



Effects of salinity on larval stages of the rhizocephalan barnacle *Loxothylacus texanus*: survival and metamorphosis in response to the host, *Callinectes sapidus*

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Abstract

It is known that the rhizocephalan barnacle *Loxothylacus texanus* infects the greater blue crab, *Callinectes sapidus*, in the Gulf of Mexico and adjacent waters, however, factors that affect the prevalence and distribution of this parasite, particularly the dispersive larval stages of this organism, are not well understood. In the current study, the effects of salinity on larval survival and the metamorphosis of *L. texanus* in response to postmolt host exoskeleton were examined. Acute and acclimated responses were similar. Larval survival was highest in the 20–35‰ range, with 100% mortality of nauplii at all salinities <20‰ and >50‰. *L. texanus* cyprids were able to metamorphose over a broad range of salinities (15–60‰). In several cases, metamorphosis was actually greatest at high salinities (40–50‰). These data predict that *L. texanus* larvae would be concentrated in portions of Gulf of Mexico waters with salinities >20‰ such as the mouths of estuaries and bays. Conversely, upper regions of estuaries may be inhospitable to the dispersive (naupliar) stage of the parasite and may serve as a refuge from infection for host crabs.

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1. Introduction

Salinity stress has been shown to affect the growth and survival of a wide range of marine invertebrate larvae and, in turn, subsequent metamorphic competence and

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juvenile survival (see Pechenik, 1987; Pechenik et al., 1998 for reviews). However, relatively few studies have directly assessed the effects of salinity on the free-living larval stages of parasites (Reisser and Forward, 1991; Walker and Clare, 1994; Measures, 1996; Walker and Lester, 1998). Many studies have focused instead on the correlation between distribution patterns of infection and salinity (Couch, 1983; Craig et al., 1989). In investigations of parasitic rhizocephalans, Reisser and Forward (1991) and Walker and Clare (1994) examined the effects of salinity on the development and survival of the free-living stages of the sacculinid rhizocephalan parasite, *Loxothylacus panopaei* (Gissler) and suggest that low salinity waters (<10‰) may provide a refuge from infection for the mud crab *Rhithropanopeus harrisi* (Gould) by *L. panopaei*. Larval *L. panopaei* have high mortality rates at salinities less than 10‰ and larval release is limited at these salinities (Reisser and Forward, 1991; Walker and Clare, 1994). Low salinities also led to high mortality of nauplii of the sacculinid, *Heterosaccus lunatus*, a species that infects the portunid crab, *Charybdis callianassa* in Australia (Walker and Lester, 1998). Preliminary studies with *Loxothylacus texanus* Boschma, a rhizocephalan barnacle that parasitizes portunids, including the greater blue crab *Callinectes sapidus* Rathbun, found in the Gulf of Mexico (Alvarez and Calderon, 1996) and the southern Atlantic coast of the U.S. north to South Carolina (Eldridge and Waltz, 1977 as cited in Shields and Overstreet in press) suggest similar effects (O'Brien et al., 1993). However, in these studies only the effects of salinities <30‰ were examined and only larval survival was assayed. The effects of salinities >30‰ on larval survival and effects of salinity on the larval metamorphosis of *L. texanus* have not been addressed.

L. texanus are sexually dimorphic, exhibiting the typical rhizocephalan life cycle (Høeg, 1995; Glenner et al., 2000; Glenner, 2001). The larvae of *L. texanus* are released as nauplii from an external sac located on the abdomen of the host crab. The sex ratio of larvae varies from release to release, with some seasonality associated with shifts in these ratios (Walker, 1987; Høeg, 1995; Walker and Lester, 2000). After approximately 72 h, the nauplii metamorphose into cyprids. It is at this stage that female larvae will settle upon their hosts (Høeg, 1995). Settlement is specific. Female cyprids use a carbohydrate-based cue associated with the soft-shell exoskeleton of the crab to identify potential hosts and do not normally metamorphose in the absence of this cue (Boone et al., 2003). Following settlement, female cyprids will molt to form kentrogon larvae (Høeg, 1995). After 60–70 h, each kentrogon releases a vermigon larva into the host's hemocoel and the crab becomes infected (Glenner et al., 2000; Glenner, 2001). The parasite subsequently develops into an interna inside the host and after five to nine molts of the host (O'Brien, 1999), a virgin externa will eventually extrude from the abdomen. Male cyprids settle on and inoculate the virgin externa, which leads to the development of the next generation of parasites (Glenner and Høeg, 1995; Glenner et al., 2000). Infection results in what is termed "parasitic castration" (Kuris, 1974) as the parasitized crabs do not become sexually mature and thus do not reproduce (Glenner et al., 2000).

The objectives of the current study were to examine the effects of salinity on the survival of nauplii and cyprids of *L. texanus* as well as the effects of salinity on the rate of metamorphosis of female cyprids of this species. Both acute and acclimation responses were examined.

2. Materials and methods

2.1. Collection and maintenance of crabs and barnacle larvae

Non-parasitized *C. sapidus* were collected at the Dauphin Island Airport Marsh, AL and held in recirculating tanks at 25 ‰ and 25 °C. Crabs were fed once a day on a diet of beef liver (O'Brien, 1999). Non-parasitized crabs were forced to autotomize six walking legs, which induced the animals to molt (O'Brien, 1999). After molting, soft-shell crabs in stages A and B of Drach's scheme (Drach, 1939), as described by Freeman et al. (1987), were stored in a -20 °C freezer until needed (Boone et al., 2003).

Parasitized *C. sapidus* were obtained from Gulf Specimens Marine Lab in Panacea, FL and collected by otter trawls in Mobile Bay, AL, and Mississippi Sound, MS. For acute experiments crabs were held in recirculating tanks at 25 ‰ and 25 °C. For acclimation experiments, crabs were acclimated at a rate of 2 ‰ per day and were held in 18-l aerated containers at 15 ‰, 25 ‰, and 40 ‰ prior to larval release by the parasite. Depending on the salinity needed, seawater was either diluted with deionized water or the salinity increased by the addition of Instant Ocean (Aquarium Systems, Mentor, OH). Parasitized crabs were fed once a day on a diet of beef liver (O'Brien, 1999). Upon release, *L. texanus* larvae were held in 18-l aerated containers that were maintained at the same temperature and salinity as the host crab.

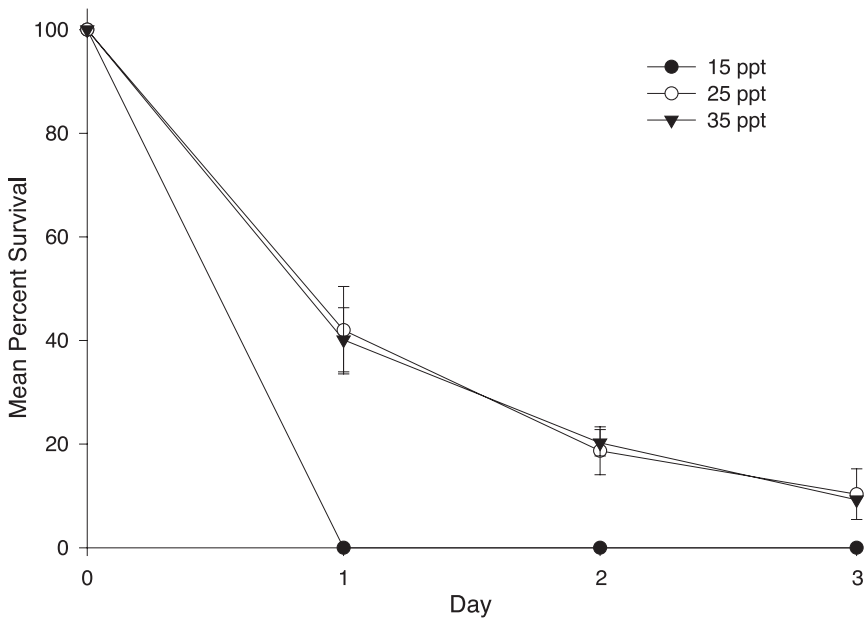


Fig. 1. Percent survival of *L. texanus* larvae from release (Day 0) through development to the cyprid stage (Day 3) at three test salinities, all acute responses. Data points are means \pm S.D., $n = 5$.

2.2. Larval survival assays

The effects of salinity on *L. texanus* larval survival were monitored in two acute response experiments and three acclimation experiments. For both acute response experiments, the host crabs were held at 25‰. Upon release, the nauplii were filtered through 32- μ m filters and placed in 11-cm diameter, 400-ml total volume glass dishes at the appropriate salinities. Dishes were covered with aluminum foil to prevent contamination. Daily inspection assured no larvae were trapped at the air–water interface. Larvae were exposed to salinities of 15‰, 25‰, and 35‰ in the first acute experiment and 20‰, 30‰, 50‰, and 60‰ in the second. For the three acclimation experiments, parasitized crabs were acclimated to salinities of 15‰, 25‰, and 40‰ prior to larval release. Larvae from each acclimated host were filtered as described above and placed in the 11-cm diameter dishes at test salinities of 15‰, 25‰, and 40‰. All survival assays were run as static experiments with five replicates per salinity and 25–58 larvae per replicate. For a given experiment, all larvae used in all treatments were from the same release batch. Larval survival was monitored daily from the release of nauplii through the cyprid stage by taking actual counts of living and dead larvae in each replicate of each treatment. The effects of salinity and day number on larval survival were examined using two-factor ANOVAs ($\alpha=0.05$; Zar, 1984).

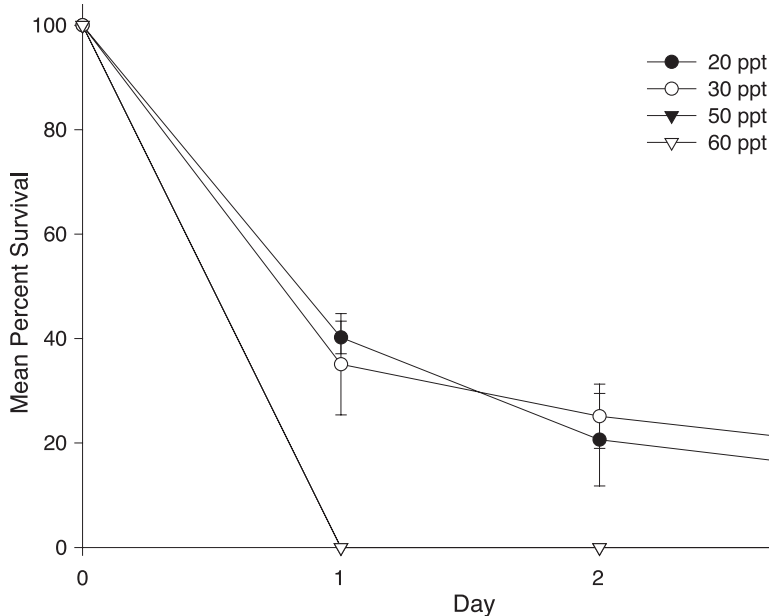


Fig. 2. Percent survival of *L. texanus* larvae from release (Day 0) through development to the cyprid stage (Day 3) at four test salinities, all acute responses. Data points are means \pm S.D., $n=5$. Note: 50‰ and 60‰ data points overlap, thus the 60‰ points obscure the 50‰ points.

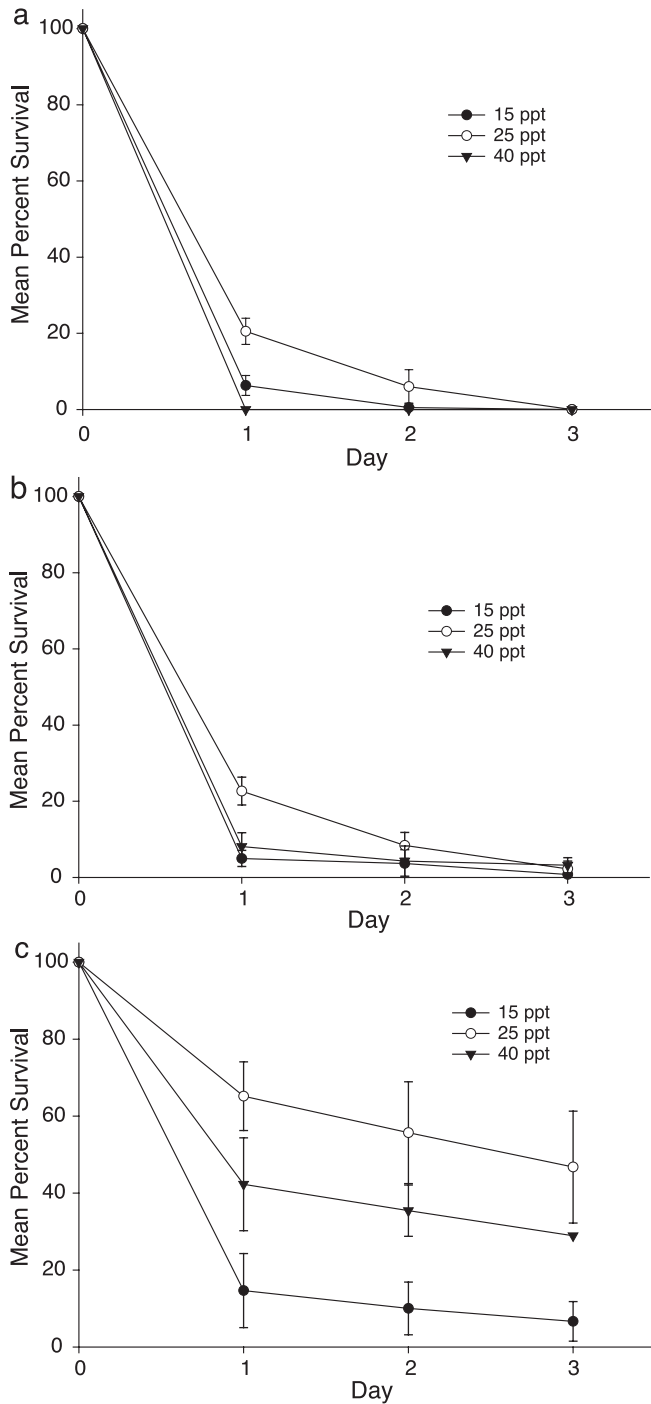


Fig. 3. Percent survival of *L. texanus* larvae from release (Day 0) through development to the cyprid stage (Day 3) at three test salinities under three acclimation conditions: (a) 15‰, (b) 25‰, and (c) 40‰. Data points are means \pm S.D., $n = 5$.

2.3. Larval metamorphosis assays

The effects of salinity on *L. texanus* larval metamorphosis from the female cyprid to the kentrogon stage were examined in two acute response and three acclimation experiments. In the first acute response experiment salinities of 10‰, 25‰, and 40‰ were tested, while salinities of 10‰, 20‰, 30‰, 40‰, 50‰, and 60‰ were tested in the second experiment. For the three acclimation experiments, parasitized crabs were acclimated to salinities of 15‰, 25‰, and 40‰, prior to larval release. Upon release, larvae from each acclimated host were then held at the acclimated salinity until development of the cyprid stage. Cyprids from each of the acclimation salinities were then tested at 15‰, 25‰, and 40‰ using strips of *C. sapidus* soft-shell carapace (5 × 10 mm) as the settlement substrates in all experiments (Boone et al., 2003). The carapace strips were placed in individual 11-cm diameter glass dishes containing water at the appropriate salinity. For all settlement tests, 200 ml of cyprid larvae were removed from holding containers, filtered through 64- μ m mesh, transferred to the treatment containers, and exposed to the substrates for 72 h (Boone et al., 2003). At 72 h, the larvae that had metamorphosed from the cyprid to the kentrogon stage were counted. All metamorphosis assays were run as static experiments with five replicates per salinity. Only cyprids that were within 24 h post-metamorphosis from the naupliar stage were used in metamorphosis assays. Only

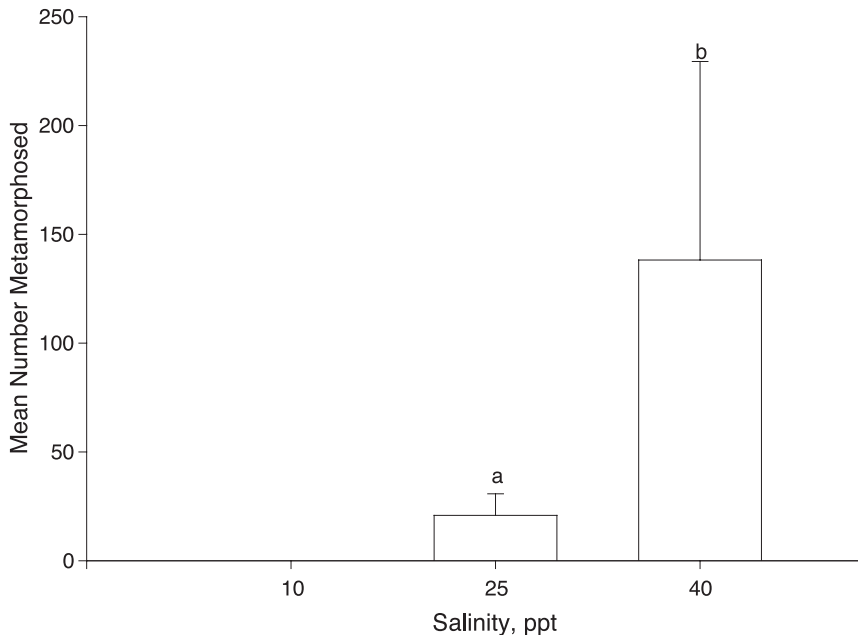


Fig. 4. Number of *L. texanus* larvae that metamorphosed from the cyprid to the kentrogon stage on *C. sapidus* soft shell at three test salinities, all acute responses. Data are means \pm S.D., $n=5$. Treatment results with the same letter above the error bar are not significantly different at $p < 0.05$.

metamorphosis of female cyprids was examined in this experiment, as they are the agent for infection. However, to account for the unknown sex ratio of each brood of larvae, all replicates in a given experiment were performed using the same batch of released larvae with the assumption that ratios remain constant within a single batch. The effects of salinity on larval metamorphosis were examined using the Mann–Whitney test or Kruskal–Wallace test and non-parametric multiple comparison tests on ranked settlement numbers ($\alpha=0.05$; Zar, 1984).

3. Results

3.1. Larval survival assays

All larvae in the 15‰ treatment in the first acute response experiment (Fig. 1) and in the 50‰ and 60‰ treatments in the second acute response experiment (Fig. 2) died during the first 24 h of exposure. These treatments were not included in the statistical analyses. For the remaining treatments, there was no significant effect of salinity and no interaction effect. There was a significant day effect with survival decreasing throughout both acute experiments (Figs. 1 and 2).

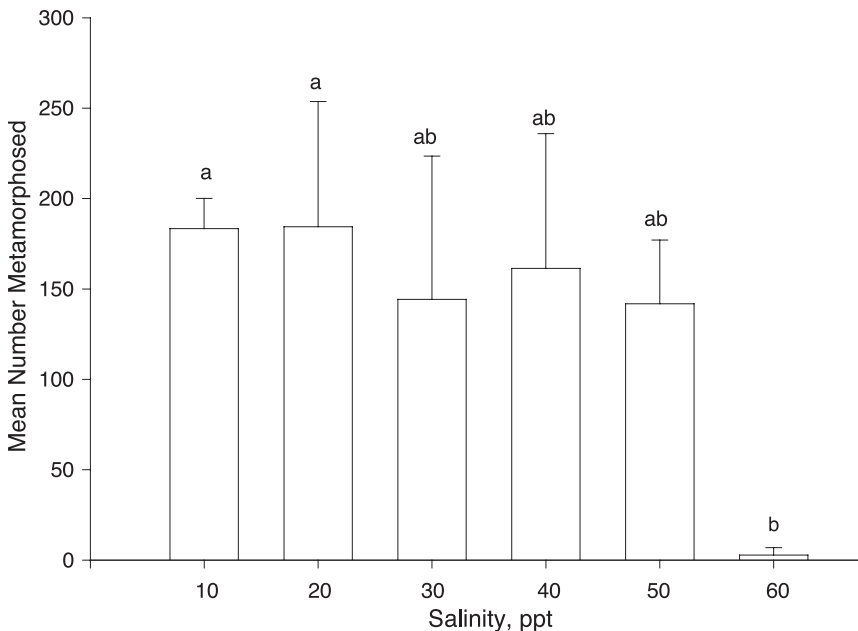


Fig. 5. Number of *L. texamus* larvae that metamorphosed from the cyprid to the kentrogon stage on *C. sapidus* soft shell at six test salinities, all acute responses. Data are means \pm S.D., $n=5$. Treatment results with the same letter above the error bar are not significantly different at $p < 0.05$.

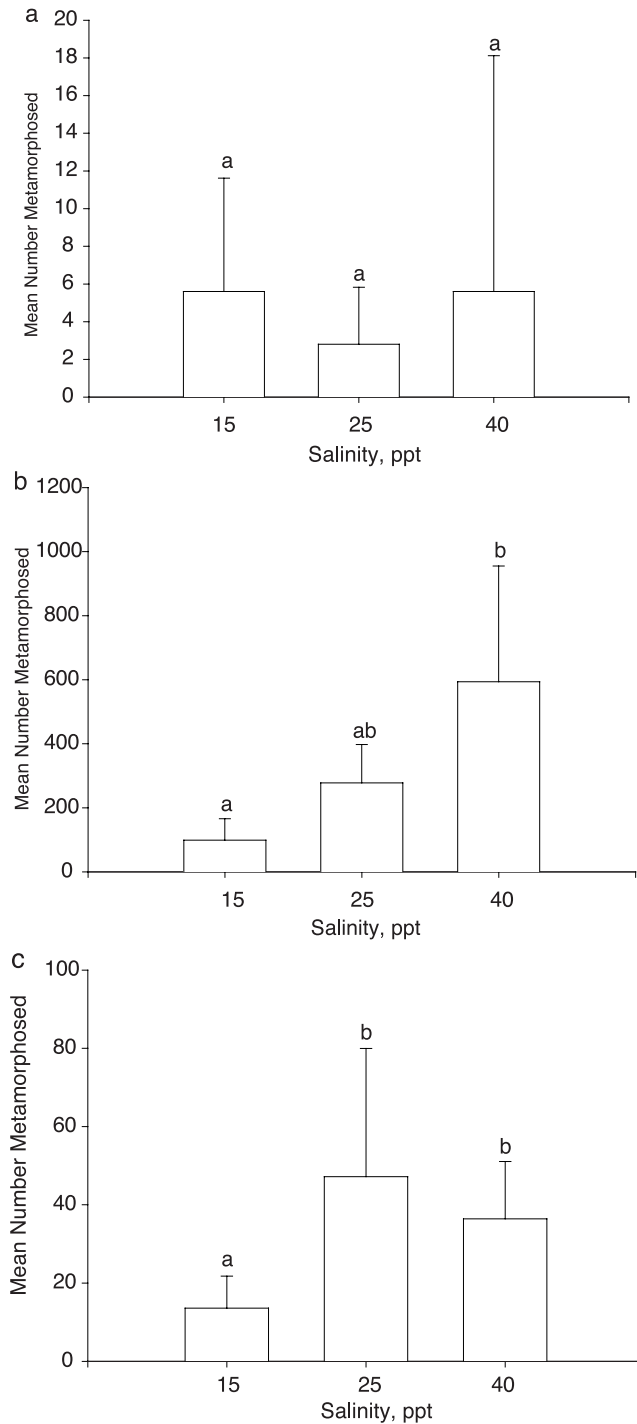


Fig. 6. Number of *L. texanus* larvae that metamorphosed from the cyprid to the kentrogon stage on *C. sapidus* soft shell at three test salinities under three acclimation conditions: (a) 15‰, (b) 25‰, and (c) 40‰. Data are means \pm S.D., $n=5$. Treatment results with the same letter above the error bar are not significantly different at $p < 0.05$.

All larvae that had been acclimated to 15‰ died during the first 24 h of the 40‰ exposure treatment (Fig. 3a), and these data were not included in the statistical analyses. For the remaining treatments in the 15‰ acclimation experiment, there were significant day and salinity effects as well as a significant interaction effect with survival decreasing with decreasing salinity and increasing day number (Fig. 3a). There were also significant day, salinity, and interaction effects for the 25‰ acclimation experiment. Survival was highest in the 25‰ treatment and decreased with increasing day number for all treatments (Fig. 3b). There were significant day and salinity effects and no interaction effect for the 40‰ acclimation experiments, with survival decreasing with increasing day number. Survival was highest at 25‰ and lowest at 15‰ (Fig. 3c).

3.2. Larval metamorphosis assays

In the first acute metamorphosis assay, there was no metamorphosis in the 10‰ treatment (Fig. 4). Data from this treatment were not included in the statistical analyses. There was significantly lower metamorphosis in the 25‰ treatment than in the 40‰ treatment (Fig. 4). However, in the second acute metamorphosis experiment no significant differences in metamorphosis were observed among the 10‰, 20‰, 30‰, 40‰, and 50‰ treatments (Fig. 5). There was significantly higher metamorphosis in the 10‰ and 20‰ treatments than in the 60‰ treatment (Fig. 5).

Low settlement was observed for all larvae acclimated to 15‰ with no significant differences among treatments (Fig. 6a). When larvae were acclimated to 25‰, significantly lower settlement occurred in the 15‰ treatment than in the 40‰ treatment (Fig. 6b). There was also significantly lower settlement in the 15‰ treatment than in either the 25‰ or the 40‰ treatments when larvae were acclimated to 40‰ (Fig. 6c).

4. Discussion

This investigation is part of a long-term effort to understand how environmental factors influence infection of blue crabs by *L. texanus* in the north-central Gulf of Mexico. Based on settlement studies of free-living balanomorph barnacles, the interactions are expected to be complex. A partial list of factors that have been identified as influencing settlement and metamorphosis of non-parasitic cirripeds includes current flow (Mullineaux and Butman, 1991), tidal height (Bertness et al., 1992), larval supply (Bertness et al., 1992; Pineda et al., 2002), extracts or pheromones originating from adult barnacles (Dineen and Hines, 1992, 1994; Matsumura et al., 1998; Khandeparker et al., 2002), microbial pre-colonization of substrates (Olivier et al., 2000), as well as temperature and salinity (Thiyagarajan et al., 2003). As complicated as settlement and metamorphosis are for free-living cirripeds, we expect that successful metamorphosis of rhizocephalan barnacles on suitable hosts will be even more complex. Parasitic castrators probably encounter defense mechanisms that provide selective advantages to hosts allowing them to avoid infection.

We have verified here (Fig. 1) what others (Reisser and Forward, 1991; Walker and Clare, 1994) have shown for the congener *L. panopaei*, i.e., nauplii of the rhizo-

cheplan genus, *Loxothylacus*, are not well adapted to low (<15‰) salinity habitats. We also demonstrated that cyprids may be unable to successfully metamorphose if suddenly exposed to a salinity of 10‰ (Fig. 4), although, the response was not consistent (see 10‰ treatment in Fig. 5). Cyprids may either be more resistant to osmoregulatory stress than nauplii or there may be significant variability in capabilities of larvae from different brood releases. In general, our results support the hypothesis first proposed by Reisser and Forward (1991) that low salinity habitats could serve as refuges for potential hosts of *Loxothylacus* spp. Our data strongly indicate that *L. texanus* can infect vulnerable hosts in habitats ranging from 20‰ to 50‰ (Figs. 4–6). One implication of these results is that the prevalence of *Loxothylacus* in host populations could be influenced by local rainfall patterns, particularly in shallow estuaries.

Substantial numbers of *L. texanus* female cyprids were able to metamorphose at salinities as high as 50‰ and a few successfully metamorphosed at 60‰ (Fig. 5). Such salinities are much higher than the organisms would be expected to encounter in the northern Gulf of Mexico near Alabama and Mississippi and are more reflective of what would be encountered in closed estuaries located in Texas and along the eastern coast of Mexico. In Mexico, Robles et al. (2002) measured oxygen consumption and Alvarez et al. (2002) examined the osmoregulatory response of *L. texanus* parasitizing a congener of the blue crab, *C. rathbunae*. Both investigations found that the energetic costs for the host of maintaining *L. texanus* increased significantly at salinities below 15‰ and the authors inferred that crabs bearing mature *L. texanus* would avoid regions of low salinity in local estuaries. This is similar to the behavior that was first noted by Rasmussen (1959) in which crabs bearing mature sacculinids migrated to the more saline regions of estuaries as did uninfected, ovigerous female crabs. Sankarankutty et al. (2000) have reported high prevalence values (45–57%) of what is probably a sacculinid rhizocephalan parasitizing the xanthid crab, *Hexapanopeus schmitti*, in an enclosed lagoon on the northeast coast of Brazil. The habitat was hypersaline with salinities consistently between 40‰ and 45‰. It appears that *L. texanus* and at least one other sacculinid rhizocephalan may be better adapted to hyper- rather than hyposaline habitats. If so, one would predict that prevalence of *L. texanus* in *Callinectes* would be much more variable (both annually and among localities) in the north-central Gulf of Mexico than elsewhere, depending upon local environmental conditions.

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