Shrinkage and condensation of spores. (14) Formation of a more definitive spore morphology, but sporoplasm material not defined yet. (15) Extension of valvogenic cells around sporoplasm and capsulogenic cells (evidenced by presence of septate junction) and maturation of sporoplasm material.



**Figs. 2-5.** Initiation of infection by *Ceratomyxa shasta* myxospores to the first stage of schizogony in *Manayunkia speciosa*. All sections were stained with May-Grünwald Giemsa protocol. Arrowhead in each figure indicates the developmental stage discussed. **Fig. 2.** Myxospore in gut cavity (GT). Photograph was taken in segment 5 of a male polychaete. Note how close the gut is located to the ventral nerve cord (VNC). **Fig. 3.** Myxospore valve in the gut cavity (GT). Myxospore separated along the suture line and released sporoplasm material between gut epithelial (GE) cells. Note dark staining material in gut epithelium adjacent to the myxospore valve. **Fig. 4.** Sporoplasm aggregate within the septal nerve (SN). Note the lytic zone surrounding the sporoplasm and trinucleated structure. **Fig. 5.** First stage of schizogony. At this stage, the actinosporean was a multinucleate structure within septal nerves (SN). Scale bar = 10  $\mu$ m.

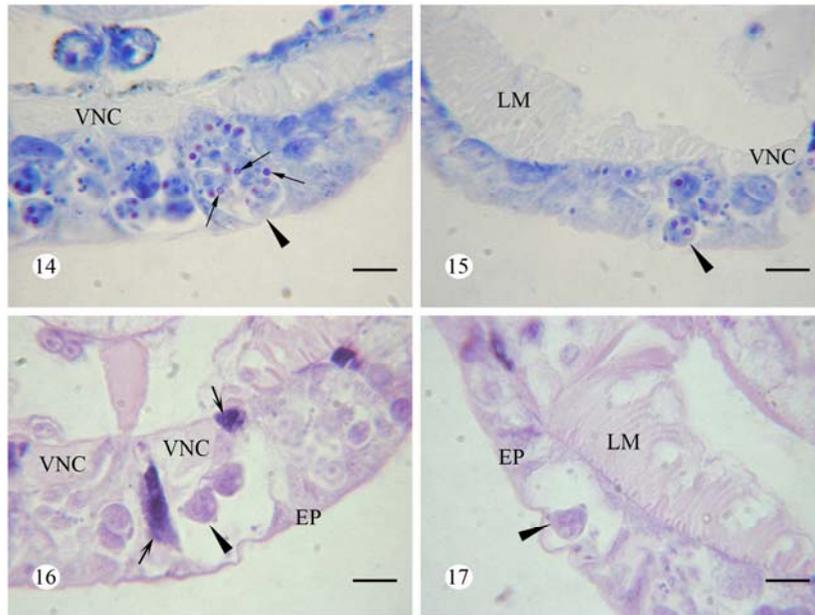


**Figs. 6-9.** Early developmental stages of *Ceratomyxa shasta* actinosporeans in *Manayunkia speciosa*. All sections were stained with May-Grünwald Giemsa protocol. Arrowhead in each figure indicates the developmental stage discussed. **Fig. 6.** Uninucleate cell of the second stage of schizogony, which developed from the dispersal of the multinucleate stage. Actinosporean was recognized by its dark-staining cytoplasm, distinct nucleus and nucleolus, and surrounding lytic zone. Note that the location of development is between the dermis (DM) and longitudinal musculature (LM). **Fig. 7.** First stage of gametogony within the epidermis (EP) formed from the fusion of two uninucleate cells. The two nuclei are destined to become different structures. **Fig. 8.** Second stage of gametogony. The binucleate cell from the first stage expanded dorsally and laterally to allow for division of the nuclei. The developing actinosporean is located right next to the ventral nerve cord (VNC) above a large secretory cell (SC). **Fig. 9.** Third stage of gametogony resulting from a fusion of the nuclei in the binucleate cell and then two mitotic divisions of the fused product. The resulting four-celled structure maintained the dark-staining cytoplasm and distinct nucleus and nucleolus that distinguished these early developmental stages. This actinosporean is also located in close proximity to the ventral nerve cord (VNC). Scale bar = 10  $\mu$ m.

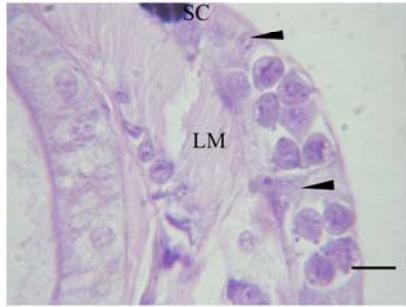


**Figs. 10-13.** Later gametogony and early sporogony stages of *Ceratomyxa shasta* actinosporeans in *Manayunkia speciosa*. All sections were stained with May-Grünwald Giemsa protocol. Arrowhead in each figure indicates the developmental stage discussed.

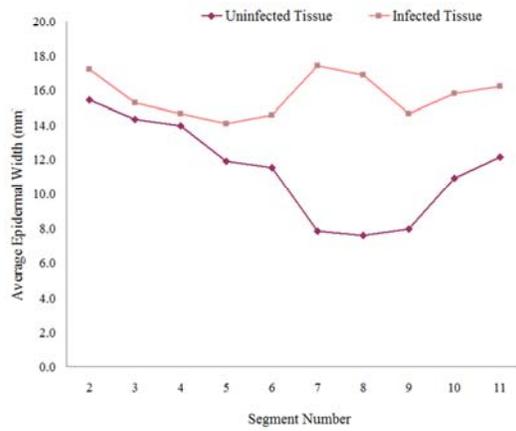
**Fig. 10.** Fourth stage of gametogony, in which the four-celled structure began to differentiate into two different types of cells: somatic and generative. The somatic cells flattened out and started to envelope the generative cells that maintained a more rounded structure. The actinosporean is located in the dorsal portion of the worm, close to the fecal groove (FG). **Fig. 11.** Fifth stage of gametogony with two somatic cells fully enveloping two generative cells. The actinosporean is located along the lateral portion of the worm. Note the longitudinal musculature (LM) layer to the right. **Fig. 12.** Sixth stage of gametogony, post mitotic and meiotic divisions. The resulting structure has 16  $\alpha$ - and 16  $\beta$ -gametocytes surrounded by polar bodies. Note the close proximity to the gut epithelial (GE) layer. **Fig. 13.** First stage of sporogony, post fusion of an  $\alpha$ - and  $\beta$ -gametocyte. The resulting structure is a pansporocyst enveloping eight zygotes with distinct nuclei. Actinosporean is located within the ventral portion of the worm in close proximity to the ventral nerve cord (VNC). Scale bar = 10  $\mu$ m.



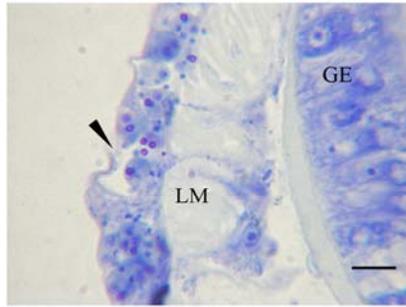
**Figs. 14-17.** Sporogonic developmental stages of *Ceratomyxa shasta* actinosporeans in *Manayunkia speciosa*. Figs. 14-15 were stained with May-Grünwald Giemsa protocol. Figs. 16-17 were stained with hematoxylin and eosin. Arrowhead in each figure indicates the developmental stage discussed. **Fig. 14.** Second stage of sporogony. This was the first stage that contained all three cell types that make up an actinospore: capsulogenic, sporoplasm, and valvogenic. Note that capsulogenic cells (arrows) were refractile, which indicated that they contained polar filaments. Valvogenic cells were present, but had not fully encapsulated the structure at this stage. Actinosporeans are located within the ventral portion of the worm near the ventral nerve cord (VNC). **Fig. 15.** Third stage of sporogony, where actinosporean material shrunk and condensed into a more typical spore. Asynchronous development was evident within a pansporocyst. **Fig. 16.** Fourth stage of sporogony. Actinosporeans expanded along the longest side and sporoplasm material condensed into the center of the structure. Actinosporean is within a pansporocyst in the epidermis (EP); however the other spores are not within the same plane. **Fig. 17.** Final mature actinospore stained with hemotoxylin and eosin. Note the suture line that divides the spore into three equal parts. Scale bar = 10  $\mu$ m.



**Fig.18.** Response of *Manayunkia speciosa* to infection with *Ceratomyxa shasta* in advanced stages of sporogenesis. Host tissue was displaced during pansporocyst development in the ventral portion of segment 7 (arrowhead). Note location of longitudinal musculature (LM) to the developing pansporocysts and limited number of secretory cells (SC). Section stained with hematoxylin and eosin. Scale bar = 10  $\mu$ m.



**Fig.19.** Average expansion of the epidermal layer of *Manayunkia speciosa*, in response to infection by *Ceratomyxa shasta*, compared to uninfected host tissue. Note that the most significant expansion occurs in segments 7 to 9, which is where the majority of actinosporean production occurs. Additionally, infection is initiated in the gut of segments 4 to 7, which is where expansion of host material begins.



**Fig. 20.** *Ceratomyxa shasta* breaking through epidermis of *Manayunkia speciosa* within segment 7 (thoracic chaetiger 6). Break in outer epidermal layer is most likely a product of increasing pressure to the breaking point as pansporocysts grow. Open channel is indicated by arrowhead. Note the close proximity to longitudinal musculature (LM) and columnar epithelial cells of the gut epithelium (GE). Scale bar = 10  $\mu$ m.

Supplemental Information

A. Summary of Expenditures. No major property was purchased during this project.

	Budget	Expenditures
SALARY		
Graduate Student Assistant	12,600	12,905
Technicians	10,920	12,516
Faculty - Hendrickson	6,136	6,136
Faculty - Cummins	4,000	3,985
TOTAL SALARY	33,656	35,542
BENEFITS		
Mandatory Benefits	5,296	3,428
TOTAL SALARY & BENEFITS	38,952	38,970
SUPPLIES	5,000	4,808
TRAVEL	2,324	2,042
HSU FEES (tuition)	1,000	1,500
LAB BENCH FEE	550	500
TOTAL DIRECT COSTS	47,826	47,820
INDIRECT TO SP FOUNDATION	7,174	7,173
TOTAL	55,000	54,993

B. Brief Description of work employed by unemployed member(s)

1. Volunteers were sometimes used on the project for field collections. This was always done under the supervision of paid project personnel.

C. In kind contributions including cash and non-cash

1. The Sponsored Programs Foundation, Humboldt State University allowed overhead at a rate of 15% of direct costs. This was below their negotiated federal rate of 45.5% of salaries, wages, and benefits. This is a match value of about \$21,681.
2. Hendrickson was one nine-month appointment with Humboldt State University Part of his work effort (1/15) during each of two academic years involved supervising the graduate student (Marlene Meaders) working on the project. This is a match value of \$10,186.

3. Hendrickson received 1 month of summer salary during 2006. He donated at least 1 month of time during 2007. This is a match value of \$6,366.
4. United States Department of the Interior, Geological Survey, California Cooperative Fishery Research Unit provided Wilzbach's salary, secretarial support, laboratory space, and space for rearing polychaetes.
5. Humboldt State University, Department of Fisheries Biology and Fish Hatchery, provided laboratory space, space for rearing fish, and fish for infection studies. Humboldt State University, College of Natural Resources and Sciences provided a technical support for designing and maintaining rearing units.
6. Volunteers were occasionally used for field work

D. Sources of funding

1. The majority of funding was provided by the United States Fish and Wildlife Service's program on Fishery Restoration Work for the Klamath Restoration Program. Additional funding was provided by the California Cooperative Fishery Research Unit and the Department of Fisheries Biology, Humboldt State University.

E. Training level of any volunteers

1. Volunteers were sometimes used for field work. All had college training in the sciences and had earned or were working on a BA or BS in Fisheries Biology or a related field. Volunteers were always under the supervision of paid project personnel.
2. We consulted with scientists at Scripps Institution of Oceanography, Oregon State University, and the US Fish and Wildlife Service's California-Nevada Fish Health Center all of whom had Ph.D.'s.
3. We also received help and advice from graduate students in microbiology at Oregon State University.