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Pathogenic effects of *Vibrio alginolyticus* on larvae and postlarvae of the red abalone *Haliotis rufescens*

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ABSTRACT: The pathogenicity of *Vibrio alginolyticus* on *Haliotis rufescens* veliger larvae and 4 d old postlarvae was tested in small-scale static bioassays with different bacterial concentrations (10^2 to 10^6 cells ml^{-1}). Larval and postlarval survival were evaluated at 24 and 48 h. Results suggest that *V. alginolyticus* can cause massive mortality in larvae of *H. rufescens* within 24 h at concentrations above 10^5 cell ml^{-1}, while a concentration of 10^6 cell ml^{-1} is required to produce the same effect in abalone postlarvae. The potential application of these results to hatchery conditions is discussed.

KEY WORDS: Abalone · Pathology · *Vibrio alginolyticus*

INTRODUCTION

Molluscs, both in culture and natural populations, can be severely affected by a wide range of microbial diseases. *Vibrio* spp. are considered some of the most common and serious bacterial pathogens in hatcheries and can cause massive mortalities of larval, postlarval and juvenile stages of several molluscs (Elston 1984, Tubiash & Otto 1986, Sindermann 1988).

Walne (1958) reported the first evidence of a pathogenic effect of bacteria on bivalve larvae. Since then, several studies have been conducted with different bivalve species to address the role of bacteria in hatcheries. Most of these studies were descriptive and observational (Tubiash et al. 1965, Brown 1973, Murchelano et al. 1975, Lodeiros et al. 1987, Jeanthon et al. 1988, Mialhe et al. 1992). Only a few experimental studies on bacterial pathogenicity (e.g. involving the manipulation of bacterial concentrations) have been reported (Guillard 1959, Elston & Leibovitz 1980, Brown 1981, Jeffries 1982, Douillet & Langdon 1993, Moore et al. 1993, Anguiano-Beltrán 1996).

In abalone *Haliotis* spp., descriptive studies have reported *Vibrio*-related mortalities during larval (Ebert & Houk 1984) and juvenile culture (Elston 1983, Elston & Lockwood 1983, Dixon et al. 1991). This contribution represents the first experimental study on the pathogenic effect of different bacterial concentrations of *V. alginolyticus* on larvae and postlarvae of the red abalone *H. rufescens*, which is the most important gastropod for commercial aquaculture in the USA and México (Ebert 1992, Pérez-Muñoz 1995).

MATERIALS AND METHODS

Early veliger larvae of *Haliotis rufescens* were obtained from a commercial hatchery (Abulones Cultivados, Eréndira, Baja California, México). Further larval culture, settlement induction with gamma-aminobutyric acid (7 d after fertilization) and postlarval culture were performed at the University of Baja California facilities at Ensenada, B.C., following methods described elsewhere (Searcy-Bernal et al. 1992). Exogenous food is not required and was not provided during abalone larval development (Ebert & Houk 1984) but a cultured benthic diatom (*Navicula incerta*) was added as postlarval food 1 d after settlement. The small-scale bioassays reported here were performed in sterile 6-well tissue culture plates (Corning) with 6 ml of autoclaved seawater per well (out of 10 ml total capacity).

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A reference strain (Gherna & Pienta 1992) of *Vibrio alginolyticus* (ATCC 17749) was plated on Zobell media and incubated at 26°C for 24 h. Bacteria were then collected, diluted and inoculated to obtain 6 treatment concentrations (0, 10^2, 10^3, 10^4, 10^5 and 10^6 cells ml^{-1}) in the wells of each of 5 plates with the aid of a spectrophotometric calibration curve at 600 nm (Anguiano-Beltrán 1996). The first treatment (without bacteria added) was considered as a control. A completely randomized block experimental design was followed considering each plate as a block into whose wells the 6 treatments were randomly assigned. The initial concentrations of bacteria after inoculation were determined by enumeration on thiosulphate citrate bile salts sucrose (TCBS) medium.

Five-day old larvae or 4 d old postlarvae (11 d after fertilization) of the same batch were added to the plates at approximate densities of 5 ml^{-1} and 2 cm^{-2} respectively. These initial densities were verified by microscopic observations and the differences among wells were less than 10%. Abalones were washed 5 times with UV-treated 0.45 µm filtered seawater before the trials. Larval and postlarval survival (%) was estimated under a dissecting microscope (100×) after 24 and 48 h. Organisms that did not show any movement or activity for 5 min were considered dead. Temperature was maintained at 16 to 18°C, water was not changed and postlarvae were not fed during the experimental period.

Treatment and block effects were tested by analysis of variance including the partition of sum of squares due to linear and quadratic effects. Second-order polynomial regressions were used to estimate the bacterial concentrations associated with a 50% mortality (referred to as threshold concentration). These analyses were performed on raw data and after a conventional arcsine transformation for percentages (Damon & Harvey 1987) These 2 approaches yielded similar statistical inferences, but only results based on raw data are given below since these can be easily interpreted.

RESULTS

The pattern of larval survival in different bacterial concentrations at 24 and 48 h is presented in Fig. 1. In both cases treatment effects were highly significant ($p < 0.001$) and no block effect was detected (Tables 1 & 2). At the highest level of *Vibrio alginolyticus* (10^6 cells ml^{-1}) all abalone larvae died within 24 h but a high mortality was also observed in the 10^5 cells ml^{-1} treatment (12.3 and 3.7% survival at 24 and 48 h respectively). The polynomial regressions fitted were similar for both evaluation times (Fig. 1) with estimates of threshold concentrations of $10^{4.7}$ and $10^{4.5}$. Linear

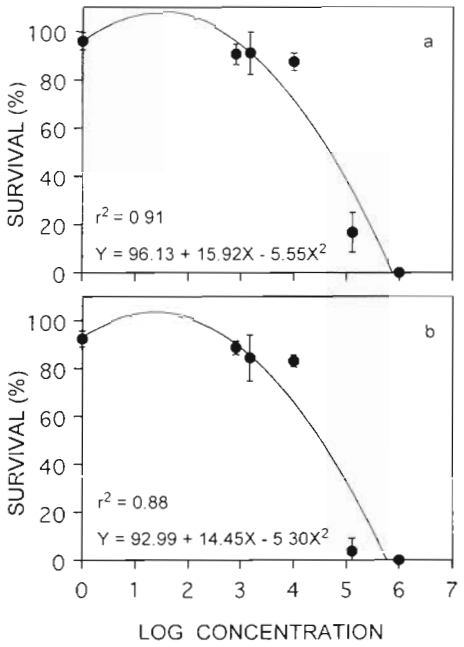

Fig. 1. *Haliotis rufescens*. Survival of larvae in different *Vibrio alginolyticus* concentrations after (a) 24 h and (b) 48 h. Vertical bars are standard errors (n = 5) and the solid line is the fitted regression equation shown

Table 1. Analysis of variance for survival of *Haliotis rufescens* larvae after 24 h of exposure to 6 concentrations of *Vibrio alginolyticus*, including the effects due to linear and quadratic regressions

Source	df	SS	MS	*F*	p
Concentrations	5	48847.05	9769.41	258.64	<0.001
Linear	1	31037.21	31037.21	24.30	<0.025
Quadratic	1	13978.84	13978.84	10.95	<0.050
Residual	3	3831.00	1277.00		
Blocks	4	49.72	12.43	0.33	0.855
Error	20	755.45	37.77		
Total	29	49652.22			

Table 2. Analysis of variance for survival of *Haliotis rufescens* larvae after 48 h of exposure to 6 concentrations of *Vibrio alginolyticus*, including the effects due to linear and quadratic regressions

Source	df	SS	MS	*F*	p
Concentrations	5	48754.90	9750.98	476.83	<0.001
Linear	1	30911.59	30911.59	18.10	<0.025
Quadratic	1	12719.93	12719.93	7.45	>0.050
Residual	3	5123.38	1707.80		
Blocks	4	176.69	44.17	2.16	0.111
Error	20	408.99	20.45		
Total	29	49340.58			

effects were significant in both cases but quadratic effects were significant only at 24 h (Tables 1 & 2). Before death, sick abalone larvae were unable to swim actively and remained on the bottom of containers.

The corresponding results for postlarval survival are shown in Fig. 2. Treatment effects were highly significant ($p < 0.001$) and a significant block effect was observed at the 48 h evaluation ($p = 0.14$) (Tables 3 & 4). Threshold concentration estimates of $10^{4.6}$ and $10^{3.4}$ for 24 and 48 h respectively were obtained and there were stronger linear and quadratic effects at the second evaluation (Tables 3 & 4). Signs preceding postlarval death included inactivity and a weak attachment by the foot. Bacterial swarming was not observed, probably due to the low magnification used; however, a mucus-like aggregation attached to the foot of postlarvae was detected in high *Vibrio alginolyticus* concentrations.

DISCUSSION

This study shows that *Vibrio alginolyticus* can cause massive mortality in larvae of *Haliotis rufescens* within 24 h at concentrations above 10^5 cells ml^{-1} (Fig. 1a). This toxic level is within the range of those reported for other mollusc species (Brown 1981, Jeffries 1982, Moore et al. 1993). However, a concentration of 10^6 cells ml^{-1} is required to produce the same effect in abalone postlarvae (Fig 2a). This result is consistent with the increased resistance to bacterial infection by older larvae of the oyster *Crassostrea virginica* (Brown 1973).

The patterns of survival as a function of bacterial concentration at 24 and 48 h were similar for larvae (Fig 1) but not for postlarvae, which showed reduced survival in higher concentrations (10^4 and 10^5) at 48 h (Fig. 2). This resulted in different regression equations, a lower threshold bacterial concentration and an increased significance of linear and quadratic effects at 48 h (Fig. 2, Tables 3 & 4). The threshold levels estimated by the quadratic regressions should be considered as a first approximation despite the high coefficients of determination (Figs. 1 & 2) since other alternative regression models were not tested.

It is difficult to explain the difference between postlarval survival at the 2 evaluation times. It might be due to the increase of bacterial concentration or toxins but these were not measured. *Vibrio* species can have very short generation times (12 to 20 min under optimal culture conditions) (Ulitzur 1974, Brown 1981). The lack of food during the 2 d experimental period probably was not an important factor since survival in controls was similar at both evaluation times (Fig. 2). In addition, recent studies suggest that *Haliotis rufescens* postlarvae can survive and develop at normal rates for

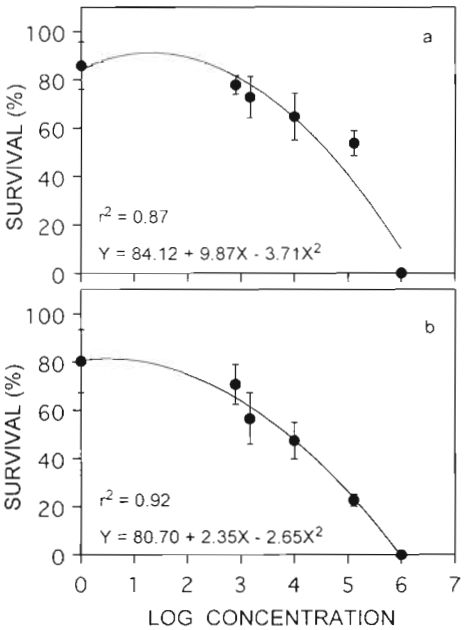

Fig. 2. *Haliotis rufescens*. Survival of postlarvae in different *Vibrio alginolyticus* concentrations after (a) 24 h and (b) 48 h. Vertical bars are standard errors (n = 5) and the solid line is the fitted regression equation shown

Table 3. Analysis of variance for survival of *Haliotis rufescens* postlarvae after 24 h of exposure to 6 concentrations of *Vibrio alginolyticus*, including the effects due to linear and quadratic regressions

Source	df	SS	MS	*F*	p
Concentrations	5	24044.14	4808.83	105.61	<0.001
Linear	1	15780.44	15780.40	23.32	<0.025
Quadratic	1	6233.75	6233.75	9.21	>0.050
Residual	3	2029.95	676.65		
Blocks	4	216.15	54.04	1.19	0.347
Error	20	910.72	45.54		
Total	29	25171.01			

Table 4. Analysis of variance for survival of *Haliotis rufescens* postlarvae after 48 h of exposure to 6 concentrations of *Vibrio alginolyticus*, including the effects due to linear and quadratic regressions

Source	df	SS	MS	*F*	p
Concentrations	5	22819.13	4563.83	98.78	<0.001
Linear	1	19346.06	19346.10	204.93	<0.001
Quadratic	1	3189.86	3189.86	33.79	<0.025
Residual	3	283.21	94.40		
Blocks	4	753.86	188.46	4.08	0.014
Error	20	924.04	46.20		
Total	29	24497.03			

at least 5 d after settlement in the absence of particulate food (Searcy-Bernal unpubl. data). However, a decreased postlarval resistance to bacterial infection after short-term starvation cannot be ruled out (Elston & Lockwood 1983, Elston 1984).

An extrapolation of these results to predict the potential effect of *Vibrio alginolyticus* on larval or postlarval abalone aquaculture would depend on the actual concentration of this bacterium in the culture systems, and also on other culture conditions. Preliminary surveys in some Mexican abalone hatcheries suggest that levels of *Vibrio* spp. in larval culture systems rarely exceed 10^3 cells ml^{-1}, which would probably be harmless to larvae. However, their concentration in the postlarval microhabitat (i.e. biofilms on surfaces of culture systems) can be several orders of magnitude higher (Lizárraga-Partida unpubl. data). In addition, the co-occurrence of sub-optimal conditions of temperature or other environmental factors might have a synergistic effect, increasing *Vibrio* spp. pathogenicity (Elston 1984). For instance, Elston (1983) reported that one of these factors may be oxygen supersaturation, which can be extremely high in the postlarval microhabitat due to boundary layer conditions (Searcy-Bernal 1996).

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