Journal of Shellfish Research, Vol. 20, No. 2, 883–888, 2001.

LIFE HISTORY OF AN EXOTIC SABELLID POLYCHAETE, *TEREBRASABELLA HETEROUNCINATA*: FERTILIZATION STRATEGY AND INFLUENCE OF TEMPERATURE ON REPRODUCTION[a]

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ABSTRACT The California abalone aquaculture industry has been struggling to rid itself of an exotic sabellid, *Terebrasabella heterouncinata* following its inadvertent introduction from South Africa in the late 1980s. The development of an effective management strategy is dependent upon understanding the life history of this sabellid, including its fertilization strategy (e.g. self-fertilization) and its generation time. In the present study, red abalone, *Haliotis rufescens* Swainson 1822, with single sabellid infestations were isolated in containers at 18°C (single host and sabellid per container). This first, parental generation (P), was held in isolation until individuals produced F_1 larvae. The F_1 larvae were subsequently isolated until individuals produced a second, F_2, generation. In a separate study, uninfested red abalone were exposed to sabellid infested abalone at 11.2°C, 15.6°C, and 20.9°C, temperatures typically encountered in California. The larvae were subsequently observed as they developed to specific life stages: the initiation of feeding, the development of all 11 setigers (which closely relates to sexual maturation) and the completion of their life cycle as recognized by the production of motile, infesting, larvae. Approximately 50% of the sabellids examined at 11.2°C, 15.6°C, and 20.9°C had developed the ability to feed by day 6, 5 and 4 ($P < 0.001$), had developed all 11 setigers by day 83, 68 and 48 ($P < 0.001$) and had produced larvae by day 298, 165 and 111 ($P < 0.001$), respectively. This research demonstrates that isolated individuals do pose the threat of producing fully functional offspring and that the generation time of *T. heterouncinata* is significantly temperature dependent.

KEY WORDS: sabellid, abalone, life history, self-fertilization, *Terebrasabella*

INTRODUCTION

The California abalone aquaculture industry is presently struggling with two serious diseases, one of which is an introduced sabellid polychaete, recently described as *Terebrasabella heterouncinata* (Fitzhugh & Rouse 1999). This sabellid was cited by California growers as their most serious "problem and constraint," reporting that the reduced growth rates and negative public perception associated with the worm were having a substantial negative impact on the industry (Mcbride 1998). *Terebrasabella heterouncinata* is believed to have been introduced from South Africa in the late 1980s, via the importation of infested research abalone (Kuris & Culver 1999, Ruck & Cook 1998). Concern exists regarding the threat that the sabellid poses to the California cultured abalone industry and to native gastropod populations if it were to become established within the state's intertidal ecosystem (Kuris & Culver 1999). Presently, there is one documented case of *T. heterouncinata* infestations in gastropods (snails and limpets) adjacent to a culture facility (Kuris & Culver 1999). Broad-scale removal of infested hosts, combined with clean-up efforts at the culture facility may have curtailed the sabellid from becoming permanently established in the intertidal environment (Kuris & Culver 1999).

Infestations occur by the unique ability of *T. heterouncinata* to overcome the host abalone's defenses and settle upon the under side of the leading edge of the shell. Normal shell deposition is disrupted as the host attempts to cover the irritant with nacre. In heavily infested abalone, the deposition of the prismatic shell layer all but ceases, resulting in the domed shell found in association with the reduced growth rates (Culver et al. 1997, Kuris & Culver 1999, Ruck & Cook 1998).

Attempts to control *T. heterouncinata* in commercial settings have included manipulations of water temperatures, the coating of abalone shells with wax, quarantine of infested stocks, and improved sanitation practices (Oakes & Fields 1996, Oakes et al. 1995, Leighton 1998). Many of these techniques have been met with limited success, allowing low-level infestations to persist. Suggested new treatments include novel therapeutic delivery systems using micro-encapsulation (Ruck & Cook 1998, Shields et al. 1998) and the use of ultrasound (Loubser & Dormehl 2000). Unfortunately, none of these techniques, to date, have been successfully applied in a culture setting.

The initial description of *T. heterouncinata* noted that individuals were simultaneous hermaphrodites, but did not indicate if they were functional hermaphrodites, capable of self-fertilization. Upon settling at the 7th setiger stage, the sabellids are covered with nacre by the host resulting in the passive formation of what becomes the adult's tube. Development is characterized by the formation of a branchial crown and four additional setigers. As the 11th setiger is formed, an alteration of the type of uncini on the 6th setiger also occurs, both events occur as individuals are recognized as sexually mature (Fitzhugh & Rouse 1999). Spermatogenesis occurs in the 8th setiger and oogenesis occurs in both the 9th and 10th setigers. The mature adult broods several embryos within its tube for an unknown, temperature dependent, amount of time. The embryos eventually mature and develop into motile larvae that subsequently emerge from the tube of the adult and seek a suitable area of attachment (Fitzhugh and Rouse, 1999).

In the following study we examined the fertilization strategies and the life history of *T. heterouncinata*. We investigated if an isolated sabellid is capable of reproduction through two generations. We assessed the reproductive capabilities of *T. heterouncinata* by quantifying generation times at three water temperatures that reflect the range of temperatures encountered in California.

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MATERIALS AND METHODS

Animals and Husbandry

Commercially reared red abalone, *Haliotis rufescens* Swainson 1822, that measured 15–25 mm in shell length, were purchased from or donated by culturists in California. All animals were held in the Pathogen Quarantine Facility at the Bodega Marine Laboratory, Bodega Bay, California. All effluent produced at this facility is chlorinated (>10 ppm Cl^- for 2–3 h) and dechlorinated prior to release. Animals were reared in flow-through, full strength, sand-filtered seawater. Experiments were conducted at average mean temperatures of ambient (12.6°C), heated (15.6°C, ~18°C and 20.9°C) or chilled (11.2°C) seawater. All abalone were fed *Macrocystis pyrifera* Agardh 1820, *ad libitum* and remained under artificial light on a 12-hour dark, 12-hour light cycle for the duration of these experiments. Strict sanitation protocols were followed to insure that no cross-contamination occurred.

Single Infestations Trial 1

Fifty uninfested red abalone were placed into a 10-l container with five red abalone, heavily infested with *T. heterouncinata*, for 24 hours. The newly infested abalone were inspected with a dissecting microscope 48 hours post-exposure and infestations were quantified. Fourteen abalone with single sabellid infestations were removed and individually isolated in 200-ml containers. Four abalone, that had multiple sabellid infestations per abalone, were isolated in a similar manner to serve as positive controls. Four uninfested abalone were isolated in a similar manner to serve as negative controls. All 22 containers received ambient seawater, ~12.6°C, and were randomly placed on a wet table.

Abalone were sampled every 14 days by gently pushing aside the epipodium and mantle tissue and inspecting the leading edge of the shell for the presence of recently settled larvae. Following the discovery of new larvae, treatments were sampled every seven days. Uninfested abalone were added to any container in which the original infested host abalone had died, providing live hosts for larval settlement. The abalone were observed for a total of 32 weeks, at which point the sabellids that had not produced larvae were carefully dissected from their tubes and observed under dissecting and compound microscopes to determine if signs of reproduction were visible.

Single Infestations Trial 2

Forty uninfested red abalone were exposed to *T. heterouncinata* infested abalone as described in Trial 1. Abalone were inspected 48 hour post-exposure and the infestations were quantified. Four abalone with single parental (P) generation infestations (Fig. 1) and four uninfested abalone (negative control) were removed and individually isolated in 700-ml containers. Animals were visually inspected weekly. Sabellid larvae settle preferentially on live hosts (C. A. Finley & C. S. Friedman, unpublished observation). To allow F_1 larvae to settle only on recipient, sabellid-free abalone, infested (P host) abalone were sacrificed after 60 days post-exposure and a single uninfested abalone (larval recipient) was placed into each tank with the infested shucked shells. Once a recipient abalone became infested with an F_1 larva, the newly infested animal was removed and isolated (single abalone with single sabellid) in a 700-ml container. Additional uninfested abalone were added to the containers with the P generation sabellids to attain multiple individuals with single F_1 infestations (Fig. 1).

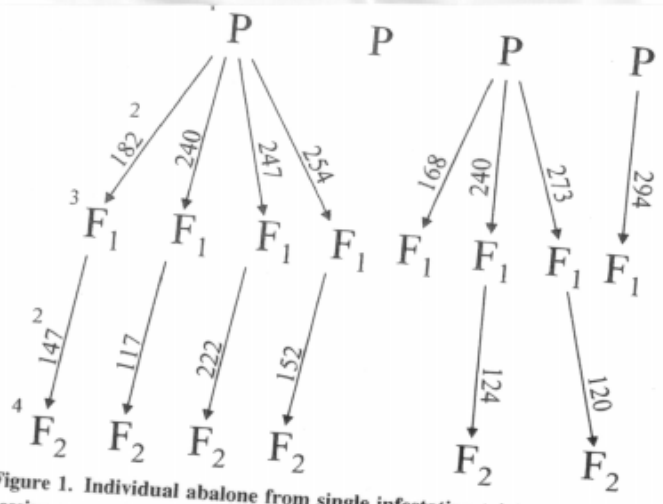

Figure 1. Individual abalone from single infestation trial 2. Two successive generations of sabellids and the number of days until offspring were produced is illustrated. Superscripts (1–4) indicate (1) the parental generation, (2) the number of days until larvae were observed, (3) the F_1 larvae produced by the parental by the parental generation and (4) the F_2 larvae produced by the F_1 generation.

Host abalone containing F_1 larvae were visually inspected for signs of F_2 larvae. All containers received heated seawater (~18°C) for the duration of this trial. The abalone were observed for a total 497 days, at which point any sabellids that had not produced larvae were carefully removed and observed for signs of reproduction.

Life History Trial

Terebrasabella heterouncinata infestations were achieved by commingling 200 uninfested and 20 infested (10 live and 10 shucked) red abalone in a 10-l container for a 24 hour period. Following the exposure the 200 newly infested abalone were divided into three replicates tanks. Exposures were conducted at 11.2°C, 15.6°C, and 20.9°C. Infestations were quantified 48 hours after the end of the exposure period. The removal of the 20 heavily infested donor abalone marked time zero and every subsequent 24-hour period was regarded as an additional day of development.

Development to the following three life stages was recorded: the ability to feed, the development of the full complement of setigers and the production of infestive, motile larvae. Replicates were sampled every 24 hours for the first eight days, followed by weekly sampling. At each sampling point, six sabellids were removed from each replicate by sacrificing the host abalone and gently breaking up the shell with a scalpel.

The ability to feed was determined by providing the sabellids with suspended stained lipid beads (LBs). Following the protocol of Shields et al. (1998) microencapsulated, sudan black-stained, tripalmatin lipid beads that ranged in size from 3 to 30 μm were produced. Several abalone, with a total of six sabellids, were removed from each replicate tank and placed into three 200–ml containers to which 0.3 g of the lipid beads were added. The seawater-lipid bead suspension was stirred every 10 minutes. After 30 minutes, the abalone hosts were sacrificed and the sabellids were excised and examined for the presence of LBs in the digestive tract using a light microscope. We enumerated the number of feeding sabellids, terminating the LB exposures when all sabellids in a given replicate were able to feed.

To identify the development of all 11 setigers, six sabellids

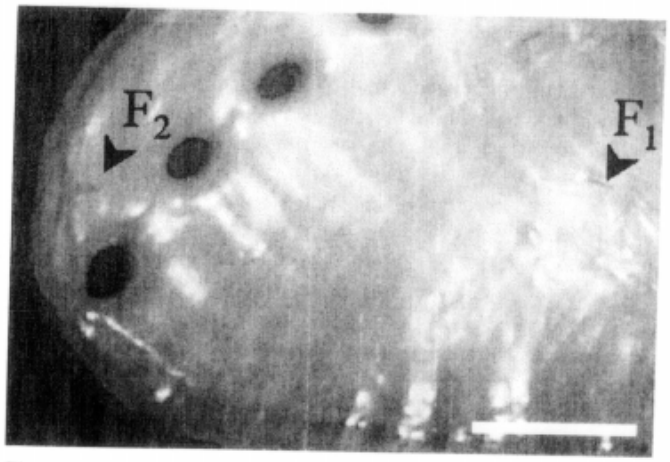

Figure 2. Abalone shell showing both the F_1 and F_2 generation, Bar = 5mm.

were removed weekly from each tank and the number of setigers was enumerated using a light microscope at 600× magnification. When 100% of the sabellids sampled from each replicate had developed a full complement of setigers, sampling was reduced to once every 1–4 weeks depending on the number of sabellids remaining and the temperature under investigation.

The completion of the life history of *T. heterouncinata* was defined by the production of either a motile, infesting larva or by a recently settled larva. Sabellids were excised as above and the presence or absence of motile or newly settled larvae was recorded. The experiment terminated when 100% of the sabellids inspected from each replicate completed their life cycle.

Statistical Analysis

With the assumptions of an ANCOVA not being met, an alternative way to analyze the data was needed. Linear regression lines were calculated for each replicate. The number of days that it took 50% of the sabellids to reach a developmental stage (T_{50}), was then calculated from the regression line using the formula $Y = bX + a$, setting the dependent variable, Y, equal to 50% and solving for X. The T_{50} estimates were then compared using one-way analysis of variance (ANOVA).

The data from the replicates at each temperature were also pooled and the number of days that it took for 50% of the sabellids to reach a developmental stage was calculated. The T_{50} estimates from the pooled replicates at a given temperature were then presented as the best estimate for the number of days post-settlement required for 50% of the sabellids to reach a given developmental stage at each temperature.

RESULTS

Single Infestations Trial 1.

Larval production by six of the 14 individually isolated sabellids began during weeks 26 to 32 (Table 1). Of the seven sabellids that had not produced any larvae, three contained well-developed internal eggs, one contained both internal and external eggs and embryos, and the remaining three tubes were devoid of sabellids at the termination of Trial 1. One abalone was contaminated by the effluent from another tank and was discarded.

Abalone with multiple sabellid infestations (positive control) also began producing larvae during week 26. Sabellids on 3 of the 4 abalone produced larvae (Table 1) and only vacant sabellid tubes were found on the fourth animal at the termination of Trial 1. No sabellid infestations were found on the negative control animals (Table 1).

Single Infestations Trial 2.

Three of the four P generation sabellids produced an F_1 generation. The first larva was observed on day 168 (Fig. 1). Subsequently, two of the P generation sabellids produced additional F_1 larvae (three and four, respectively). Six of the F_1 generation produced an F_2 generation. The first larva appeared 117 days after the F_1 parent had settled (Fig. 1). Figure 2 illustrates a host shell with both F_1 and F_2 sabellids.

Life History

Recently settled larvae that had fed on stained lipid beads were easily distinguished from individuals that had not fed (Fig. 3). The amount of time required for the sabellids to develop the ability to feed increased significantly as the temperature decreased (ANOVA, $p < 0.001$). The pooled replicates indicated that 50% of the sabellids reached this developmental stage at 20.9°C, 15.6 °C, and 11.2°C by 3.7, 4.9 and 6.0 days post exposure, respectively (Fig. 4).

The rate at which the sabellids developed a full complement of setigers was also affected by temperature. The number of days required for the sabellids to develop all 11 setigers required significantly more time as the temperature decreased (ANOVA, $p < 0.001$). Analysis of the pooled data indicated that 50% of the newly settled larvae developed a complete complement of setigers at 20.9°C, 15.6°C, and 11.2°C by 47.7, 68.4 and 83.1 days post exposure, respectively (Fig. 5). The majority of the sabellids observed had developed both eggs and sperm in conjunction with the development of all 11 setigers.

The number of days required for the sabellids to produce motile, infesting larvae or newly settled larvae required significantly more time as the temperature decreased (ANOVA, $p < 0.001$). Analysis of the pooled data indicated that 50% of the newly settled larvae had completed their life history at 20.9°C, 15.6°C, and 11.2°C by 110.8, 165.0 and 297.8 days post exposure, respectively (Fig. 6).

DISCUSSION

Self-fertilization is not considered the predominant mode of fertilization for many simultaneous hermaphrodites due to the del-

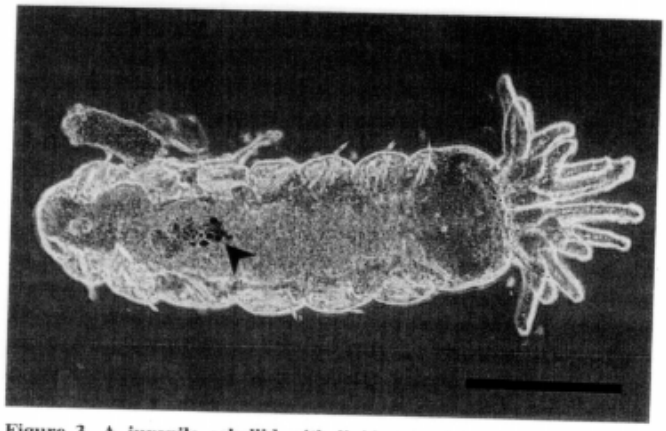

Figure 3. A juvenile sabellid with lipid-stained beads visible in the digestive tract (arrow). Scale bar = 100 μm.

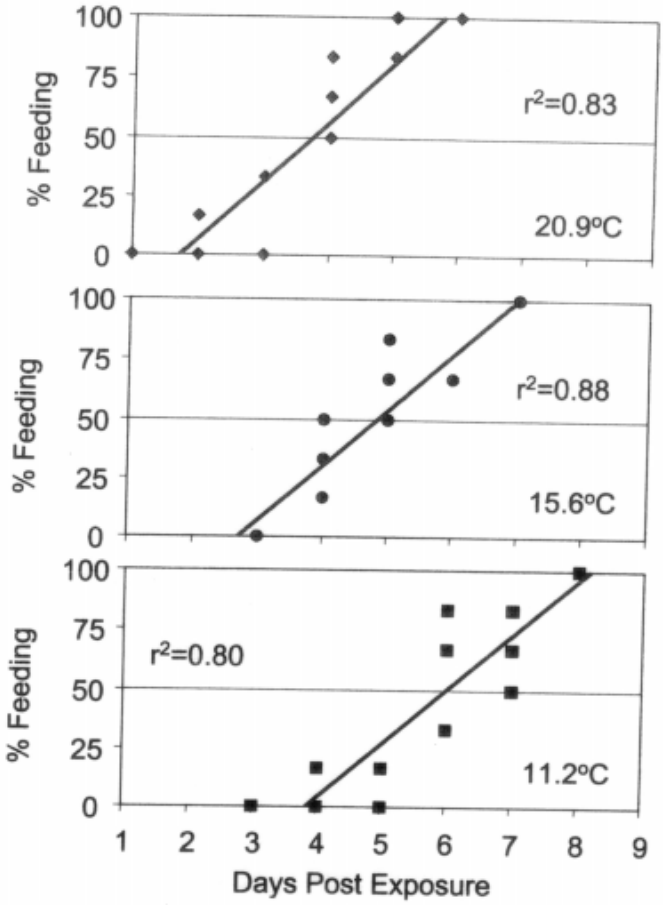

Figure 4. Percentage of feeding sabellids in pooled replicates at each of three temperatures investigated. Regression lines indicate that 3.7, 4.9 and 6.0 days post-exposure, 50% of the sabellids had developed the ability to feed at 20.9°C, 15.6°C, and 11.2°C, respectively.

eterious effects believed to be associated with inbreeding (Heath 1977, Beckwitt 1982, Michiels & Streng 1998). There are, however, several examples of successful self-fertilization among marine invertebrates (Ghiselin 1974, Beckwitt 1982, Knowlton & Jackson 1993, Scharer & Wedekind 1999), some of which include members of the class Polychaeta (Ghiselin 1969, Hsieh 1997). Hsieh (1997), for example, found no deleterious effects of self-fertilization in the simultaneous hermaphrodite *Laonome albicingillum*, also a sabellid polychaete.

Most polychaetes broadcast spawn small ova that are capable of being distributed great distances from the parent. In contrast, *T. heterouncinata*, produces a relatively small number of large eggs that are brooded within their tube and lack a pelagic stage. Combined, these characteristics make *T. heterouncinata* a likely candidate for self-fertilization (Knowlton & Jackson 1993). In addition, adult sabellids are sessile and rely solely upon their short infesting motile stage for the distribution of progeny to other hosts. Given this life history, one would predict that if the benefits of reproductive assurance associated with self-fertilization outweighed the deterrents of "selfing," the requirement of cross-fertilization would be selected against (Jarne & Charlesworth 1993). For a sessile parasite such as *T. heterouncinata*, that invests a relatively large amount of energy into a limited number of eggs combined with a lack of control over accessing a partner, self-fertilization would be a successful strategy to assure that a founding sabellid on a host abalone would remain capable of producing progeny.

Self-fertilization is one plausible explanation for the production of the F_1 and F_2 generations that we observed, though alternative fertilization strategies, such as parthenogenesis, cannot be ruled out. These experiments were designed to investigate whether a single isolated sabellid has the ability to produce functional progeny or if a minimum population number is necessary to assure cross-fertilization. Our research indicates that a single sabellid is capable of founding a viable population.

Following its accidental introduction in the late 1980's, *T. heterouncinata* was able to quickly spread throughout the state's farmed abalone industry. This rapid spread was due, in part, to the interdependency within the industry on seed transfers, in conjunction with the early misidentification of the sabellid as a native polychaete. Following the discovery of the sabellid as an exotic species, the California Department of Fish and Game (CDF&G) recognized the organism as a threat to the state's natural resources and aquaculture industry. In an attempt to control the further spread of the polychaete, the CDF&G, consulting with the industry-initiated Abalone Sabellid Worm Advisory Committee, developed a policy that all abalone transfers by farms be inspected and deemed to be sabellid-free (Aquaculture Disease Committee, July 6, 1995; Thoesen 1994). This sampling regime was selected, in part, because a minimum population density of sabellids was believed to be required to establish a viable population. Our research demonstrates that low-level infestations (or even a single founding

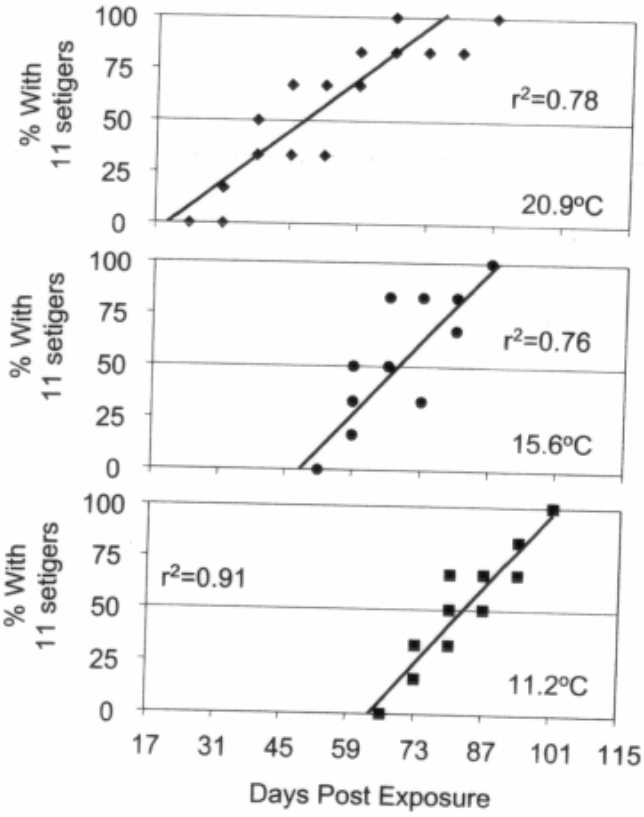

Figure 5. Percentage of sabellids that had developed a full complement of setigers (n = 11) in pooled replicates at each temperatures investigated. Regression lines indicate that 47.7, 68.4 and 83.1 days post-exposure, 50% of the sabellids had developed 11 setigers at 20.9°C, 15.6°C, and 11.2°C, respectively.

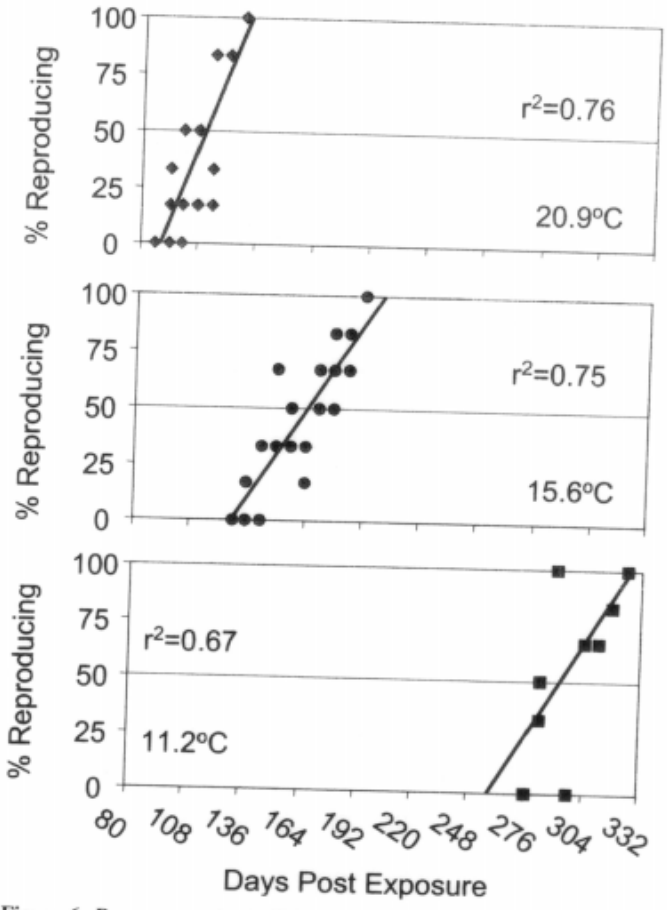

Figure 6. Percentage of sabellids that had produced motile, infestive larvae or newly settled larvae in pooled replicates at each temperature investigated. Regression lines indicate that after 110.8, 165.0 and 297.8 days, 50% of the sabellids had completed their life history at 20.9°C, 15.6°C, and 11.2°C, respectively.

sabellid) could pose a significant threat not only to the aquaculturist, but to the marine intertidal ecosystem.

Many polychaetes demonstrate positive relationships between environmental temperature, within the physiological ranges, and the rate at which they reach maturity (Cha et al. 1997, Olive et al. 1997, Qiu & Qian 1997, Qiu & Qian 1998). *Terebrasabella heterouncinata* appears to follow this trend. Previous research has reported that reproductive maturity in *T. heterouncinata* can occur in approximately one month (Kuris & Culver, 1999) or in three months (Ruck & Cook 1998) when animals were held at undefined ambient temperatures. Our controlled laboratory studies found that the most rapid development occurred at the highest of the three temperatures investigated (20.9°C), where average development of a full complement of setigers (n = 11) took 47.7 days. This decrease in time to maturity in association with elevated seawater temperatures would be expected to result in increased infestation levels, an observation that agrees with what has been experienced by California growers (B. Beede pers. comm.). Higher infestation levels are typically observed in the warmer waters of the southern part of the state, particularly in association with El Niño events.

Previous studies on polychaetes have demonstrated that at lower temperatures (again within physiological ranges) development would be prolonged, but that one would expect to observe a significant number of embryos and larvae developing into mature adults (Qiu & Qian 1997). Our research indicates that the development of all 11 setigers and the age at maturity is indeed prolonged at lower temperatures and would be expected to result in lower infestation levels. *Terebrasabella heterouncinata* does, however, appear to be capable of completing its life history at the lower temperatures encountered in the northern part of the state where annual averages range from 8–13°C (Mcbride 1998). This portion of the state should not be viewed as a thermal refuge from *T. heterouncinata*, although its life history will take longer to complete and less obvious, low level infestations, may result.

ACKNOWLEDGMENTS

We appreciate the editorial comments of James D. Moore. This work was supported, in part, by the Saltonstall-Kennedy Programs of the National Oceanic and Atmospheric Administration, U.S. Department of Commerce and the National Sea Grant College under grant numbers NA96FD0206 and NA66RG0477, Project No. R/A-32PD, respectively. Additional support was provided by the California State Resources Agency, California Department of Fish and Game and the University of California, Davis. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its sub agencies. The U.S. Government is authorized to reproduce and distribute this work for governmental purposes.

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Journal of Shellfish Research, Vol. 20, No. 2, 867–874, 2001.

TRANSMISSION OF THE RICKETTSIALES-LIKE PROKARYOTE "*CANDIDATUS XENOHALIOTIS CALIFORNIENSIS*" AND ITS ROLE IN WITHERING SYNDROME OF CALIFORNIA ABALONE, *HALIOTIS* SPP.

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ABSTRACT Withering syndrome (WS) is a chronic, wasting disease responsible for mass mortality in southern California populations of black abalone *Haliotis cracherodii* and responsible for significant losses of cultured red abalone *H. rufescens*. Ongoing studies in our laboratory indicate that a recently described gastrointestinal Rickettsiales-like prokaryote, "*Candidatus Xenohaliotis californiensis*" (RLP) is the etiologic agent of WS. Here we describe attempts to experimentally transmit the RLP and demonstrate its role in WS. In two preliminary experiments, RLP-infected black abalone postesophagus homogenate (IPEH) was injected into the foot or orally administered to RLP-free black abalone. No RLPs were detected 8 wk after pedal injection. Low rates of transmission were observed 8 to 12 wk after oral inoculation, although an RLP-positive animal was also detected in a negative control group inoculated with filtered seawater. In a separate, 16-wk study, RLP infections were detected in red abalone that received effluent from a tank of infected red abalone while control animals that received direct-source seawater remained RLP-free. A fourth, long-term study delivered IPEH or seawater to RLP-free red abalone with either bath exposure or intra-digestive gland injection. An additional treatment to test a potential viral etiology for WS consisted of intra-digestive gland injection with a 0.1–0.2 μm filtrate of IPEH and subsequent treatment with antibiotics. Each treatment was administered six times over a 16-wk period. Cohabiting RLP-infected black abalone with RLP-free red abalone provided a positive control treatment. At wk 63 post-initiation, untreated, saline injected and IPEH filtrate injected groups had low cumulative mortality (0–20%), while mortality in the IPEH bath, IPEH injection and cohabitation treatment groups was 70–90%. There were statistically significant relationships between experimental treatment, RLP burdens and signs of WS. Low-level RLP infections of uncertain origin were observed in one each of the duplicate tanks of the negative control and the IPEH filtrate-injected animals. An absence of WS signs in recipients of the IPEH filtrate provides strong evidence that the agent of WS is non-viral. Collectively these studies provide solid evidence that the RLP is the etiologic agent of WS.

KEY WORDS: *Haliotis rufescens, cracherodii,* Rickettsiales, withering syndrome, transmission, abalone

INTRODUCTION

The black abalone *Haliotis cracherodii* is one of several haliotid species found on the Channel Islands and mainland of southern and central California, USA. This species lives in the intertidal zone and is considered less desirable for human consumption than several subtidal species, but became an important component of the commercial fishery in the 1970s when stocks of subtidal species declined, presumably due to increasing recreational and commercial fishing pressures (Davis et al. 1992). Resource managers and abalone fishermen first noticed large numbers of dead and dying black abalone on the Channel Islands in the mid-1980s (Haaker et al. 1992). The term Withering Syndrome was coined to describe the affected abalone, characterized by lethargy and a greatly reduced pedal mass (Haaker et al. 1992). Southern California black abalone populations have essentially collapsed, with mortality greater than 90% in many areas (Haaker et al. 1992, Richards & Davis 1993). Stocks of subtidal red (*H. rufescens*) and pink (*H. corrugata*) abalone also declined during the 1980s (Davis et al. 1992) but the role of fishing pressure, climatic events and WS in those declines remains unclear.

Although the appearance and severity of WS outbreaks appeared to be enhanced by warm water temperatures (Steinbeck et al. 1992, Tissot 1995), the epizootiology of the disease indicated involvement of an infectious agent (Lafferty & Kuris 1993). WS spread throughout the Channel Islands followed by movement to the California coastline, where it advanced northward during the early 1990s (Lafferty & Kuris 1993, Alstatt et al. 1996). An initial investigation of nine black abalone with WS and five without signs of the disease reported the presence of several parasites and a gastrointestinal Rickettsiales-like prokaryote (RLP), although none occurred in all affected abalone while being absent in healthy-appearing abalone (VanBlaricom et al. 1993). The RLP formed large intracellular inclusions in gut epithelium, causing hypertrophy but little other cytopathology. RLPs are common endosymbionts in molluscs, often being unassociated with disease (Sparks 1985, Elston 1986). Gardner et al. (1995) examined black abalone from both where WS occurred and where it did not. They found that the RLP was present only in abalone from the WS-endemic site, and noted pathological changes in the digestive gland that could be due to the RLP. Subsequently, Friedman et al. (1997) examined starvation, temperature, the renal coccidian *Pseudoklossia haliotis* and the gastrointestinal RLP as causes of WS. While eliminating the coccidian, starvation and elevated temperature as being directly responsible for WS, complex relationships between the RLP and temperature warranted further investigation. Friedman et al. (this volume) transmitted WS and the RLP between black abalone. Groups of initially RLP-free black abalone that contracted the RLP by cohabitation with RLP-positive animals had a higher proportion of animals with signs of WS and higher proportion that died than did unexposed RLP-free control animals. However, the authors also observed a lack of significant correlations between WS signs and RLP infection intensity, further indicating a complex relationship in black abalone.

The RLP is not restricted in host species to the black abalone.

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The RLP is present in most or all abalone aquaculture facilities in California, which raise red abalone to a market size of approximately 9 cm in land and cage-based operations throughout the state (McBride 1998). This is due to its increasing range in wild populations and the widespread distribution of infected seedstock prior to it being recognized as a potentially significant pathogen (C. S. Friedman, unpublished observations). During the 1997–1998 El Nino, red abalone aquaculturists in the southern and central portions of the state began observing sharply elevated frequencies of animals showing WS signs in association with elevated water temperatures. Recently, Moore et al. (2000) demonstrated that farmed red abalone with RLPs can show little or no signs of WS at lower temperatures (14.7°C), but elevation of temperature to 18.5°C caused elevated mortality, expression of WS signs and increased RLP burdens. With increasing evidence for its role in WS, Friedman et al. (2000) recently provided a description of the RLP, designated "*Candidatus Xenohaliotis californiensis*." The term "*Candidatus*" in the taxon indicates that the species was described largely on morphological and DNA sequence-based data and still requires the serological and biochemical analyses that would be performed if the species could be cultured *in vitro*.

The coinciding geographic boundaries and correlation between WS and the RLP do not alone demonstrate that this agent causes the disease. An alternative hypothesis suggests that the RLP proliferates in host animals debilitated by WS, itself caused by an unidentified, possibly viral agent. Our current studies, while limited by the difficulty of obtaining and maintaining RLP-free abalone, were designed to experimentally transmit the RLP and examine links between severity of RLP infection and expression of WS signs.

MATERIALS AND METHODS

The experimental designs of the four consecutive experiments conducted are shown in Figure 1. Experiments 1–3 were designed to investigate transmission of the RLP. Animals were therefore sacrificed at time periods of several weeks to several months, generally before expression of WS occurred. Experiment 4 was designed to allow sufficient time for development of WS and was terminated following extensive mortality in treatment groups 15 mo post-initiation.

Animals and Animal Husbandry

All experiments were conducted in the Pathogen Containment Facility at the Bodega Marine Laboratory, from which all effluent is treated with 10 mg/L chlorine for two hours and dechlorinated with sulfur dioxide before release. Lidded, 8-L tanks with outflows situated so that each tank held 5 L were used in Experiments 1–3. In Experiment 4, lidded 58-L tanks with standpipes situated to hold 50 L were used. All tanks received sand-filtered, aerated seawater and were supplied with kelp (*Macrocystis pyrifera*) several times per month. Care was taken to prevent cross contamination between tanks, including submersing gloved hands, measuring tools etc. in a tamed iodine solution (Prepodyne) and spraying surfaces with 70% ethanol as necessary. Animals were tagged by inserting either a numbered stainless steel washer on stainless steel wire or a numbered vinyl sleeve (Floy Tag, Seattle) on a cable tie through the first and second most recently formed respiratory pores. Benzocaine (Sigma, 40 mg/L, 15 min baths) was used as an anesthetic to dislodge animals for transfer or treatment.

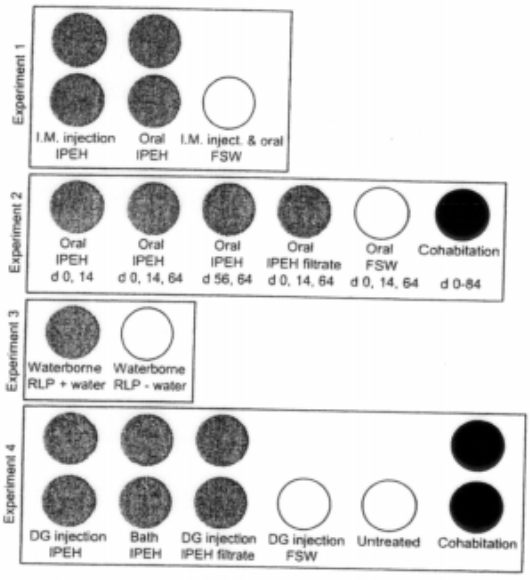

Figure 1. Schematic diagrams of the experimental units in Experiments 1 through 4. Circles represent the numbers of tanks in each treatment group, with experimental tanks shaded, negative control tanks white and positive control tanks black. The first line of text indicates the method of delivery of the material shown in the second line of text. For Experiment 2 the third line of text shows the days on which animals were treated; the first group was sacrificed at day 28 and the remainder on day 84. IPEH: RLP-infected black abalone postesophagus homogenate; FSW: Filtered seawater; DG: Digestive gland.

Histology: Quantification of RLP Infection Intensity and WS Signs

Animals that died during each experiment and all survivors at experiment termination were processed for histological detection of RLP burden. Animals in Experiment 4 were also assessed for WS-associated pathomorphological changes in the digestive gland and foot. Tissue sampling and histological processing was performed as previously described (Moore et al. 2000). Davidson's-fixed (Shaw & Battle 1957), hematoxylin and eosin-stained 5 μm paraffin tissue sections containing postesophagus, digestive gland, foot muscle, kidney and gonad were prepared for each animal, and slides were encoded to prevent bias during assessment. RLP infection intensity was estimated for the postesophagus and digestive gland (the two tissues in which RLPs can be found at high densities, Friedman et al. 2000). RLP burdens were quantified in each tissue using the scale of Friedman et al. (1997) based on the average number of RLP inclusions per 200× magnification field of view: absent = (0), 1–10 = (1), 11–100 = (2), or greater than 100 = (3). Four different disease signs of withering syndrome were assessed using integral scales from 0–3, modified from Moore et al. (2000). For all four parameters, (0) represented a normal healthy appearance. For body shrinkage, (1), (2) and (3) represented slight, moderate and severe shrinkage respectively; for digestive gland metaplasia or digestive gland atrophy, (1), (2) and (3) denote 5%–10%, 11%–25%, and greater than 25% of the digestive gland being comprised of transport duct epithelia or connective tissue, respectively. Foot degeneration scores of (1), (2) and (3) denote muscle fibers comprising 76%–90%, 51%–75% and less than 50% of the foot muscle, respectively. A condition index, CI = total weight, g/(shell length, cm)^3, was also used to assess body shrinkage. The presence of the renal coccidian *Pseudoklossia haliotis* (Friedman et al. 1995) was also noted.

Preparation of Infected Postesophagus Homogenate (IPEH)

Experiments 1, 2 and 4 attempted transmission of the WS-RLP using several methods of delivering homogenized, RLP-infected postesophagus tissue excised from adult black abalone (IPEH). Black abalone donor animals were collected from Vandenberg Air Force Base, California, where WS has been endemic since approximately January 1994 and RLP prevalence was approximately 90% during the collection period (C. S. Friedman & J. D. Moore, unpublished observations). Animals were collected several times per year during 1996–1998, held in 9–13°C seawater and fed kelp *ab libitum*. Postesophageal tissue (including the "crop" as described by Bevelender, 1988) from three to eight animals was pooled and minced in 0.2 μm filtered seawater (FSW) on ice. The tissue was homogenized with a 7-ml Tenbroeck homogenizer. The tissue homogenate contained large pieces of connective tissue and other debris which were removed by gentle centrifugation (100-250 × *g* for 2–5 min). The clarified homogenate was transferred to a new tube and diluted with FSW as necessary to obtain required volumes as described below.

Experiment 1: RLP Transmission by Intramuscular Injection or Oral Inoculation

In July 1997, black abalone were collected from Sobranes Point (Carmel), California, a location where WS had not been observed. One month after collection, the animals were randomly distributed to 5 tanks (4–5 abalone per tank) and acclimated from 14°C to 19°C over 1 wk. The treatment groups are shown in Figure 1. The IPEH consisted of 1.745 g of postesophagus from four black abalone diluted to 15 ml. The IPEH was administered at a rate of 6.8 μl IPEH/ g whole animal weight to four animals in each of two duplicate tanks by either intramuscular injection in the foot with a 23-gauge needle or oral delivery using a 23 gauge animal feeding tube inserted into the mouth. A single tank (n = 5 animals) served as a negative control; animals were treated with both oral delivery and intramuscular injection of FSW. All animals were sacrificed on day 60 post-initiation and examined for the presence of RLPs by histology.

Experiment 2: RLP Transmission by Oral Inoculation

In August 1997, black abalone were collected from Carmel Point (Carmel), California, where WS had not been observed. In March 1998 the abalone were randomly distributed into six tanks (n = 6 animals/tank). Temperatures during the experiment ranged from 11.5°C–15°C until day 40, after which temperatures were elevated to 17°C–20°C, with an overall mean ± s.d. of 15.6°C ± 2.8°C. Treatments (Fig. 1) consisted of multiple oral inoculations with IPEH or a 0.2 μm filtrate of IPEH at a rate of 3.0 μl/g whole animal weight on days 0, 14 and 64 post-initiation. The IPEH on each day of treatment consisted of 5.2–7.5 g of postesophagus from three or four black abalone brought to a volume of 15 ml with FSW. To prepare IPEH filtrate, IPEH was centrifuged (400 × *g*, 5 min) and then filtered twice through 0.2 μm syringe filters. As a positive control, three black abalone from Vandenberg Air Force Base were added to one tank of the Carmel Point abalone and maintained until termination of the experiment. On day 56, after discovering negative results in the IPEH treated group that was sacrificed at day 28, a previously untreated group was inoculated with a more concentrated IPEH (2.8 g postesophagus in 5 ml FSW without centrifugation). One IPEH treated group was sacrificed at day 28 and all other groups were sacrificed on day 84 post-initiation and examined for the presence of RLPs by histology.

Experiment 3: Waterborne RLP transmission

In March 1998, adult red abalone were collected by scuba from Caspar Cove and Mill Cove, Mendocino County, California where WS had not been observed. In August 1998, 14 animals were divided equally into two groups and placed in 8-L tanks. Each of the two tanks was connected to its own intermediate 8-L tank by approximately 25 cm of tubing. The inflow to the intermediate tank of one treatment was connected to an 18°C water source (= control). The inflow to the other intermediate tank was connected to the outflow of a 30-L tank that contained approximately 60 RLP-positive red abalone 2–10 cm in length and was supplied with the same seawater source. The animals were sacrificed 111 days post-initiation and examined histologically for the presence of RLPs.

Experiment 4: WS Development Following RLP Transmission by Digestive Gland Injection or Bath Exposure

Experiment 4 utilized animals from the same collection as Experiment 3. In July 1998 one hundred animals were tagged, equally distributed among ten tanks and allowed to acclimate for 29 days, during which period the water temperature was raised from 15°C to 18°C. Animals were treated on days 0, 21, 35, 56, 91 and 109 of the study with the treatments shown in Figure 1. On each treatment date, IPEH was prepared from 6.5–7.9 g of postesophagus tissue excised from six to eight black abalone and brought to a final volume of 160 mL with FSW (FSW/3% heat-inactivated fetal bovine serum on day 0). The IPEH filtrate was prepared by successive filtration of IPEH through 8 μm, 0.8 μm, 0.2 μm and 0.1 μm filters, except on day 0, when 0.1 μm filters were unavailable and the 0.8 μm filtrate was put through 0.2 μm filters twice. For all digestive gland injection treatments (FSW, IPEH, IPEH filtrate), animals were injected with 3.6 μL/g whole animal weight in the posterior ventral portion of the digestive gland using a 23 gauge needle. Recipient animals ranged in weight from 221 g to 1,360 g resulting in injection volumes ranging from 0.8 ml to 4.9 ml. For the bath treatment, tanks were drained and brought to a volume of 30 L, to which 25 mL of IPEH was added. Tanks were then maintained statically with aeration for two hours, after which flowing seawater was returned. The cohabitation positive control replicates were each supplied on day 0 with three Vandenberg black abalone showing variable degrees of body shrinkage. Black abalone that died were replaced until day 104, at which time all black abalone were removed.

After the discovery that an animal in the IPEH filtrate treatment that died on day 91 was infected with the RLP, a decision was made to inject these animals with antibiotics. This was based on the design of this treatment to allow a potential viral pathogen to infect recipients in the absence of the RLP. These abalone were injected in the foot with oxytetracycline (10 mg/ml in 2% saline) at a rate of 21 mg/kg tissue weight. Injections were made every 48 h for a total of three injections, followed by two to three weeks without injections and repeating the series two more times for a total of nine injections over 47 days.

Five feeding trials were conducted to measure the amount of kelp consumed by abalone in each treatment. An amount of kelp equal to 15% of the total body weight in each tank was added with the amount remaining after 24 h being reclaimed and weighed.

The experiment was terminated on day 446 post-initiation when mortality in the bath treatment approached levels seen in the cohabitation and IPEH injected groups. Statistical analysis of the results from Experiment 4 included a Chi Square contingency table analysis comparing IPEH injected, IPEH bath, cohabitation and IPEH filtrate-injected treatment groups to the negative control group ($\alpha = 0.05$). Data for WS signs (body shrinkage, digestive gland metaplasia, digestive gland atrophy or foot degeneration) were condensed into low (score of 0–1) or high (score of 2–3) categories of severity and 2 × 2 contingency tables compared observed vs. expected frequencies in each treatment group and the negative control group. Similarly, frequencies of low vs. high levels of each WS sign were compared in animals with low-level RLP infections (RLP intensity score 0–1) vs. high level infections (RLP intensity score 2–3) for the postesophagus and digestive gland separately. When the frequency in any cell was less than five observations a Fisher Exact test was employed.

RESULTS

Experiments 1 and 2: RLP Transmission by Intramuscular Injection or Oral Inoculation

In Experiment 1, 60 days after a single injection or oral inoculation with infected black abalone postesophagus homogenate (IPEH) or filtered seawater, one individual that received IPEH orally contained a single focus of two RLP inclusions in the postesophagus (RLP intensity = 1). In Experiment 2, no RLPs were seen among the six animals sacrificed at day 28. Upon termination at day 84, RLPs were detected in postesophagi of two of the six individuals orally inoculated with non-clarified IPEH on days 56 and 64, and in one individual in the FSW-injected negative control group. The RLP inclusions in these animals were small and very infrequent (RLP intensity = 1). Five of the six cohabitation positive control animals had low RLP intensity scores (RLP intensity = 1 in postesophagus, digestive gland, or both) and in one animal no RLPs were detected.

Experiment 3: Waterborne RLP Transmission

Histological analysis of survivors in each tank at day 111 revealed the RLP to be present in 100% (6/6) of the animals receiving effluent from the RLP-positive tank, while those receiving direct-source seawater had no RLP infections (0/6). One animal in each tank died prior to termination and in each case tissues were too necrotic for histological analysis. Intensities of postesophagus and digestive gland infections in the six RLP-positive animals ranged from relatively mild (1) to severe (3).

Experiment 4: WS Development Following RLP Transmission by Digestive Gland Injection and Bath Exposure

No significant difference in condition index was observed between tanks at day 0 (ANOVA, $p = 0.29$) and only one individual (in one of the replicates to receive IPEH by bath treatment) showed slight body shrinkage. Mortality in the group injected in the digestive gland with IPEH began during wk 27 and reached 90% at termination (Fig. 2). Mortality began during wk 33 in the tanks to which infected black abalone were added (positive control) and cumulative mortality also reached 90%. Mortality in the IPEH bath treatment group began during week 35 and steadily increased to 70% by termination of the study. Nearly all of the animals that died in the positive control, IPEH injected and IPEH bath treated groups showed signs of WS and the presence of RLPs (see below). One animal in the control, FSW-injected group was sacrificed on day 44 post-initiation due to morbidity related to shell damage. Microscopic examination of stained tissue sections indicated an absence of RLPs in this animal. One animal in the IPEH filtrate-injected group was sacrificed on day 91 post-initiation due to the presence of an extensive shell fungal lesion (not seen in any other animals throughout the experiment). Histological examination indicated the presence of several small foci of RLP inclusions in the postesophagus. An antibiotic treatment regimen was initiated in this treatment group to eliminate any possible infections in the remaining animals. This allowed for continuity of the treatment group as a test for whether the pathogen causing WS could be a viral agent. Several wk before termination of the experiment two animals died in the negative control treatment group and histological examination revealed an absence of RLPs.

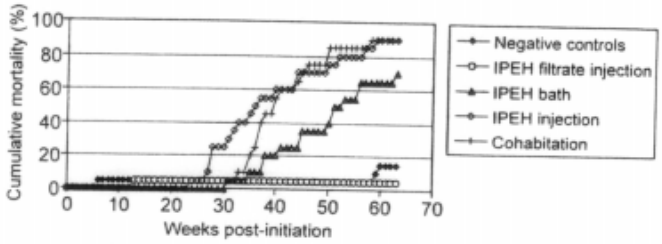

Figure 2. Cumulative mortality in Experiment 4. Treatment groups consist of duplicate tanks containing ten abalone each; the negative controls group consists of one tank of untreated abalone and one in which digestive glands were injected with filtered seawater. IPEH: RLP-infected black abalone postesophagus homogenate.

Nearly all of the animals that died had moderate to severe signs of WS. Figure 3 illustrates that mean values for body shrinkage, digestive gland metaplasia, digestive gland atrophy and foot degeneration were all higher for animals that died than those that survived. Coinciding with these elevated signs of WS, animals that died had mean RLP burdens more than four times those of survivors (RLP intensity = 2.3 ± 1.0 vs. 0.50 ± 0.8 in postesophagi, 1.8 ± 1.0 vs. 0.30 ± 0.6 in digestive glands for animals that died vs. survivors respectively, mean ± s.d.).

Histological examination of survivors of the experiment revealed that eight of the nine animals in the untreated negative control tank contained RLPs, including seven with mild infections (RLP intensity = 1 in postesophagus, digestive gland, or both) and one with a moderate infection (RLP intensity = 2 in both the postesophagus and the digestive gland). One of the tanks of IPEH filtrate-injected animals contained an individual with a light RLP infection of the postesophagus. Table 1 indicates that, despite these

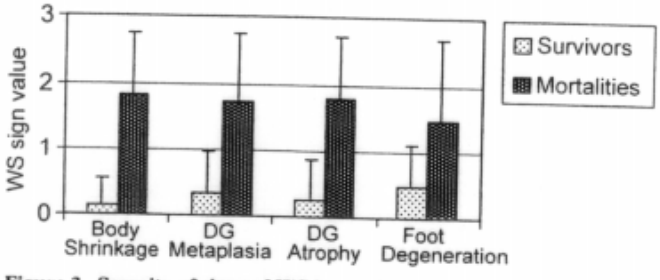

Figure 3. Severity of signs of WS in survivors and animals that died in Experiment 4. The range for each sign was from 0 (normal, healthy) to 3 (severe) (see Methods). DG: Digestive gland. Mean + s.d.

TABLE 1.

Experiment 4, mean values for RLP infection intensity and WS signs in duplicate tanks of each treatment.[1]

Treatment	Postesophagus RLP Intensity	Digestive Gland RLP Intensity	Digestive Gland Metaplasia	Digestive Gland Atrophy	Foot Degeneration	Body Shrinkage	Condition Index
Negative controls[2]	0.9, 0	0.6, 0	0.1, 0.5	0.5, 0.5	0.3, 0.9	0.3, 0.3	0.155, 0.173
IPEH filtrate injection	0, 0.2	0, 0	0.1, 0.1	0, 0.1	0.1, 0.4	0, 0.2	0.148, 0.159
IPEH bath	2.1, 1.6	1.4, 1.7	1.1, 1.8	1.3, 1.4	0.6, 1.3	1.0, 1.3	0.130, 0.131
IPEH injection	2.2, 3.0	1.5, 2.2	1.7, 2.2	1.6, 1.9	1.5, 2.2	1.9, 2.3	0.108, 0.115
Cohabitation	2.2, 2.4	1.8, 1.9	1.6, 1.6	1.7, 1.8	1.2, 1.6	1.2, 2.1	0.104, 0.122

[1] Definitions and scales are described in Materials and Methods section.
[2] First replicate = animals injected with filtered seawater, second replicate = untreated.

infections, the negative control and IPEH filtrate-injected groups had dramatically lower RLP infection intensities and signs of WS than the IPEH bath, IPEH injected and positive control groups. The mean values for RLP burden and all WS signs were highest in the cohabitation positive control group and IPEH injected group, and lower in the IPEH bath treatment group (Table 1). Table 2 shows the results of contingency table analyses comparing RLP burdens and severity of WS signs in the treatment and positive control groups to the negative controls. The IPEH injected, IPEH bath and cohabitation exposures resulted in significantly increased RLP infection intensity and severity of WS signs while the IPEH filtrate injected animals were not different from the controls.

The relationship between WS signs and RLP burdens was addressed by comparing the severity of WS signs in animals with different RLP infection intensities. Figure 4 illustrates that animals with higher RLP burdens had more severe signs of WS. This association was statistically examined by contingency table analyses which compared the frequencies of low vs. high RLP burdens in animals having low vs. high levels of WS signs. Body shrinkage, digestive gland metaplasia, digestive gland atrophy and foot degeneration all showed highly significant relationships with postesophagus and digestive gland RLP infection intensities ($p < 0.001$ for all comparisons).

The amount of food eaten on a per-weight basis by the animals in each tank was measured at five time points during the experiment. By wk 30 post-initiation, the amount of kelp eaten by the cohabitation and IPEH injection groups was lower than that of the other groups (Fig. 5). These low rates remained fairly consistent over time as severely affected animals died and others developed more severe signs of WS. Feeding rates in the IPEH bath treatment group declined prior to onset of mortality with high RLP burdens.

The renal coccidian, *Pseudoklossia haliotis* was present in only five animals with no relation to treatment across all treatment groups.

DISCUSSION

Withering syndrome appears to be directly caused by a gastrointestinal Rickettsiales-like prokaryote that has been designated "*Candidatus Xenohaliotis californiensis*" (Friedman et al. 2000). These studies demonstrate that the organism can be transmitted by experimental methods and that successful transmission appears requisite for development of WS in previously healthy red abalone.

Despite rigorous preventative efforts, we experienced apparent contamination of tanks with the RLP or the inadvertent use of infected recipient animals, as evidenced by discovery of the RLP in negative control animals in Experiments 2 and 4. In Experiment 2, the single positive animal in the FSW-administered negative control group may have resulted from infection prior to collection from the wild population; 15 months after collection of the animals in this experiment the RLP was found in a high percentage of animals at a nearby site (C. S. Friedman & C. A. Finley, unpublished observations). The two animals inoculated with IPEH that were found to be RLP-positive could have acquired infections by that treatment or via the method of the contaminated control animal. In Experiment 4, infections in the untreated negative control tank apparently developed very late in the study and were likely due to recent contamination from adjacent tanks, equipment, water

TABLE 2.

Experiment 4, significance values for Chi Square or Fisher Exact tests using dichotomous categories for each parameter (scores of 0 or 1 vs. 2 or 3) and comparing treatment groups shown to negative control groups.[1,2]

Treatment	Postesophagus RLP Intensity	Digestive Gland RLP Intensity	Survival[3]	Digestive Gland Metaplasia	Digestive Gland Atrophy	Foot Degeneration	Body Shrinkage
IPEH filtrate injection	1.0	1.0	0.605	[4]	0.231	0.487	0.487
IPEH bath	<0.001*[5]	0.005*	0.003*	<0.001*	0.251	0.052	0.117
IPEH injection	<0.001*	<0.001*	<0.001*	<0.001*	0.009*	0.003*	<0.001*
Cohabitation	<0.001*	<0.001*	<0.001*	<0.001*	0.022*	0.018*	0.003*

[1] The Chi square analysis defaulted to Fisher exact test if the number of observations in any cell was less than five.
[2] Definitions and scales are described in Materials and Methods section.
[3] Survivors vs. animals that died during the experiment.
[4] All values for both treated and control groups fell into the category of low severity.
[5] Asterisks indicate significant p values ($p < 0.05$).

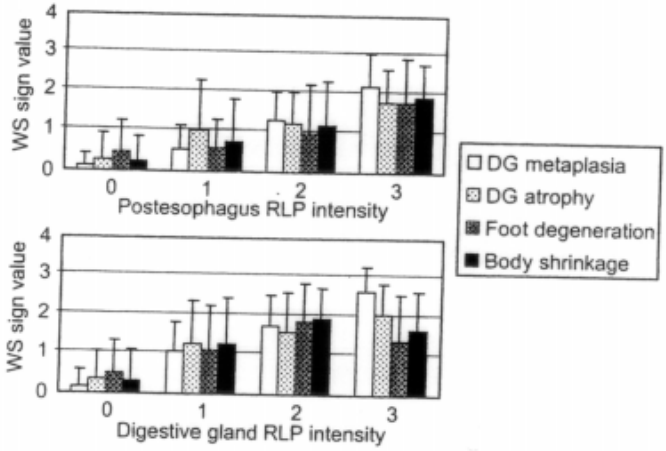

Figure 4. Severity of various WS signs for animals in Experiment 4 grouped by intensity of postesophagus or digestive gland RLP infection. The range for each sign was from zero (normal, healthy) to 3 (severe) (see Methods). DG: Digestive gland. Mean + s.d.

source or food supply. The water source (BML) and site of kelp collection (Bodega Bay) are believed to have been RLP-free throughout the study period. The two animals in one tank of the IPEH filtrate-injected treatment of Experiment 4 could have obtained infections via the routes noted above or by passage of the RLP through the filters used to create the filtrate. As commonly reported for RLPs of molluscs, "*Candidatus Xenohaliotis californiensis*" is pleomorphic with spherical to rod-shaped forms. The smallest diameter of the rod-shaped form measured 145 nm (Friedman et al. in press) and therefore the RLP may have passed through the 0.2 μm filters used on day 0.

Evidence for the RLP being the Etiologic Agent of WS

Our experiments clearly show that the agent of WS was present in RLP-infected black abalone postesophagus tissue homogenate (IPEH). Although contamination of negative control tanks prevents concluding with certainty, the dynamics of infection and mortality in the bath and injection treatments of Experiment 4 indicate that the RLP infections and WS signs were due to our experimental transmission. The onset of mortality in the IPEH-injected group before the cohabitation group suggests that this experimental treatment was more effective in transmitting the WS-associated pathogen than natural exposure. Furthermore, the lack of expression of WS in animals receiving injections of the IPEH filtrate, in contrast to those receiving unfiltered IPEH, indicate that the etiologic agent of WS is probably not a virus. These observations, considered with the relationships between RLP intensity and severity of WS signs, provide strong evidence linking the RLP directly to WS.

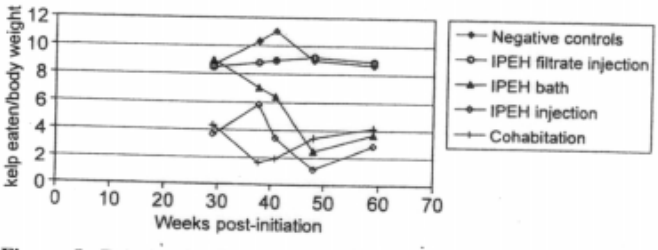

Figure 5. Relative feeding rates of Experiment 4 treatment groups measured at five timepoints. Each point is the average of values for two tanks. Abscissa scale is equal to that of Figure 1. IPEH: RLP-infected black abalone postesophagus homogenate.

WS Signs

Body shrinkage, foot muscle atrophy and to some extent digestive gland atrophy are generic signs of starvation and not specific to WS. Shrunken abalone with soft flesh have been reported in association with apparent poor food supply or environmental conditions (Young 1964, MacGinitie & MacGinitie 1966) and diseases other than WS (Nakatsugawa et al. 1999). In contrast, digestive gland metaplasia, in which functional tissue including secretory cells is replaced with cells similar in appearance to those of transport ducts, appears to be pathognomonic for WS. One fascinating aspect of this alteration is that the RLP infects duct-like tissue of the digestive gland and not terminal tubules; thus, infection with the RLP results in morphological changes which provide more tissue of the type which the pathogen can infect. Moore et al. (2000) recently reported that WS-positive animals ate less than animals without overt signs of the disease. Based on those findings we initiated feeding studies during wk 29 of Experiment 4, and the five trials suggest that a sharp drop in feeding rate precedes death in animals with signs of WS. These observations suggest that although the RLP results in pathologic changes to gastrointestinal epithelia, malabsorption resulting from these changes is not solely responsible for body shrinkage, and decrease in the ability or desire to consume food also plays a role. Diminished feeding rate may be one of the earliest indicators of adverse health in animals that acquire withering syndrome.

RLP has Low Host Species Specificity

Transmission of the RLP by cohabitation of black abalone donors and recipients was reported by Friedman et al. (this volume). Our Experiment 2 had similar results and Experiment 4 showed for the first time that the agent can be transmitted by cohabitation of black abalone donors and red abalone recipients, while Experiment 3 demonstrated transmission between red abalone. These findings agree with a wealth of morphological (Friedman et al. 1997, Moore et al. 2000), ultrastructural (Friedman et al. 2000, C. S. Friedman & J. D. Moore, unpublished observations) and DNA-based studies (Andree et al. 2000, Antonio et al. 2000) indicating that the RLP found in red and black abalone does not differ between species. RLPs identical in appearance and tissue location by histology have also been observed in California green (*H. fulgens*) and pink (*H. corrugata*) abalone (C. S. Friedman, unpublished observations).

Modes of RLP Transmission

Intramuscular injection and oral inoculation were investigated as methods of transmitting the RLP in Experiments 1 and 2. Lack of detection of the RLP following intramuscular injection agrees with observations that infected tissue appears to be completely restricted to specific cell types in the epithelium lining the digestive tract. However, Gulka and Chang (1984) reported successful transmission of a gill epithelium-specific RLP in the scallop *Placopecten magellanicus* by intramuscular injection of the adductor muscle with RLP-infected gill homogenate. The limited success of oral inoculation in our experiments may have been affected by their short duration (60 and 84 days for Experiments 1 and 2 respectively), since histological detection of the RLP was based on examination of a single 5 μm tissue slice for each potentially

affected organ. Prior to this study, transmission of the RLP had only been achieved by cohabitation. Abalone are gregarious animals, and during cohabitation frequently come in direct physical contact with each other. The results from Experiment 3 suggest that transmission of the RLP can survive in seawater and does not require close contact for transmission.

Many if not all Rickettsiales-like prokaryotes of marine invertebrates and fish are capable of direct horizontal transmission from host to host. This is in contrast to most Rickettsiales-like organisms with mammalian primary hosts, which require a parasitic arthropod host for dispersion (Marchette & Stiller 1982). The requirement for parasitic arthropods (lice, ticks, fleas) for transmission of mammalian RLPs is related to their lack of a sporogonic stage and incapability of surviving desiccation. Ingestion of RLP-contaminated food is likely the predominant mode of RLP transmission in the marine environment. Transmission of *Piscirickettsia salmonis* has been reported for coho salmon held in aquaria with infected conspecifics for one month (Cvitanich et al. 1991). Horizontal transmission of RLPs by feeding infected tissue of conspecifics has been reported for 'Stained prawn disease' of *Pandalus platyceros* (Bower et al.1996) and for transmission of an RLP from the red shrimp *Penaeus marginatus* to the blue shrimp *P. stylirostris* (Brock et al. 1986). Ingestion of contaminated food as a mode of pathogen entry and fecal/oral spread as a mode of dispersal are likely operative for the RLP associated with withering syndrome in abalone.

We found that injecting the digestive gland with infective material was an efficient mode of transmission of the RLP. Sites of injection healed rapidly with no associated morbidity or mortality. The digestive gland of abalone is a large organ containing transport ducts that are host tissue for the RLP. These open into the stomach, which receives food from the postesophagus, while the distal portions lead to terminal tubules where nutrient absorption and enzyme secretion occur (Bevelender 1988, Campbell 1965, Voltzow 1994). Among IPEH-injected animals, 16 out of 18 animals for which histological sections of both organs were available had RLPs present in both the digestive gland and postesophagus, indicating that the infections were able to spread from the former to the latter tissue. In the remaining two animals the RLP was detected in postesophagus and not digestive gland tissue, possibly due to the pathogen spreading to the postesophagus before spreading completely throughout the digestive gland. Thus, our studies provide further evidence that '*Candidatus Xenohaliotis californiensis*' is the etiologic agent of WS, a disease capable of affecting multiple haliotid species.

ACKNOWLEDGMENTS

This work was supported by the Saltonstall-Kennedy Program of the National Oceanic and Atmospheric Administration (NOAA), U. S. Department of Commerce, grant NA76FD0046. Additional support was provided by the California State Resources Agency, Department of Fish and Game. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its sub agencies. The U. S. Government is authorized to reproduce and distribute this work for governmental purposes. We would like to thank Pete Haaker, Pete Kalvass, Kon Karpov and Ian Taniguchi of the California Department of Fish and Game for assisting in collection of abalone.

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