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Host specificity of *Sacculina carcini*, a potential biological control agent of the introduced European green crab *Carcinus maenas* in California

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Abstract

The European green crab, *Carcinus maenas*, is an introduced marine predator established on the west coast of North America. We conducted laboratory experiments on the host specificity of a natural enemy of the green crab, the parasitic barnacle *Sacculina carcini*, to provide information on the safety of its use as a possible biological control agent. Four species of non-target, native California crabs (*Hemigrapsus oregonensis*, *H. nudus*, *Pachygrapsus crassipes* and *Cancer magister*) were exposed to infective larvae of *S. carcini*. Settlement by *S. carcini* on the four native species ranged from 33 to 53%, compared to 79% for green crabs. Overall, cyprid larvae tended to settle in higher numbers on individual green crabs than on either *C. magister* or *H. oregonensis*. However, for *C. magister* this difference was significant for soft-shelled, but not hard-shelled individuals. Up to 29% of the native crabs arrested early infections by melanizing the rootlets of the parasite. Most native and green crabs settled on by *S. carcini* became infected, especially when settled on by >3 cyprids. Infected green crabs died at more than twice the rate of uninfected green crabs. In contrast to green crabs, all infected native crabs died without producing an externa (reproductive sac). At high settlement intensities, infected native crabs frequently exhibited neurological symptoms (twitching, loss of movement) before death. These results indicate that use of *S. carcini* as a biological control agent could result in the death of native crabs. The magnitude of this effect would be proportional to the density of infected green crabs in the environment and the probability that cyprids would contact native crabs in the wild. Potential benefits of biological control should be assessed in relation to these potential non-target effects.

Introduction

Classical biological control has been successfully employed in agricultural and freshwater ecosystems (Caltagirone 1981; Greathead 1995; McFadyen 1998). Adverse effects (Howarth 1991; Simberloff and Stiling 1996a, b; Barratt et al. 1997; Boettner et al. 2000; Louda 2000; Henneman and Memmott 2001) deriving from a lack of host specificity (Louda et al. 2003) have not been evaluated in comparison to the benefits of pest reduction. Non-target impacts can be avoided by careful host-specificity testing (Greathead 1995; McFadyen 1998; Thomas and Willis 1998; Strong and Pemberton 2000). One tractable standard for safety is an inability to

complete development and reproduce in non-target hosts (Sands 1998).

The use of biological control in marine ecosystems has only recently been proposed (Lafferty and Kuris 1996; and see review by Secord 2003). Lafferty and Kuris (1996) developed a theoretically based strategy for the use of natural enemies as biological control agents against introduced marine pests in general and the European green crab, *Carcinus maenas*, in particular. The rhizocephalan barnacle, *Sacculina carcini* (Figure 1), a parasitic castrator of *C. maenas* in Europe, is a candidate biological control agent of introduced green crab populations because of theoretical (Kuris and Lafferty 1992) and empirical (Torchin et al. 2001) evidence that it can reduce green crab biomass.

Carcinus maenas is introduced to both the east and west coasts of North America, and to southern Australia, Tasmania, and South Africa (Cohen et al. 1995; Grosholz et al. 2000; Yamada 2001). It has spread along the west coast of North America to most of the estuaries between Morro Bay, California, and Vancouver Island, British Columbia, (Grosholz et al. 2000; Yamada 2001). Green crabs are hardy generalist predators with a preference for bivalves, gastropods, and polychaetes (Glude 1955; Ropes 1968). They are pests for mariculture (Grosholz et al. 2000) and may impact native predators such as shorebirds and the economically important dungeness crab, *Cancer magister* (Grosholz and Ruiz 1996; Grosholz et al. 2000). Efforts to trap, fence, and poison crabs are either labor intensive, expensive or have effects on non-target species (Glude 1955; Hanks 1961; Walton 1997).

The host specificity of *S. carcini* is far from certain. Unlike its host, *S. carcini* has not expanded beyond it native range (Torchin et al. 2001). In its native range, *S. carcini* parasitizes only species of Portunidae (including the green crab) and one species in the closely related Pirimelidae (Høeg and Lützen 1985). Although often present, and sometimes common in the same habitats, *S. carcini* does not successfully parasitize European crabs closely related to the native California crabs (for example, *Cancer pagurus* and *Pachygrapsus marmoratus*). However, these

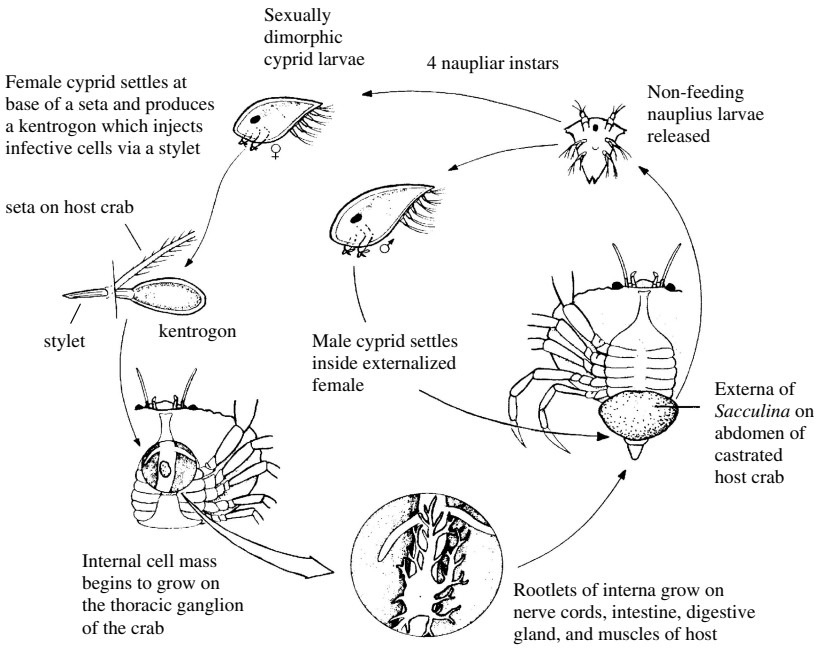

Figure 1. Life cycle of *Sacculina carcini* modified from Hickman et al. (1988).

crabs are parasitized by other species of *Sacculina* (*S. triangularis* and *S. benedeni*, respectively). *S. carcini* has been observed to settle on non-host crabs in the laboratory (Høeg et al. 1997; Thresher et al. 2000).

Materials and methods

The goal of this study was to test the host specificity of *S. carcini* against California native crab species. Normally, this would involve selecting species closely related to the pest (a portunid brachyuran). Because native portunids (and the closely related pirimelids) do not overlap with green crabs in California, we chose ecologically or economically important brachyurans that have habitat overlap with green crabs (as these are valued and would likely be exposed to the parasite). In our experiments we attempted to maximize the opportunity for *S. carcini* to successfully infect California native crabs, based on the results of other studies of rhizocephalans. Accordingly, we used small, postmolt animals (Glenner and Werner 1998), abnormally high infective cyprid dosages (often thousands per liter), small exposure volumes, and the presence of susceptible green crabs to provide necessary host recognition cues (Boone et al. 2003). Specifically, we asked the following questions: (1) Can larval *S. carcini* settle on native California crabs? (2) Do they show a preference for native crabs vs introduced green crabs? (3) If *S. carcini* settles on native crabs, can it penetrate their cuticle and begin development of its root like interna? (4) What effects does the growth of the interna have on host survival? (5) Does this vary when native crabs are compared to its usual hosts? (6) Can *S. carcini* develop to maturity (i.e., produce its reproductive externa) in the native species, and therefore use them fully as alternate hosts?

Procedures

Carcinus maenas has been experimentally infected in the laboratory using competent cyprids of *S. carcini* (Rubiliani et al. 1982). However, these procedures were conducted on a small scale using few replicates. To conduct host-specificity tests it was necessary to establish a repeatable and reliable experimental system. Previous studies of *S. carcini* infectivity demonstrated that brood size, sex ratio, and quality varied greatly among barnacle broods (Walker 1987, 1988). In addition to these undesirable and still insufficiently understood sources of experimental variability, our protocol required certain host characteristics. For each simultaneous exposure trial (described below), we sought small (immature) postmolt green crabs and one of the experimental (native) species, matched for size. This necessitated holding large numbers of small individuals of each species of crab, monitored daily for ecdyses. To fully satisfy the protocol, at least one postmolt individual of both species had to be available when a hatch including competent female cyprids was also available. Frequently, the temporal confluence of these events was not achieved. When numerous competent cyprids were available and we did not have at least a pair of postmolt crabs, we used hard crabs of each species (matched for size). Finally, exposed crabs had to be held for several months until externas were produced or the animals died. Because of these and other logistic constraints (seasonality of host ecdysis, *S. carcini* sex ratio, brood production), these experiments took 2 years (1998–1999). The experimental sources of variability (host size, molt stage, and number of settled cyprids) were considered in the analyses.

Three California native crabs served as test species, the grapsids *Hemigrapsus oregonensis*, *H. nudus*, and *Pachygrapsus crassipes*, based on their ecological overlap with the green crab on the west coast, and a fourth, the Dungeness crab *Cancer magister*, for both its ecological overlap and economic importance. Ecological overlap greatly increases the likelihood of encounter between host and parasite (Combes 2001). The *Hemigrapsus oregonensis* and *P. crassipes* came from the mouth of Carpinteria Salt Marsh in southern Santa Barbara County, California, USA; *H. nudus* came from Bodega Harbor, California, USA; and juvenile *C. magister* came from Coos Bay, Oregon, USA. Juvenile green crabs known to be unexposed to *S. carcini* were collected from New England (outside the range of *S. carcini*, Torchin et al. 2001) for use in both the larval preference trials and as standards in the infection experiments.

As a source of *S. carcini* larvae, adult green crabs parasitized by *S. carcini* with well-developed

externae were maintained in the laboratory. Collectors at marine laboratories obtained parasitized crabs in Great Britain in the summer and Spain and Portugal in the fall and winter, and then shipped them by air in damp containers (as per California Department of Fish and Game importation permit). Owing to the seasonal variation in sex ratio of the broods of *S. carcini* with latitude (Walker 1987, 1988), we therefore had a source of female cyprid larvae most of the year.

Green crabs infected with mature *S. carcini* were held in 70-l aquaria with running, filtered seawater at ambient temperatures (12–19 °C). The native crabs and control green crabs were kept individually in compartmentalized plastic boxes perforated to allow water circulation. To prevent accidental release of the parasite or its host all aquaria containing *S. carcini*, green crabs, or crabs exposed to the larvae of *S. carcini* (see below) were held in a restricted access room with the seawater outflow filtered down to 90 μm by redundant sand and mesh filters (as approved by the California Department of Fish and Game). Crabs were fed 2 or 3 times a week with fresh mussel tissue (*Mytilus* spp.).

The externae of adult *S. carcini* were examined two or three times per week for the presence of broods close to hatching (revealed by dark tan externas). These crabs were placed in individual plastic 500 ml containers in aerated seawater filtered to 2 μm and checked once or twice daily until release of *S. carcini* nauplii. Broods of newly released nauplii were separated in 500 ml containers with 85 μm nylon mesh bottoms, open tops, and a 1 ml/sec overhead stream of filtered seawater, in a culture system adapted from Høeg (1984b). Cultures were examined daily until nauplii had metamorphosed into cyprids. The larvae were sexed using the morphological criteria described by Walker (1987), using samples of 20–30 cyprids which had been anesthetized in 57% seawater. For the settlement trials, we used broods containing more than 10% female larvae. To be sure that female cyprids were competent to settle, broods containing female larvae were held for 2–3 days after metamorphosis before being used in settlement trials (as per Høeg 1984a, 1995).

Settlement preference of S. carcini

For the larval preference (choice) experiments, *C. magister* or *H. oregonensis* were placed with green crabs of similar size and molt cycle stages (see below) and held together with competent female cyprids of *S. carcini* in 500 ml containers maintained in the above culture system. Because *S. carcini* cyprids more often settle on postmolt than on hard-shelled green crabs (Glenner and Werner 1998; our unpublished observations), we used postmolt crabs in these experiments whenever possible. The number of crabs exposed in a single container varied from 2 to 5, and the number of containers used in each settlement experiment varied from 1 to 4, depending on the size of the larval brood and the number of available postmolt crabs. Most of the larval preference trials simultaneously exposed a green crab and one of the four native species. For each crab, we recorded its sex (*C. maenas* and *H. oregonensis* only), carapace width (at the widest point, excluding lateral spines), and stage in the molt cycle. The molt cycle was subdivided between molt stages C2 and C3. If the cardiac region could be depressed by gentle thumb pressure, the crab was in postmolt stage C2 (or earlier). If not, it was considered hard (late postmolt, intermolt or premolt) (Drach 1939; Hiatt 1948; Kuris 1971).

In addition to the above preference tests, we conducted single-host larval settlement trials solely for the purpose of obtaining infected native and green crabs. The following size range (carapace width) of crabs was used in the preference and non-preference settlement trials: *C. maenas*, 7–22 mm; *C. magister*, 10–18 mm; *H. oregonensis*, 7–19 mm; *H. nudus*, 9–12 mm; *P. crassipes*, 7–29 mm.

Broods of *S. carcini* containing female larvae were distributed equally among containers in each trial, with densities of larvae ranging from 1 to 75 cyprids/ml, depending on the size of the brood. Crabs were exposed for 1–2 days and then, using a dissecting microscope, the number of settled cyprids on the surface of each crab was counted. Each exposed crab was isolated in an individual compartment, checked daily, and fed pieces of mussel three times a week. Deaths and abnormal behavior (see below) were recorded.

Infection outcome

We determined whether exposed crabs were parasitized by dissecting them and examining the digestive gland, intestine, and thoracic ganglion for the presence of the interna of the parasite. Fresh tissue or tissue from freshly frozen crabs was examined. The rootlets of the interna are distinctive in their branching pattern, irregular diameter, bluntly rounded tips, and lipid content (see Figure 2). Crabs were scored as either infected, uninfected, infection arrested (when all observed *S. carcini* rootlets were melanized by a host immune response), or no data (when a crab died less than 3–4 weeks after being settled on by *S. carcini*, too early to allow recognition of the parasite, though we acknowledge that such death could have been caused by the parasite).

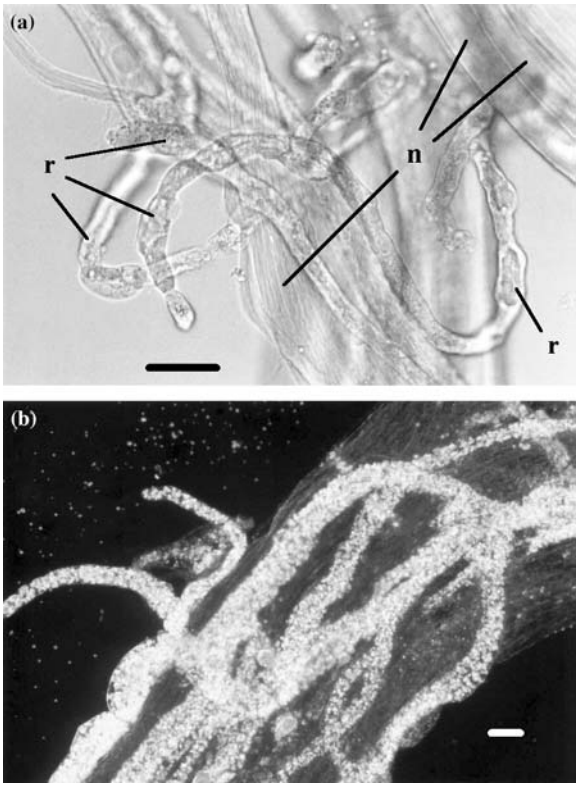

Figure 2. Roots of *Sacculina carcini* and nerve cords from the walking legs of juvenile green crabs infected in the laboratory; scale bars = 75 μm. Unless noted otherwise, all photomicrographs were of fresh tissue and employed bright field microscopy; mr = melanized rootlets, n = nerve cords, r = roots. (a) Roots 237 days after settlement by four cyprids on a 9.0 mm (carapace width) crab. (b) Roots (light-colored) packed with lipid droplets growing on nerve cords (gray-colored) 153 days after settlement by approximately 100 cyprids on a 17.7 mm crab; dark field microscopy.

The state of infection was 'early' if roots were found only on the thoracic ganglion, 'spreading' if they had reached the intestine, 'extensive' if they had reached the digestive diverticulae, and 'disseminated' when roots surrounded the stomach, had largely supplanted the digestive gland and invaded the abdomen. Crabs were held between 5 and 22 weeks before sacrificing them for dissection. Any crabs that died before their scheduled sacrifice were dissected within a day. Voucher specimens and samples for future studies were preserved in 70 or 95% ethanol or Bouin's fixative and archived in our laboratory. In addition to examining crabs for the presence of the roots of *S. carcini*, we noted any abnormalities in the tissues examined, including the presence of melanization (indicating a host immune response).

To determine the probability of infection by a single cyprid for each crab species, as well as to evaluate the possibility that a fraction of the hosts were resistant, we developed a model, $I = S - S(1-P)^C$, where I was the probability that an exposed crab would be infected (alternatively it would be the proportion of a sample of crabs that would be infected), S was the proportion of susceptible crabs in the population, P was the per-cyprid probability of an infection, and C was the number of cyprids that settled on a crab. A computerized, iterative search was used to determine the values of S and P that minimized the sum of the squared deviations of the expected probability of infection for a trial vs the actual outcome of a trial (infected or uninfected, classifying arrested infections with the latter category). Using these parameters, we then plotted I as a function of C for each species.

Survival of infected crabs

We compared the survivorship of infected crabs to the survivorship, during the same period, of crabs either not exposed to *S. carcini*, or exposed to but not settled on by the parasite. All of these crabs were maintained in parts boxes as described earlier. The effect of the number of settled cyprids,

or settlement intensity, on the longevity of infected host crabs was examined using a multiple regression analysis (see below).

Data analyses

For the settlement preference experiments, we compared, for each pair of crab species, (1) the proportion of crabs on which cyprids settled, and (2) the number of cyprids settled per crab (settlement intensity). Owing to the unequal and variable number of crabs used in the exposure containers, as well as potential variability within and among broods of *S. carcini* larvae, the analysis treated each container (not each crab) as a replicate. To exclude incompetent broods, only those trials in which *S. carcini* settled on at least one individual crab were analyzed. For the first comparison, we calculated the proportion of individuals of each species settled on by *S. carcini* in each container and then calculated, for each container, the ratio of those proportions, using the proportion of native crabs as the numerator, and that for green crabs as the denominator. We then tested the difference of the mean of these ratios from an expected value of one (indicating no difference between the proportion of native and green crabs on which cyprids settled) using a two-tailed Wilcoxon signed rank test.

A similar analysis for the second comparison was conducted using the ratio of the mean number of cyprids settled per crab for each species in each container (providing a relative measure of settlement that was independent of the density of competent barnacle larvae), and then testing the difference of the mean of those ratios from an expected value of one. For the first comparison, a G-test of independence (Sokal and Rohlf 1981, p. 737) was also conducted on the number of crabs settled on, or not, by *S. carcini* for each pair of crab species. In this test, exposure containers were also treated as replicates, and the number of containers in which at least one individual of that species was settled on by *S. carcini* and the number lacking settlement on crabs of that species was tallied for each species.

We used a full factorial, ordinal logistic regression model to examine the effects of number of settled cyprids, crab size (as carapace width), molt stage and species on the outcome of the infections for crabs on which cyprids settled. These included 28 *C. magister*, 26 *H. oregonensis*, 7 *H. nudus*, 1 *P. crassipes* and 34 green crabs (sex was not recorded for *C. magister* and therefore, could not be included in this analysis). In this model, crabs were scored as 1 if uninfected, 2 if the infection had been arrested by a host immune response, and 3 if they became successfully parasitized. We then sequentially removed non-significant factors and interactions until the best fit model had been obtained.

To determine if individual host survival was affected by the intensity of settlement by *S. carcini* cyprids (and presumably the number of developing internae), multiple regression analyses of survival (in days) of infected crabs as a function of the number of settled cyprids and crab size (as carapace width) were performed. In these analyses, the survival time of crabs that had died within 3–4 weeks after settlement by *S. carcini* (too early to detect infection by dissection) was also included. We did separate analyses for *C. magister*, *H. oregonensis* and green crabs, and another for all California native crabs combined, with species as an additional independent variable. The logarithm of the number of settled cyprids per crab was used in all regression analyses to normalize the distributions of the residuals.

Results

Overall settlement of S. carcini

Sacculina carcini settled on all four of the California native crabs (Table 1). For those trials ($n = 49$) with cyprid settlement on at least one crab, the total percentage of native individuals settled on averaged 46.5% (range, 33–53%), compared to 79% of green crabs (Table 1). Specifically, settlement was higher on green crabs than on *C. magister* ($G_{adj} = 14.680$, $P < 0.005$), *H. oregonensis* ($G_{adj} = 11.810$, $P < 0.005$), *H. nudus* ($G_{adj} = 4.780$, $P < 0.05$) and *P. crassipes* ($G_{adj} = 4.856$, $P < 0.05$).

Table 1. Settlement and infection results for *Carcinus maenas* and four species of California native crabs exposed to cyprid larvae of *Sacculina carcini*.

Species	Crabs exposed[a]			Crabs settled on by *Sacculina*			Infection outcome, % of settled crabs			
	No.	Mean carapace width ± SE	% with post-molt shells[b]	% of crabs exposed	Mean intensity of cyprids per crab ± SE (n)		Not infected	Infection arrested	Successfully infected	(N)[c]
					Postmolt crabs[b]	Hard crabs[b]				
Carcinus maenas	97	13.5 ± 0.4	62	79	42.7 ± 10.8 (50)	41.5 ± 18.6 (27)	36	0	64	(55)
Cancer magister	62	14.4 ± 0.2	66	50	5.5 ± 1.5 (21)	49.3 ± 12.3 (15)	7	11	82	(28)
Hemigrapsus oregonensis	53	12.6 ± 0.4	55	53	25.4 ± 15.1 (14)	42.5 ± 28.0 (14)	15	19	66	(26)
Hemigrapsus nudus	14	11.1 ± 0.2	0	50		51.0 ± 20.4 (7)	14	29	57	(7)
Pachygrapsus crassipes	6	12.3 ± 1.3	100	33	9.0 ± 3.0 (2)		0	0	100	(1)

[a] Crabs exposed during trials in which *Sacculina* settled on at least one crab.
[b] Postmolt includes Drach molt stages A_1 through C_2; hard includes Drach stages C_3 through D_2 (see Materials and methods).
[c] Sample sizes are smaller than the product of columns 2 and 5, owing to early mortality, sacrifice of crabs for other observations, or accidental loss.

Settlement preference of S. carcini

Sacculina carcini settled preferentially on green crabs compared to *C. magister*, but this difference depended on molt stage. In 17 trials with larval settlement, *S. carcini* settled on both species but in lower numbers on individual *C. magister* than on individual green crabs (Table 2). The mean ratio of the number of cyprids settled on *C. magister* over those that settled on green crabs was 0.60, which is significantly different from 1 (Wilcoxon signed-rank test, $P = 0.037$, soft and hard-shelled crabs combined). This preference for green crabs was especially pronounced for soft individuals (mean ratio = 0.038, $P = 0.000$) but was non-existent for hard crabs (mean ratio = 1.945, $P = 1$).

Sacculina carcini also settled preferentially on green crabs compared to *H. oregonensis*. Cyprids settled on all but one green crab, whereas they only settled on 50% of the exposed *H. oregonensis* (Table 3) ($G_{adj} = 7.612$, $P < 0.01$). This difference was also significant when only postmolt crabs were compared ($G_{adj} = 5.917$, $P < 0.02$). No statistical comparison was made for hard crabs since there were only two trials of this type. Comparing postmolt crabs, cyprids settled in similar numbers on green crabs and *H. oregonensis* but this comparison suffered from a low sample size and an outlier (Table 3).

In all preference and non-preference settlement trials combined, the mean number of *S. carcini* settled per crab did not differ significantly by molt stage for either green crabs or *H. oregonensis* (Table 1; Wilcoxon signed-rank test, $P = 0.29$ and 0.24, respectively). However, higher numbers of cyprids settled on hard individuals of *C. magister* compared to postmolt individuals (Table 1; Wilcoxon signed-rank test, $P = 0.0003$). Only postmolt or hard individuals of *H. nudus* and *P. crassipes* were exposed to *S. carcini*, precluding similar comparisons for these two species. We did not design the settlement trials in this study to test the preference of the cyprids for molt cycle stages of individuals within a species, and the above *a posteriori* comparisons are made primarily to note the unexpected result for higher settlement on hard vs postmolt *C. magister*.

Infection outcome

Infection rates for the crabs settled by *S. carcini* are shown in Table 1. Sixty-four percent of the green crabs settled on by *S. carcini* became parasitized. Similarly high (but not statistically different) proportions of *C. magister*, *H. oregonensis*,

Table 2. Settlement by *Sacculina carcini* on postmolt or hard *Carcinus maenas* and *Cancer magister* exposed to *S. carcini* together in the same container. Two to five crabs were exposed in each trial (both species combined).

Molt stage	Number of trials	*Carcinus maenas*			*Cancer magister*		
		Percentage of crabs settled on	(Total # of crabs)	Mean # of settled cyprids	Percentage of crabs settled on	(Total # of crabs)	Mean # of settled cyprids
Postmolt	12	100	(21)	39.8	54	(22)	3.8
Hard	5	100	(10)	101.1	100	(9)	61.4

Table 3. Settlement by *Sacculina carcini* on postmolt or hard *Carcinus maenas* and *Hemigrapsus oregonensis* exposed to *S. carcini* together in the same container. Two to five crabs were exposed in each trial (both species combined).

Molt stage	Number of trials	*Carcinus maenas*			*Hemigrapsus oregonensis*		
		Percentage of crabs settled on	(Total # of crabs)	Mean # of settled cyprids	Percentage of crabs settled on	(Total # of crabs)	Mean # of settled cyprids
Postmolt	9	100	(13)	32.3 (18.3*)	55	(9)	23.5 (1.3*)
Hard	2	67	(3)	133.7	33	(3)	3.7

*Mean with outlier trial removed.

and *H. nudus* also become infected. The interna roots were first detected on the thoracic ganglion. As infections developed, the roots followed the nerves radiating from this ganglion into the chelipeds and walking legs. Roots also spread along the gut and into the digestive gland. We observed the roots of the interna of *S. carcini* in green crabs as early as 30 days after cyprid settlement and as early as 22 and 48 days in *C. magister* and *H. oregonensis*, respectively. Anecdotally, the roots in early infections appeared, by visual inspection, to have a consistently smaller diameter and a lower density of lipid droplets compared to roots of mature *S. carcini* in *C. maenas*, but otherwise were similar in morphology (Figures 2 and 3).

Individual *C. magister*, *H. oregonensis,* and *H. nudus* often showed signs of an early infection that had possibly been arrested (Table 1). These were characterized by a host response of darkened, hardened, presumably melanized structures of irregular shape, often branching in the form of roots on the thoracic ganglion, intestine or digestive diverticulae of exposed native California crabs (12, 23, and 33%, respectively, of infected *C. magister*, *H. oregonensis*, and *H. nudus*). In arrested infections, these melanized bodies were only found in or on the thoracic ganglion (Figure 4). With the exception of one *H. nudus* that developed an effective host response after being settled by 51 cyprids, and one *H. oregonensis* settled on by 13 cyprids, this cellular immune response was otherwise successful in stopping infections only in crabs settled on by 10 or fewer

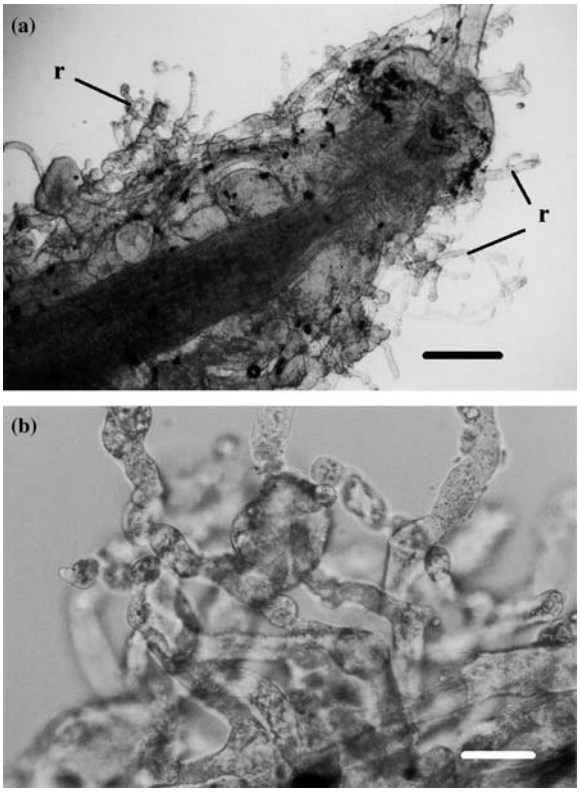

Figure 3. (a) Roots of *Sacculina carcini* on the intestine of a 14.2 mm *Cancer magister* found paralyzed and sacrificed 32 days after being settled on by 180 cyprids in the laboratory; scale bar = 400 μm. (b) Detail of (a); scale bar = 75 μm. Abbreviations as in Figure 2.

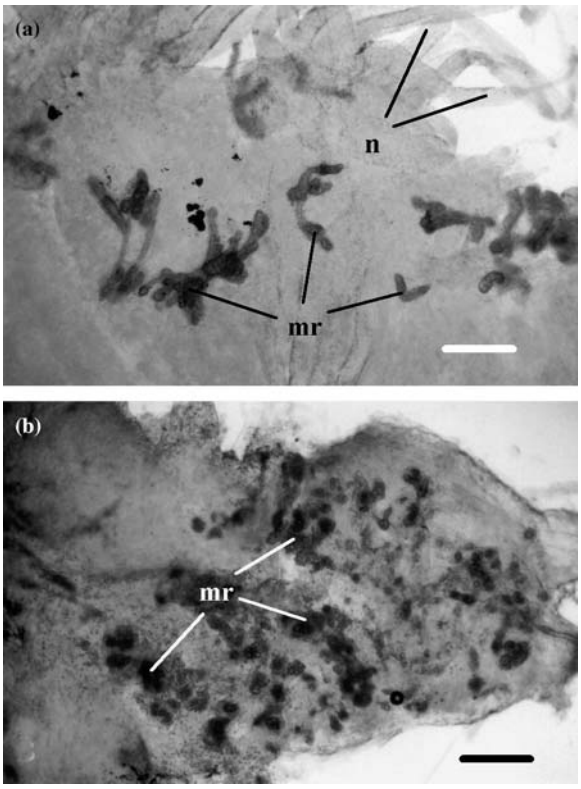

Figure 4. Melanized rootlets of *Sacculina carcini* in the thoracic ganglion of native California crabs infected in the laboratory. Dorsal views, scale bars = 150 μm. (a) Melanized rootlets in the left side of the thoracic ganglion of a 14.9 mm *Cancer magister* sacrificed 47 days after settlement by two cyprids. (b) Melanized rootlets in the anterior thoracic ganglion of a 13.9 mm *Hemigrapsus oregonensis* sacrificed 113 days after settlement by two cyprids. Abbreviations as in Figure 2.

cyprids. Branching melanized bodies were not observed in any infected green crabs or in unexposed native California crabs, but were often found on the thoracic ganglion, intestine or digestive diverticulae of native California crabs settled on by more than 10 cyprids. These bodies were usually in close proximity to, or continuous with, seemingly normal *S. carcini* roots.

Using the data for green crabs and for all four native California species, infection outcome was best modeled in a logistic regression analysis as a function of species, logarithm of the number of settled cyprids and molt stage (soft or hard) (whole model $X^2 = 44.55$, $P < 0.0001$, $R^2 = 0.283$, $n = 96$). Species and number of cyprids were significant contributors, but molt stage was not ($X^2 = 13.71$, $P = 0.008$, $X^2 = 21.08$, $P < 0.0001$, $X^2 = 2.37$, $P = 0.123$, respectively).

We used 2×2 contingency tables to examine more closely the association between infection outcome (parasitized or not, combining uninfected crabs with those that had arrested infections) and molt stage of crabs settled on by *S. carcini*. There was no significant difference in the likelihood that postmolt or hard individuals were infected ($G_{adj} = 0.842$, 3.333, and 1.456; $P > 0.1$, 0.05, and 0.1) for green crabs, *C. magister* and *H. oregonensis*, respectively). However, there was a trend in all three species, especially *C. magister*, toward infection of more hard than postmolt crabs. In *C. magister,* this trend appeared to be the result of higher settlement intensity on hard than on postmolt crabs (Table 1), combined with the positive relationship between settlement intensity and chance of infection (see below).

The relationship between percentage of crabs infected and number of settled cyprids is shown in Figure 5. Settlement by three or fewer cyprids usually did not result in successful parasitization of green crabs, usually did in *C. magister*, and had intermediate results in *H. oregonensis*. At the highest levels of cyprid settlement, all native crab individuals, but not all green crabs, became infected. These uninfected green crabs ($n = 4$) were settled upon by 40–100 cyprids and survived 41–195 days after exposure. They included postmolt and hard crabs at exposure. At postmortem examination, no evidence of interna roots was detected in them.

A least squares model of parasitization as a function of number of cyprids settled per crab revealed different estimates of susceptibility to *S. carcini* infection for different species of crabs (Figure 6). The best fit for *C. magister* was for nearly all crabs (97%) to be susceptible to infection by *S. carcini*, with a 56% chance of interna establishment of susceptible crabs per settled cyprid. The best fit for *H. oregonensis* was for a similarly high proportion of susceptible crabs (94%) but with a lower per cyprid chance of infection (24%) for those crabs. For green crabs, the best fit estimated that a lower proportion of the crab population was susceptible (84%) and these crabs had a 24% chance of infection per cyprid. These estimates are shown in Figure 6, which compares the association between the number of settled cyprids per crab and the expected proportion of the three species of crabs that became infected by *S. carcini*.

Survival of infected crabs

We compared survivorship curves for infected and uninfected crabs. Parasitized green crabs survived longer than did parasitized *C. magister*, but *S. carcini* clearly had a large effect on survivorship of both species (Figure 7). All infected *C. magister* were dead within 97 days of exposure,

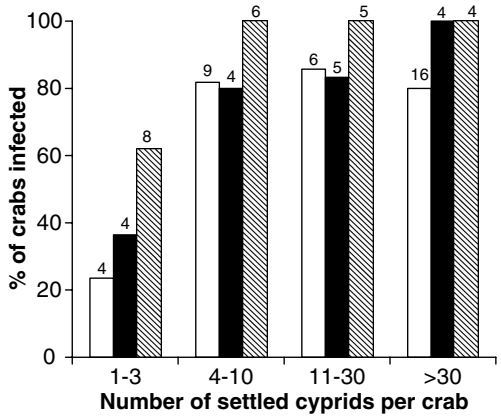

Figure 5. Percentage of *Carcinus maenas* (open bar, $n = 55$), *Hemigrapsus oregonensis* (solid bar, $n = 26$) and *Cancer magister* (shaded bar, $n = 28$) infected by *Sacculina carcini* vs. intensity of settlement by *S. carcini*. Values above bars are numbers of parasitized individuals (arrested infections not included).

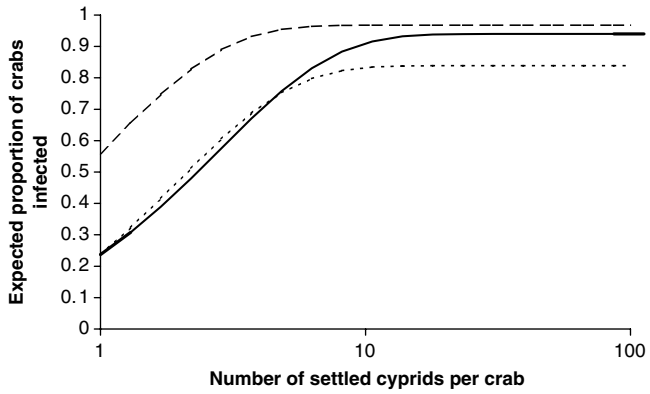

Figure 6. Association between the expected proportion of infected crabs and the number of cyprids settled per crab for green crabs (dashed line) and two species of native California crabs, *Hemigrapsus oregonensis* (solid line) and *Cancer magister* (dotted line) (exposed to *Sacculina carcini* in the laboratory. The relationship was fit with a least squares model and data on number of settled cyprids and parasitization outcome (infected vs. not infected or infection arrested).

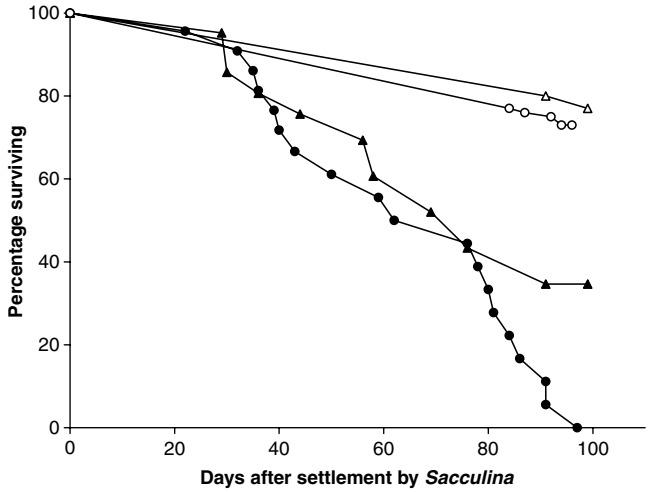

Figure 7. Survivorship of *Carcinus maenas* (solid triangles, $N = 21$) and *Cancer magister* (solid circles, $N = 23$) parasitized by *Sacculina carcini*, compared to the survivorship of uninfected crabs (*Carcinus maenas*, open triangles, $N = 90$; *Cancer magister*, open circles, $N = 105$) over the same time. Infection status was determined by presence of an interna of *S. carcini* in crabs dissected post-mortem. All infected green crabs were sacrificed to detect infection status within 120 days after exposure.

compared to 65% of the infected green crabs. Survivorship of both species did not begin to markedly decline until about 1 month after settlement by *S. carcini*, about the time we were first able to detect the roots of the interna. All seven parasitized *H. oregonensis* died within 120 days of exposure to *S. carcini*, while only 24% of the unexposed crabs died over the same time (Figure 8). Of the four infected *H. nudus*, three died between 60 and 78 days after settlement, and one died after 128 days. The single parasitized *P. crassipes* died 32 days after settlement by *S. carcini*.

Green crabs exposed to and parasitized by *S. carcini* lived up to 355 days after settlement by the parasite. In cases where the infection was successful, it appeared to follow normal development for the parasite. Although our trials were not intended to raise *S. carcini* to maturity, two infected green crabs produced (virgin) externas, one 142 days after settlement, the other after 198 days. In contrast, *S. carcini* did not follow

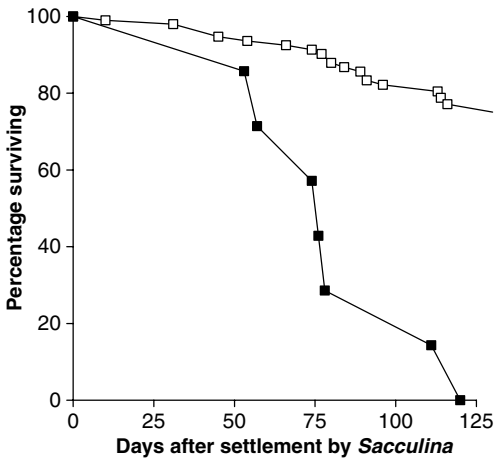

Figure 8. Survivorship of *Hemigrapsus oregonensis* infected by *Sacculina carcini* (closed squares, $n = 7$) compared to the survivorship of uninfected crabs (open squares, $n = 99$) over the same period. Infection status of exposed crabs determined by presence of an interna of *S. carcini* in crabs dissected postmortem.

normal development in native crabs and none of the exposed native crabs produced an externa. The furthest that *S. carcini* developed in native California crabs was to an abnormally early, but highly differentiated externa primordium measuring up to 265 microns in diameter. This occurred in a *C. magister* (settled on by 375 cyprids and dead after 23 days), and in a *H. oregonensis* (settled on by 100 cyprids with no host response and dead after 53 days). In both crabs, the externa primordium was ectopic, situated over the thoracic ganglion in one, and amongst digestive diverticulae in the other, rather than the normal position near the point of externa emergence on the ventral surface of the abdomen (Høeg and Lützen 1995). J. Høeg confirmed their developmental status.

Of the native crabs that died after parasitization by *S. carcini*, three *C. magister*, three *H. oregonensis*, and two *H. nudus* presented slow and uncoordinated movements, were unable to right themselves or were paralyzed up to 7 days before they died. As for other infected crabs, the thoracic ganglia of these individuals were infiltrated with the roots of the parasite. Further, in most of these crabs, the nerve cords radiating from the thoracic ganglion to the limbs, abdomen, and cerebral ganglion were severely atrophied or entirely missing (Figure 9). This pathology was not evident in other parasitized crabs, in all of which the thoracic ganglion was enveloped and infiltrated by the roots of the interna. Similar nerve damage was observed in two other *C. magister* that did not exhibit abnormal behavior before they died. The movement of one *C. maenas*, upon which 400 cyprids had settled, was also observed to be slow and uncoordinated 2 days before death.

Multiple regression analyses of the effects of crab species, crab size and the abundance of cyprids that settled on crabs in all exposure experiments revealed that, for all four native California crabs, all three variables were significant contributors to survival of parasitized crabs (Table 4); together accounting for 59% of the variance. Cyprid abundance provided the stron-

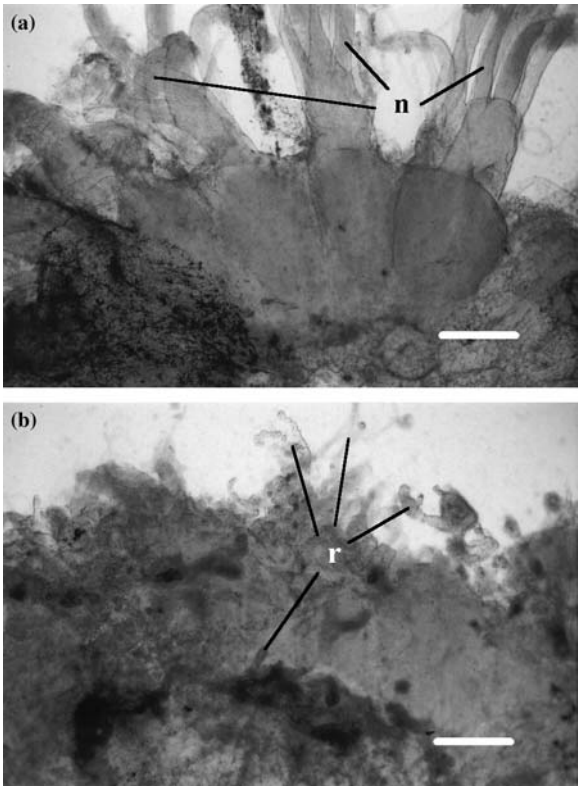

Figure 9. Comparison of the left sides of thoracic ganglia (a) a *Hemigrapsus oregonensis* not exposed to *Sacculina carcini* and (b) a *Hemigrapsus oregonensis* 52 days after settlement by over 100 cyprids in the laboratory. Note the missing and damaged lateral nerve cords in (b). Dorsal views, scale bars = 400 μm. Abbreviations as in Figure 1.

Table 4. Multiple regression analyses of survival (in days) of infected crabs as a function of the number of settled cyprids per crab, crab size (as carapace width) and, for all California native species combined, species. In these analyses, we also included crabs on which *Sacculina carcini* cyprids had settled but where the crab died within 6 weeks after exposure.

Species	Whole model			Probability of effects		
	R^2	N	P	Log (# settled cyprids)	Carapace width	Species
Cancer magister	0.42	26	**0.002**	**0.008**	**0.018**	–
Hemigrapsus oregonensis	0.37	14	0.079	0.060	0.209	–
All four California native spp.	0.59	46	**< 0.0001**	**0.0002**	**0.010**	**0.0003**
Carcinus maenas	0.08	32	0.312	0.430	0.197	–

gest effect, crab size the weakest. For analyses by species, both cyprid abundance and crab size were significant contributors to the model for *C. magister,* with the former having the stronger effect. The same pattern was seen for *H. oregonensis,* but neither effect was statistically significant (cyprid abundance almost reached the 5% level), likely due to loss of power associated with modest sample size (14). In marked contrast, the multiple regression analysis for *C. maenas* revealed no effect for either cyprid abundance or crab size ($R^2 = 0.08$, $P > 0.05$). The lack of a relationship between survivorship and number of settled cyprids was particularly evident.

Discussion

Under favorable laboratory conditions, *S. carcini* settled on and infected all four native California crab species. However, the infection process in California crabs differed dramatically from that in the natural host. Although post-molt green crabs received proportionally and numerically more settlement by cyprids, suggesting preference for the co-evolved host, hard-shelled *C. magister* were settled on by as many cyprids as hard-shelled green crabs. Initial development of the parasite was seen in all species exposed. Sixteen percent of green crabs, but few, or no, California crabs, appeared to be innately resistant to the barnacle. However, a majority of *H. oregonensis* and a minority of *C. magister* did produce a successful immune defense against the barnacle when few cyprids settled. If many cyprids settled, development of the interna did not proceed normally in native crabs, and the subsequent pathology led to increased mortality rates and eventual death of the crab and parasite. *S. carcini* was unable to produce an externa in California crabs.

Thresher et al. (2000) observed settlement by *S. carcini* in the laboratory on *C. maenas*, four species of non-host portunid crabs and one non-host grapsid crab. They found internas in three green crabs, but not in any of the non-host crabs. Genetic screening correctly identified the barnacle in the three green crabs and also revealed a weak signal for *S. carcini* in two individuals of the Australian portunid, *Ovalipes australiensis*. The latter survived 6 and 19 months after exposure to *S. carcini*, but never produced externas. Thresher et al. (2000) inferred that the infection in these *O. australiensis* were either weakly developed or 'stopped altogether'. Our results on arrested infections in native California crabs suggest that the latter outcome was more likely in *O. australiensis*, especially given their longevity after the experimental exposure to *S. carcini* cyprids.

The damage to the nervous system and the high mortality we observed in native California crabs infected by *S. carcini* was unexpected. This may have an explanation in the context of the profound effects the barnacle has on the nervous and endocrine systems of its usual green crab hosts (reviewed by Høeg 1995), compounded by the lack of evolutionary history of the parasite with California crabs. These effects include: regression or complete autolysis of the Y-organ, hypertrophy followed by degeneration of the androgenic gland, loss of function of the sinus gland, and extensive lysis of secretory regions of the central nervous system and the thoracic ganglion. Most of these effects are produced by substances released from the roots, and are not due to direct contact by the roots themselves (Høeg 1995). The composition, amount and timing of

release of these substances must represent, at least in part, adaptations by *S. carcini* for controlling its usual host. Therefore, we might expect these substances to produce variable pathology in non-host species lacking previous association with the parasite and likely differing from normal host crabs in details of their physiology and biochemistry. Moreover, if the amount of these substances is important to control of the host, we might expect pathology, and perhaps survival, to vary with settlement intensity and the resulting number of developing internas. Indeed, all of the crabs with damaged nervous systems had been settled on by high numbers of *S. carcini* cyprids (a scenario produced by our laboratory setting). Further, we found that the duration of survival of infected native crabs, but not of green crabs, was significantly related to settlement intensity. The lack of association between settlement intensity and length of survival in parasitized green crabs suggests that there is little or no dosage-dependent effect of *S. carcini* internas on survival of its usual host.

Higher levels of innate resistance in green crabs than in native California crabs might be expected given the evolutionary history of green crabs with *S. carcini*. However, we used green crabs from populations in the northeastern USA that have not been exposed to *S. carcini* since their introduction to the western Atlantic, approximately 200 years ago (Cohen et al. 1995). Since there is a general trend towards evolutionary loss of defenses in the absence of natural enemies (Thompson 1994), we might expect European populations of green crabs (and their more recently introduced offshoots) to have even higher levels of resistance to *S. carcini*, depending on the coevolutionary dynamics of this host-parasite interaction. This could increase the efficacy of the barnacle as a green crab biological control agent on the East Coast. We do not know why some green crabs appear to be resistant to *S. carcini*, but it appears that *C. maenas* can occasionally resist an early infection of *S. carcini*. We observed small, hardened, translucent, not evidently melanized bodies on the thoracic ganglia of two crabs upon which cyprids settled but that never developed internas. A genetic analysis of these two specimens detected a faint DNA signal for *S. carcini* (N. Murphy and R. Gurney, personal communication), supporting our interpretation that these infections were somehow eliminated at a very early developmental stage.

Arthropods respond to foreign tissue by enclosing the immunogenic material with blood cells followed by deposition of melanin. None of the experimentally infected green crabs produced a melanization response to these developing *S. carcini* internas, even though they do mount a very extensive response to the remaining interna after the externa dies (Veillet 1945, personal observations). In contrast, many individuals of the infected native species did respond to infections of *S. carcini* with melanization. However, when more than 10 cyprids settled, this response was usually incomplete (parts of the internas were still evidently growing) and failed to prevent a fatal outcome for these crabs. In a study of the host range of a parasitoid introduced to control non-native weevils, Barratt et al. (1997) reported that the embryos of the parasitoid wasp, *Microctonus aethiopoides,* were similarly melanized in the non-target native weevils, but not in the target weevil (which is also the natural host for the wasp). Our recent experiments with a European crab (*P. marmoratus*) sympatric with *C. maenas* demonstrate that it mounts a fully effective host response to *S. carcini*, employing early melanization (Kuris et al. submitted).

These experimental results have an important implication for the consideration of *S. carcini* as a potential biological control agent for pestiferous introduced green crab populations. Here, the lack of development to maturity of *S. carcini* in California crabs is a key finding (Sands 1998). Although *S. carcini* did not fully develop and reproduce in California crabs, our ability to infect and kill native California crabs, including the commercially important *C. magister,* indicates the potential non-target effects of *S. carcini* as a biological control agent. Our results do not indicate how extensive these impacts would be because our experimental protocol maximized the possibility of infection. The non-target impacts in nature would be a function of four sequential steps: (1) the density of infected green crabs, and (2) the probability that a female cyprid would successfully settle on a Californian crab, (3) develop and (4) evade the host response. Step 1, the density of infected green crabs, is likely to

reach a low equilibrium due to epidemiological processes (in Europe, crab densities are low where the barnacle is prevalent, Torchin et al. 2001). Step 2, the encounter of California crabs by *S. carcini* cyprids will certainly be lower than in our laboratory experiments. In particular, it will necessitate that the cyprid find the microhabitat of California native crabs which often differ from green crab habitats (McDonald et al. 2001; Jensen et al. 2002). Concurrent work indicates that an estuarine grapsid crab (*P. marmoratus*) sympatric with infected green crabs can be infected using our laboratory procedures. However, it avoids infection in nature (Kuris et al. submitted), indicating that step 2, encounter of California crabs, could be very low in nature (a true evaluation of this hypothesis would require either an experimental release of *S. carcini* or the placement of sentinel California crabs in Europe). Steps 3 and 4 indicate that *C. magister* would have a 56% chance and *H. oregonensis* would have a 24% chance of being infected and killed if settled on by a single cyprid.

In a cost-benefit analysis, these costs, plus those estimated for other species of crabs not included in our testing, would be weighed against the benefit of controlling green crabs. The most likely benefit of control would come in the form of reduced predation on mollusks, polychaetes, and other invertebrate prey of green crabs. This benefit is difficult to compare with the costs associated with potential impacts from the barnacle to California native crabs. Studies of the impact of the green crab on native California crabs would be helpful for evaluating more comparable costs and benefits of the use of *S. carcini* as a natural enemy. At this point, evidence indicates that where they overlap in distribution, green crabs prey on, displace and compete with native crabs for food; the outcome of a particular interaction varies among crab species and is affected by relative body sizes (Grosholz et al. 2000; McDonald et al. 2001; Jensen et al. 2002). The cost of mortality of native crabs due to *S. carcini* parasitization could be directly weighed against the benefit of releasing California native crabs from interactions with *C. maenas*.

A worst-case scenario would be one in which the parasite does not control the pest but has large non-target impacts. Here we can only speculate, but we assume that the impacts of *S. carcini* on native crabs would be directly proportional to the density of infected green crabs (which should theoretically be relatively low at equilibrium whether the parasite controls the crab population or not). Because the effect of a parasitic castrator on host population density seems likely to have a time delay associated with time to maturity of the host, there is the potential for an initial period where a large proportion of the pest population could be infected while it was still at high density. This would result in a relatively high density of barnacle cyprids in the water, but such an effect would be transitory or, at worst, cyclical.

Since biological control agents have never been deployed against invasive marine pests, the regulatory environment is not well defined. Presumably, regulations will crystallize in the context of a proposed candidate natural enemy. In the USA, this will involve a wide array of stakeholders including environmentalists, conservationists, marine biologists and ecologists, fishers, aquaculturists, managers, and regulators.

Significant information exists on the green crab's economic and ecological impacts, and now, on the safety and efficacy of *S. carcini* as a potential biocontrol agent. However, two elements still need to be addressed to generate a sufficiently realistic evaluation for a safe and efficacious release of *S. carcini*. Realistic field data for encounter rates by the cyprid larvae must be generated. A distinctive life history element of rhizocephalan barnacles opens up possibilities for mesocosm or even field experiments on the ability of *S. carcini* cyprids to locate and infect native crabs. Although rhizocephalans have separate sexes, only female cyprids can infect host crabs (Høeg and Lützen 1995; Figure 1). Male cyprids must await the presence of infected crabs with virgin female externas, locate such hosts and mate with them. Only then can *S. carcini* reproduce. Cyprid infectivity is ephemeral, lasting 1–2 weeks (Walker 1988), but it takes 6 months to a year for *S. carcini* development to proceed to the emergence of virgin externas. Hence, a release of cyprids to estimate attack rates can safely be done because any male cyprids released at that time will necessarily fail to locate virgin externas (Thresher 1996). Such cyprid release

experiments could be repeated for at least several weeks without any risk of establishing rhizocephalans in field populations. This life history feature also makes mesocosm experiments with diverse and naturalized biotas more tractable because, again, over a limited time span, large volumes of outflow would not have to be decontaminated. Of course, any such field or mesocosm experiments would require the biology summarized here to be fully documented, including determination of the maximum possible length of the larval period of *S. carcini*. They would also necessitate regulatory approval with careful oversight.

There is no need for biological control unless the adverse effects of the pest can be sufficiently quantified to permit a cost benefit analysis with a risk assessment. Here, the relationship between green crab density and its impact on native species (so far best described for *H. oregonensis*, Grosholz et al. 2000) should be more comprehensively documented. Density of both the green crab and native species is critical because the magnitude of any non-target impacts of *S. carcini* will depend on the abundance of the infected green crabs. Modeling these interactions and impacts should also incorporate body size data. Green crabs are significantly larger in their introduced range than in their native range (Torchin et al. 2001; Grosholz and Ruiz 2003), and the average size of a native individual is inversely proportional to the prevalence of *S. carcini*. High prevalence of *S. carcini* can, therefore, reduce the ecological impacts of green crabs since, for crabs, body size strongly affects the predatory and competitive abilities of crabs (Minchin 1997).

Finally, other natural enemies might, in theory, have the ability to control green crabs. These include the entoniscid isopod parasitic castrator, *Portunion maenadis*, the fecampiid flatworm, *Fecampia erythrocephala*, and the nemertean symbiotic egg predator, *Carcinonemertes carcinophila*. Some is known about the host specificity of these agents. *C. carcinophila*, like its congeners, attacks a wide range of crab species (Torchin et al. 1996). In this regard, it should be noted that *Carcinonemertes epialti*, which is native to the northeastern Pacific, has already expanded its host range in California to include introduced green crabs (Torchin et al. 1996). *F. erythrocephala* utilizes cancrid hosts, specifically *Cancer pagurus*, and has also been reported from hermit crabs (Kuris et al. 2002). The entoniscid isopod, seems likely to be more host specific and, therefore, may hold promise as a safe candidate if details of its culture and life history could be better understood (Høeg 1997).

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