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Comparison of Methods for Sampling Ectoparasites from Coral Reef Fishes

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Abstract. Methods for sampling ectoparasite assemblages were compared using 7 species of coral reef fishes (*Acanthochromis polyacanthus*, *Thalassoma lunare*, *Ctenochaetus striatus*, *Chlorurus sordidus*, *Scolopsis bilineatus*, *Hemigymnus melapterus*, and *Siganus doliatus*). Estimates of total numbers and composition of ectoparasites were dependent on post-collection handling techniques and the method of ectoparasite removal. Fish were enclosed within plastic bags under water at the point of capture. Filtration of water from the plastic bags revealed a large number of parasites (mainly gnathiid isopods) that had detached from the host on capture. A subsequent sea-water rinse removed a large number of ectoparasites, but further treatment with the anaesthetic chloretone released additional individuals. The few remaining parasites were removed by visual inspection. A chloretone bath was more effective than a sea-water bath at removing parasites. The species composition of parasites recovered by a chloretone bath plus a visual survey was different from that recovered with a sea-water bath and visual survey; this suggests that traditional scanning techniques may not detect all parasites.

Introduction

Parasites of fishes are increasingly being used as tools for analysis of host biogeography (Byrnes and Rohde 1992) and host evolutionary relationships (Brooks and McLennan 1993), for discrimination of fish stocks (Lester 1990), and for validation of host demography studies (Lester *et al.* 1985). Accurate estimates of parasite assemblages are of particular importance for such studies, but recent work has cast doubt on the reliability of traditional fish parasite surveys (Williams *et al.* 1991).

Parasite sampling programmes generally follow three steps that vary widely: host collection (Nagasawa 1985; Whittington and Kearn 1993), post-collection handling (Collins 1984; Byrnes and Rohde 1992), and parasite removal and quantification (Cowell *et al.* 1993; Whittington and Kearn 1993).

A few recent studies suggest that internal and external parasites may be lost during the collection of the host (Nagasawa 1985; Williams *et al.* 1991), during post-collection handling (Möller 1976; Hine 1980), and during the detection of parasites (Gaida and Frost 1991). However, only two of these studies have concerned ectoparasites, one comparing nets (Nagasawa 1985) and the other detection methods (Gaida and Frost 1991). Furthermore, only one parasite species was involved in each case. Despite increasing evidence for sampling biases in counting parasites, the issue is seldom addressed in parasitological studies.

Host collection may be subject to sampling error such as spatial and temporal variation that may confound results (Grutter 1994). Using seven common coral reef fishes of varying morphology and ecology, the present study examined sampling biases during post-collection handling and parasite removal and quantification and developed a procedure, based on six parasite recovery comparisons, for measuring parasite loads.

Materials and Methods

The fish species used in the six comparisons of methods were *Acanthochromis polyacanthus* (Pomacentridae), *Ctenochaetus striatus* (Acanthuridae), *Scolopsis bilineatus* (Nemipteridae), *Siganus doliatus* (Siganidae), *Chlorurus sordidus* (Scaridae), *Thalassoma lunare* (Labridae), and *Hemigymnus melapterus* (Labridae). They were selected because they coexist in similar habitats on the reef and are abundant and relatively easy to capture. In total, 90 fish were collected from four sites off Lizard Island, Great Barrier Reef, Australia (14°40′S,145°26′E). The sites are in shallow fringing coral reefs with different degrees of wave exposure.

Collection and Handling of Fish

Fish were captured with a 15-m × 1·6-m barrier net with a 20-mm mesh. Fish were herded into the net one at a time and captured with a hand-net. All fish except those to be transported alive in containers were placed in a quick-seal 2-L plastic bag as quickly as possible (15–60 s), then enclosed in a second plastic bag and kept underwater in a mesh bag for up to 1 h. Fish died quickly from lack of oxygen. Fish in bags were placed in a shaded 40-L plastic container of sea water for up to 1 h and taken to the laboratory. Ice was added to the water supporting the bags, and the water and fish were refrigerated for 2–10 h.

Parasite Recovery from Fish

All fish, except those collected for the parasite recovery comparisons, were removed from plastic bags and the contents of the plastic bags set aside for filtration. The whole fish, with each operculum slit at the base and pried open, was covered in a solution of 0.4% chloretone (BDH Chemicals, England) in 57-μm-filtered sea water for 30–60 min as described by Hargis (1953). Fish were then rinsed thoroughly with filtered sea water. During rinsing, the body surface, fins, gills, buccal cavity, lips, eyes, and nares were gently scraped with the nozzle of a squirt bottle. This scraping removed most of the mucus released post mortem. All plastic bags, filters, and containers were rinsed three times. All liquids were filtered (nylon plankton

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mesh). Parasites were removed from the filter and placed in vials containing 4% formaldehyde in 57-μm-filtered sea water. The gills of *H. melapterus* contain many *Hatschekia hemigymni* (Copepoda) after the chloretone bath (Grutter, unpublished observation), so the gills from all *He. melapterus* specimens were removed before the chloretone bath and fixed for parasite counts.

Counting of Parasites

The vials containing fixed parasites were allowed to settle for a minimum of 30 min and the excess liquid was decanted. No parasites were found in this liquid. The gills of *He. melapterus* were cut into separate arches, fixative was added, and the contents of the vials were shaken, then rinsed three times. Blood cells associated with these gills were removed by shaking the vials, allowing parasites to settle for 30 min, and decanting the suspended blood cells (less than 1% of the total parasites were present in decanted material). The remaining material was examined with a sorting tray under a stereomicroscope (×35) and sorted into broad taxonomic categories (Table 1).

Some copepods were larvae or males and could therefore not be identified to species. Owing to difficulties in separating males of *Orbitacolax* sp. A and *Acanthocolax* sp. A, these were combined. Because gnathiid isopods can be identified to species only from adult males (Holdich and Harrison 1980), these were reared from larvae found on *Si. doliatus* and *He. melapterus* (Grutter, unpublished data). Reared adult males were identified as a new species of *Gnathia* (B. Cohen, personal communication). Fixed larvae belonged either to this new species or to at least one other species of *Gnathia* (B. Cohen, personal communication).

Transport of Live and Dead Fish

To quantify parasite losses during the transport of live fish in containers, *He. melapterus* specimens ($n = 7$) were captured in August 1992 and placed in 20-L plastic bags to reduce handling stress. Fish were taken directly to the boat and placed in separate covered containers (20 L sea water) for 2 h during transport to the laboratory. Fish were then removed from containers, killed with a blow to the head, and placed in plastic bags and refrigerated. To determine how many parasites are lost when fish are transported dead in plastic bags, *He. melapterus* specimens ($n = 8$) were collected and placed in 2-L plastic bags, where they died. The contents of containers and bags were filtered with a 200-μm filter and fixed. The parasites in the fluids of the container and bag were compared with those recovered from fish.

Table 1. Codes of categories used for classifying ectoparasites from seven coral reef fishes

Voucher specimens have been deposited in the Australian Museum, Sydney

Copepoda
HatH = *Hatschekia hemigymni* (280 μm–1 mm)
HatA = *Hatschekia* sp. A (1·9–2·3 mm)
Orbi = *Orbitacolax* sp. A females (830 μm–1·3 mm)
Acan = *Acanthocolax* sp. A females (1·1–1·2 mm)
Bomo = *Orbitacolax* sp. A and *Acanthocolax* sp. A males (490–710 μm)
Cali = *Pseudocaligus* sp. A (1·9 mm)
CalL = Caligidae larvae (230–570 μm)
Naup = nauplii (140–200 μm)
UCop = unidentified copepods (290 μm–1·6 mm)

Isopoda
Gnat = *Gnathia* spp. larvae (540 μm–2·3 mm)

Monogenea
Anop = *Anoplodiscus* sp. (310 μm–1·3 mm)
Bene = Benedininae spp. (1·1 mm)
Dact = Dactylogyridea spp. (170–600 μm)

Digenea
TraL = *Transversotrema licinum* (370 μm–1·9 mm)
UDig = unidentified larvae and *Gyliauchea* sp. (170μm–1·3 mm)

Turbellaria
Turb = *Ichthyophaga* and *Paravortex* spp. (110–770 μm)

Platyhelminthes
UFla = unidentified flatworms (430 μm–3·7 mm)

Parasite Recovery

In January 1993, all seven fish species were used to investigate the parasite recovery process (n = 4–8 fish per species), the same individuals being used in the three comparisons. Fish were removed from the 2-L plastic bags and the contents of each plastic bag were set aside.

Rinse v. chloretone. Each fish was rinsed thoroughly and the rinse was added to the contents from the plastic bag. All fish, except for *Ch. sordidus* (see below), were then soaked in chloretone and rinsed.

Filter pore size. The chloretone and rinse solutions were filtered first with a 200-μm filter and then with a 57-μm filter, and the filtrates were kept separately.

Visual survey. To quantify how many parasites remain on the fish after the chloretone bath, the entire body surface and gill and buccal cavity of the above specimens were inspected under a stereomicroscope (×16–×20). Specimens of *Ch. sordidus* were scanned under a microscope rather than soaked in chloretone to recover parasites, as the thick mucus in their gills and body surface blocks filters. The remaining parasites found with the scan were compared with those already recovered (bag, rinse, and chloretone). Only a subsample of the fish used in the rinse and filter comparisons were scanned; this resulted in different fish sample sizes and size ranges for this comparison.

Effectiveness of Chloretone

In November 1993, specimens of *Sc. bilineatus* were soaked in either chloretone ($n = 11$) or sea water ($n = 11$) for 30 min and parasites were recovered. The fish were also scanned for remaining parasites. The parasites from the soak and the scan were summed and compared among treatments. Both filtrates (200 and 57 μm) were combined.

Statistical Analyses

When data were not normal, non-parametric analyses of variance by rank (Kruskal–Wallis test) were used. The Kruskal–Wallis test was used to compare transport of live fish and dead fish, to test for differences among fish species in the proportion of parasites that were removed by a rinse, by the 200-μm filter, and by a rinse plus a chloretone bath, to investigate whether *Ha. hemigymni* and *Gnathia* spp. (the two most abundant parasite categories on *He. melapterus*) dropped off fish in different proportions when transported in containers or bags, and to test for differences among parasite categories in the proportion of parasites removed by a rinse or by the 200-μm filter. For the last test, only species with relatively large numbers of parasites were tested (*Sc. bilineatus*, *Si. doliatus*, and *He. melapterus*); this involved three to four of the most abundant parasite categories and only individuals with those parasite categories present. Fish with no parasites were omitted from the above tests.

The proportions of total parasites (arcsine transformed to satisfy the assumption of normality) recovered with a sea-water bath and with a chloretone bath were compared by a t-test. The numbers of parasites per category were tested for differences between baths by a multivariate analysis of variance (MANOVA). To test if the parasites recovered with a bath plus a scan were the same between baths, the total numbers of parasites per category from bath plus scan were tested for differences with a MANOVA. A canonical discriminant analysis was used to discriminate among treatments when MANOVAs were significant. Data were transformed by $\ln(x + 1)$ to satisfy the assumptions of the MANOVA. The multivariate test statistic, Pillai's Trace, was used in the MANOVAs because it is more robust to heterogeneity of variance and is less likely to involve Type I error than are comparable tests (Green 1979).

Results

Fish Transport

Transport method had no significant effect on the proportion of total parasites that dropped off *Hemigymnus melapterus* or on the proportion of *Gnathia* spp. that dropped off the fish (Fig. 1). However, a higher proportion of *Hatschekia hemigymni* dropped off dead fish than off live fish (Kruskal–Wallis test: 4·364, $P = 0{\cdot}037$) (Fig. 1).

Rinse v. Chloretone

A thorough rinse of the body surface and gills removed a large number of parasites from all seven fish species, but additional parasites were recovered when fish were then soaked in chloretone (Fig. 2). The proportion of parasites removed with the rinse was not significantly different among fish species. Among the host species with abundant parasites, the proportion of parasites per parasite category removed with a rinse plus chloretone was significantly different from the proportion removed with a rinse alone for *He. melapterus* (Kruskal–Wallis test: 6·705, $P = 0{\cdot}035$) but not for *Si. doliatus* or *Sc. bilineatus* (see Figs 3*e*–3*g* for parasite categories tested). The parasites that were almost always completely removed with the rinse were all copepods (Fig. 3).

Parasites removed with chloretone were often species found in gills (unidentified flatworms and Dactylogyridea spp.) or under scales and in epidermal pockets (Turbellaria) or were possibly internal parasites released post mortem (unidentified Digenea spp.; T. H. Cribb, personal communication) (Fig. 3).

Filter Pore Size

The 57-μm filter was easily blocked with mucus and debris and required frequent cleaning. However, additional filtering at 57 μm revealed additional parasites for all fish species except *Ch. sordidus*. Only the plastic-bag contents were filtered for *Ch. sordidus* parasite species (Fig. 4), but some turbellarians, which are small parasites (Table 1), were later recovered by scanning (Fig. 5*d*). The proportion of total parasites removed by the large filter was not

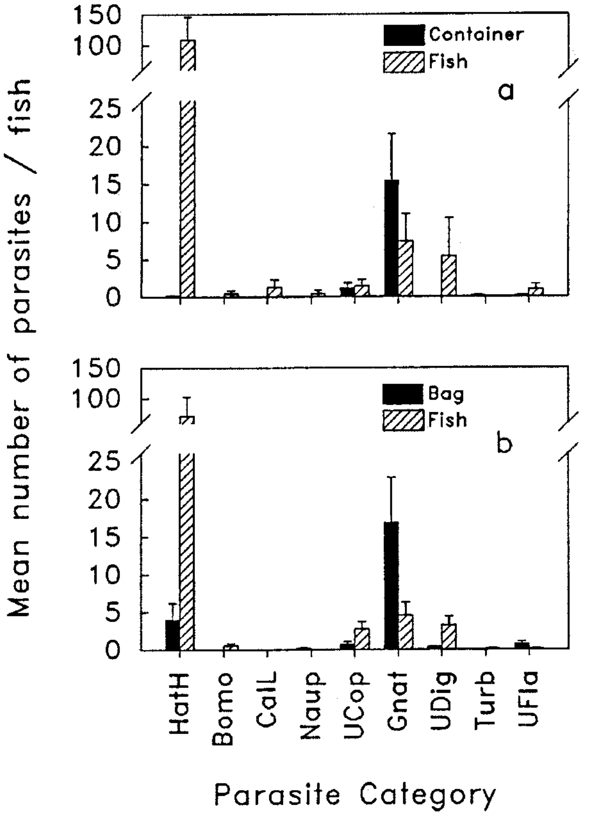

Fig. 1. Mean number of parasites (± s.e.) found in the containment liquid (container or bag) compared with those found on *Hemigymnus melapterus* after transport. *(a)* After fish were held alive in container for 2 h. *(b)* After fish were held dead in bag for 2–10 h. See Table 1 for parasite categories.

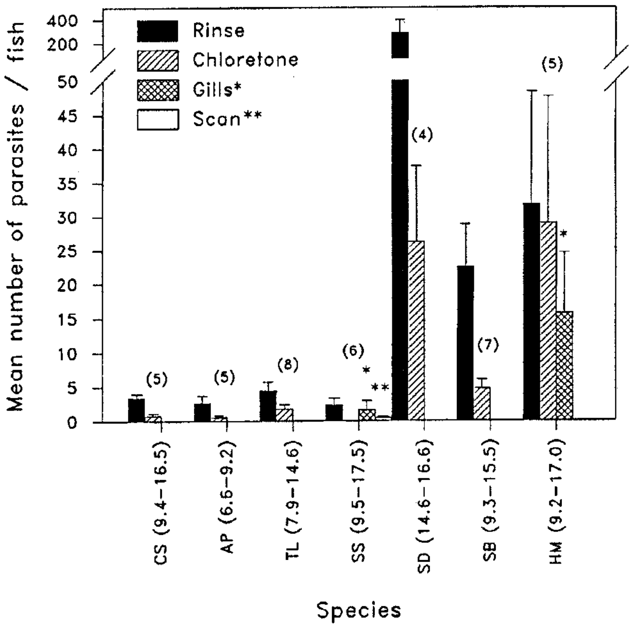

Fig. 2. Mean total number of parasites (± s.e.) removed with a sea-water rinse compared with additional parasites recovered after a chloretone bath. Rinse includes contents of plastic bag. Sample sizes in parentheses above the columns. Fish species: CS, *Ctenochaetus striatus*; AP, *Acanthochromis polyacanthus*; TL, *Thalassoma lunare*; SS, *Chlorurus sordidus*; SD, *Siganus doliatus*; SB, *Scolopsis bilineatus*; HM, *Hemigymnus melapterus*. The range in standard length (cm) is given next to the fish species code. Gills were assessed separately only for SS and HM. Scan was performed only on SS.

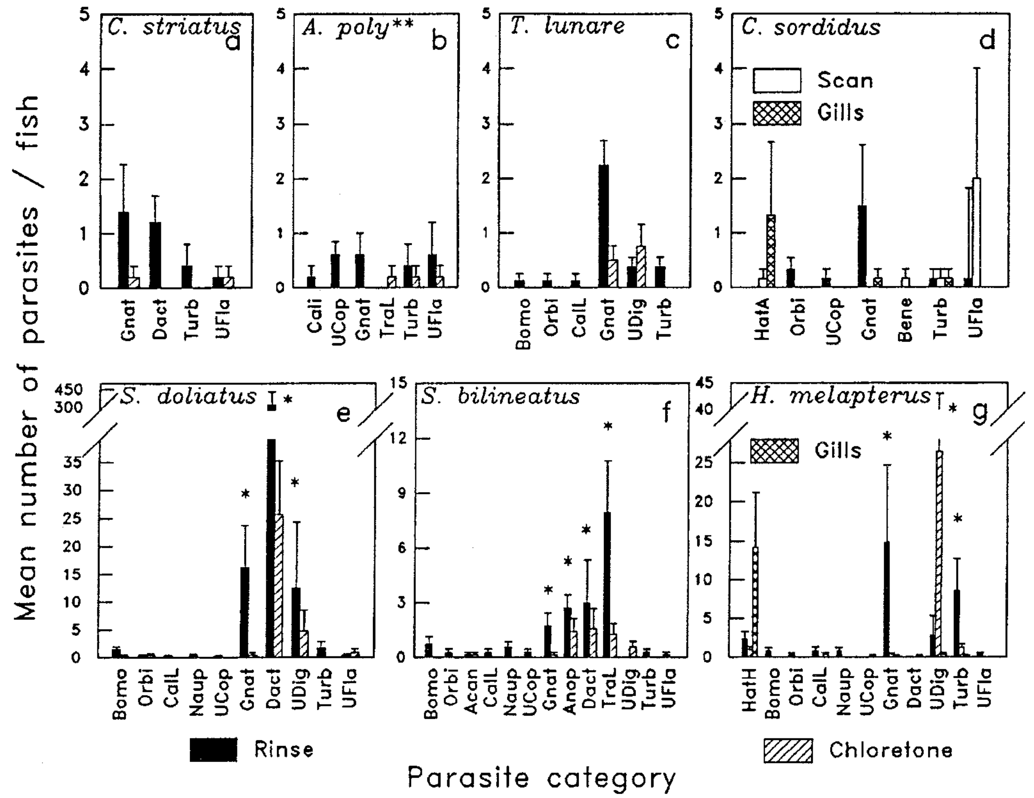

Fig. 3. Mean number of parasites (± s.e.) in each category removed with a sea-water rinse compared with additional parasites recovered after a chloretone bath. Parasites recovered separately in gills and by scanning are labelled separately. ***Acanthochromis polyacanthus*. See Table 1 for definitions of parasite categories. *Parasite categories tested for differences in the proportion removed with a rinse.

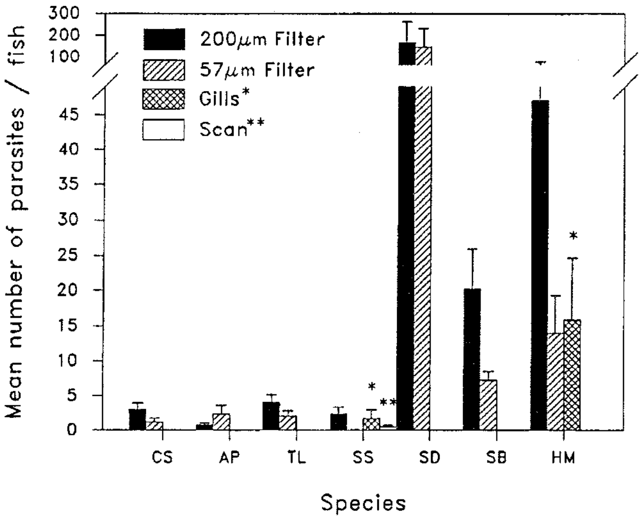

Fig. 4. Mean total number of parasites (± s.e.) removed first with a 200-μm filter and then with a 57-μm filter. Parasites recovered separately in gills and by scanning are labelled 'gills' and 'scan' and apply only to species labelled with * or **. Species names, sample sizes, and size ranges as in Fig. 2.

significantly different among fish species (Kruskal–Wallis test: 6·306, $P = 0·390$). Among parasite categories that were abundant, the proportion of parasites removed by the large filter was significantly different for *Sc. bilineatus* (Kruskal–Wallis test: 15·5, $P = 0.001$) and for *He. melapterus* (7·519, $P = 0·023$) but not for *Si. doliatus* (see Figs 5*e*–5*g* for parasite categories tested). Copepod nauplii (Table 1) were mostly recovered with the small filter (Figs 5*e*–5*g*). A proportion of Turbellaria passed through the 200-μm filter for all fish species (Fig. 5). The only categories that were always fully recovered with the 200-μm filter were the relatively large (Table 1) *Orbitacolax* sp. A females, *Acanthocolax* sp. A females, and Caliginae spp. (Fig. 5). Many gnathiid isopods were also recovered by the large filter (Fig. 5).

Visual Survey

Although many parasites were removed by rinsing, soaking the fish in anaesthetic, and filtering all liquids with the 200-μm and 57-μm filters, a visual survey revealed some parasites remaining on the fish (Fig. 6), most

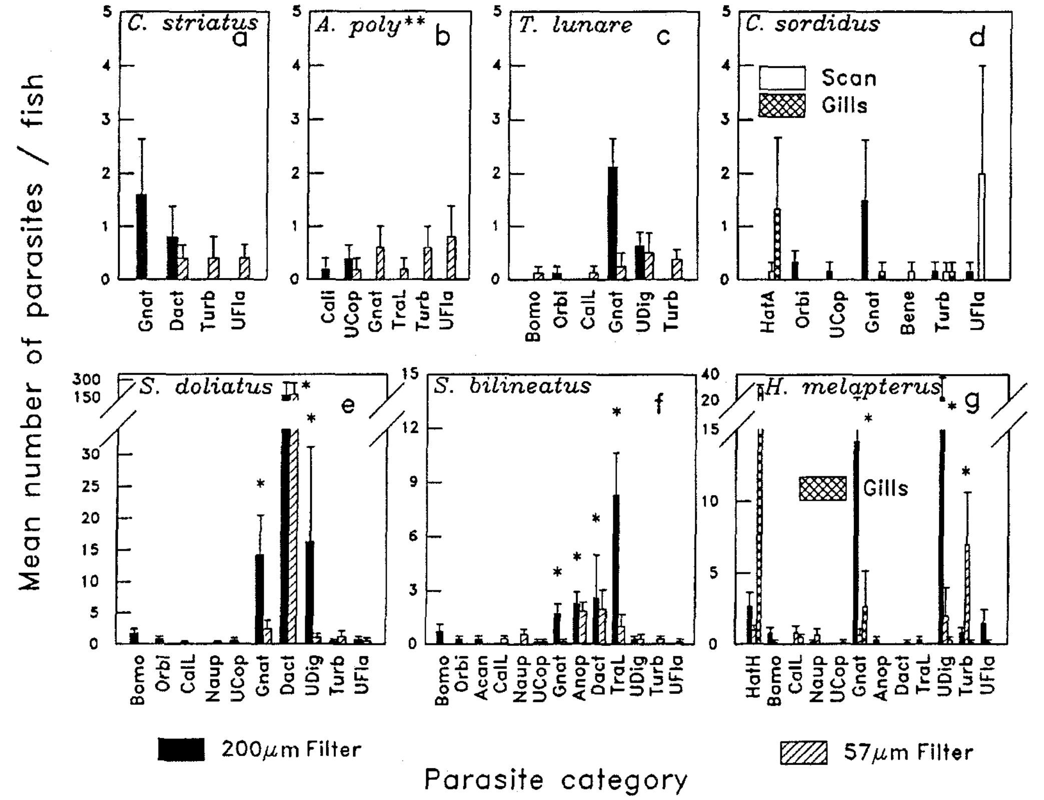

Fig. 5. Mean number of parasites (± s.e.) in each category removed first with a 200-µm filter and then with a 57-µm filter. Parasites recovered separately in gills and by scanning are labelled separately. **Acanthochromis polyacanthus*. See Table 1 for parasite categories.

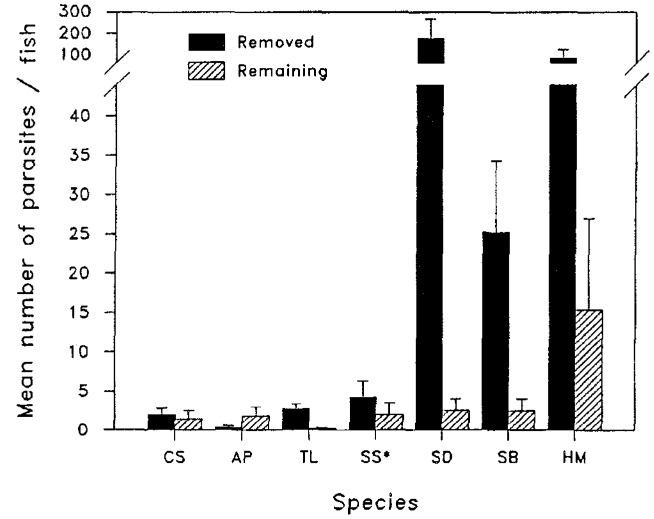

Fig. 6. Mean number of parasites (± s.e.) removed with a chloretone bath compared with parasites remaining on fish after the chloretone bath. Remaining parasites were found by scanning fish under a microscope. Species abbreviations as in Fig. 2. *Scanned under a microscope rather than soaked in chloretone.

commonly unidentified Digenea spp., Turbellaria, and unidentified flatworms (Table 2). The proportion of parasites removed was not significantly different among fish species.

Effectiveness of Chloretone

The chloretone bath removed many more parasites than did the sea-water bath (Fig. 7). The percentages of total parasites recovered with a chloretone bath (mean ± s.e., 88% ± 2·8%) and with a sea-water bath (37% ± 8·7%) were significantly different ($T = 5{\cdot}52$, d.f. = 20, $P < 0{\cdot}001$). The species composition of recovered parasites was different between treatments, with the chloretone treatment characterized by many *Transversotrema licinum* and *Anoplodiscus* sp. and to a lesser degree by Dactylogyridea spp. (MANOVA Pillai's Trace: 0·868, $F = 5{\cdot}996$, d.f. = 11,10, $P = 0{\cdot}004$). If all parasites not removed by a bath were recovered by a scan, then the species composition of all parasites collected (bath plus scan) would be the same. This was not the case, as the species composition of all parasites (bath plus scan) was significantly different among

Table 2. Mean number of parasites remaining on fish, found by scanning fish under a stereomicroscope, after fish were soaked in chloretone

Chlorurus sordidus was not soaked in chloretone but scanned under a microscope for parasites. Standard errors are in parentheses. Parasite categories as in Table 1

Fish species [sample size]	UCop		Dact		TraL		UDig		Turb		UFla	
Ct. striatus [5]	0		0		0		1·20	(1·20)	0·20	(0·20)	0	
A. polyacanthus [5]	1·20	(1·20)	0		0		0		0		0·60	(0·60)
T. lunare [5]	0		0		0		0·20	(0.20)	0		0	
Ch. sordidus [5]	0		0		0		0		0·20	(0·20)	0·20	(0·20)
Si. doliatus [5]	0		0·80	(0·80)	0		1·00	(0.77)	0·20	(0·20)	0·60	(0·40)
Sc. bilineatus [4]	0		0		0·50	(0·29)	0		0·75	(0·48)	1·25	(1·25)
H. melapterus [5]	0		0		0		12·60	(9.28)	2·80	(2·33)	0	

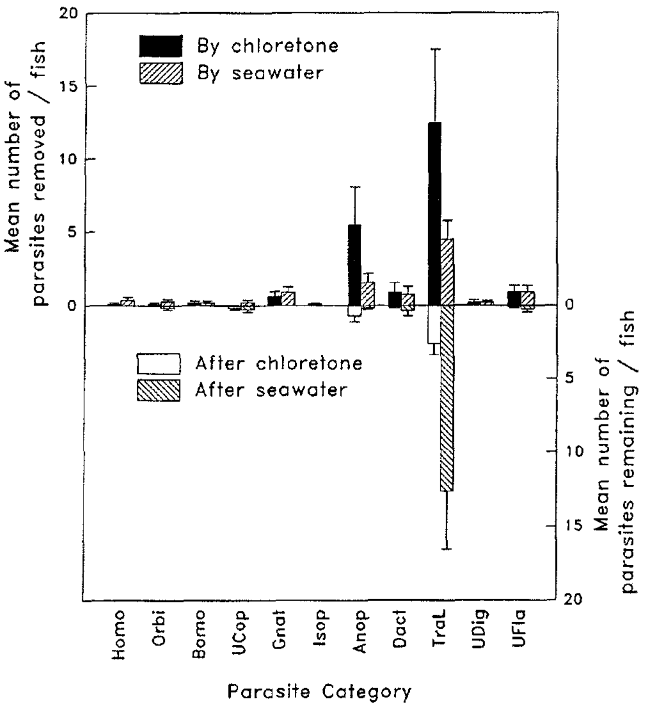

Fig. 7. Mean number of parasites (± s.e.) removed from and remaining on *Sc. bilineatus* soaked in either 0·4% chloretone or sea water only. The mean standard length (cm) (s.e.) of chloretone-soaked fish is 12·3 (0·7) and that of sea-water-soaked fish is 11·7 (0·7). See Table 1 for definitions of parasite categories.

treatments and was mainly due to more *Anoplodiscus* sp. being removed by the chloretone treatment and scan (MANOVA Pillai's Trace: 0·872, $F = 6{\cdot}193$, d.f. = 11,10, $P = 0{\cdot}004$).

Discussion

The study demonstrates that the method used to transport fish and remove ectoparasites can have a large influence on the number and species composition of recovered parasites. Two general categories of parasites are apparent: mobile crustaceans, which vacated their host on disturbance, and cryptic parasites, which remained on the host through most protocols but were recovered by the anaesthetic. Mobile parasites, particularly gnathiid isopods, that dropped off fish during transport of live and dead fish were recovered by retaining and filtering all transport liquids. It is likely that the stress of transport resulted in this loss. Several studies have shown that transportation (Specker and Schreck 1980), handling (Pickering *et al.* 1982), and capture (Perrier *et al.* 1978) of fish results in biochemical changes; these are likely to be detected by parasites. Davies and Johnston (1976) found that the capture of a blenny with a hand-net disturbed ectoparasites, including a gnathiid, and they used anaesthetics to capture the fish and decrease gnathiid loss. Thus, methods that maintain a low degree of stress during capture and handling of fish will likely result in lower parasite losses.

The method described here lowers capture-related parasite loss by using a net with a small mesh that reduces entanglement of fish and abrasion of parasites. Handling time is decreased by using SCUBA, by capturing one fish at a time, and by placing fish into plastic bags as quickly as possible. Surprisingly, plastic bags have only occasionally been used to reduce parasite losses (Hobson 1971; Losey 1974; Gorlick *et al.* 1987).

Enclosure in a bag and rinsing may be useful for recovering some types of mobile copepods, because this method mainly removed copepods that have retained the ability to swim (Yamaguti 1963). However, if all parasites are sought, especially flatworms, which were often found alive on fish dead for several hours, an anaesthetic soak is more effective. Not only were more parasites recovered by the chloretone bath than by the sea-water bath, but also a different species composition was obtained when the combination of anaesthetic bath and visual scan was used. This suggests that some parasites may remain undetected by visual scanning alone.

That there were no significant differences among fish species in the proportion of total parasites removed by only a rinse, by only a large filter, and by all treatments without a scan indicates that these sampling biases did not differ among fish species. However, there was some variation in the proportion removed in some parasite categories, and many parasite categories were missed completely by the rinse and large filter. Thus, although the method used to recover parasites may not have an effect on the relative differences in parasite loads among fish species, it does influence the apparent composition of parasites on fish.

The most efficient method for obtaining reliable estimates of the ectoparasites of the fish species investigated appears to be the following: fish are placed in plastic bags as quickly as possible, preferably underwater, and all liquids are retained; for fish species with many gill-inhabiting copepods, the gills are removed and fixed separately; fish species with high mucus loads are not soaked in anaesthetic; all liquids should be filtered with a 57-μm filter; and finally, a subsample of fish should be scanned to check for any remaining parasites.

The benefit of this method over other methods is that it reduces field laboratory time because all parasites are removed from the fish and fixed; parasites can therefore be sorted and identified at a later date. The need for modifications suggests that either the method of parasite quantification must be extremely rigorous or an optimal method must be identified for each parasite species of interest. It is only then that reliable estimates of parasite loads will be obtained.

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