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Limb Autotomy as an Investigatory Tool: Host Molt-Stage Affects the Success Rate of Infective Larvae of a Rhizocephalan Barnacle[1]

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SYNOPSIS. **D. M. Skinner was the first biologist to systematically investigate the effect of multiple limb autotomy on ecdysis in decapod crustaceans. She proposed the existence of (1) limb autotomy factor anecdysis (LAF_{an}) which initiates precocious molting, and (2) limb autotomy factor proecdysis (LAF_{pro}) which postpones proecdysis. Mean time to ecdysis did not differ significantly among groups of small juvenile *Callinectes sapidus* with zero, two, four, and six limbs removed. Variance was significantly less for the group missing six limbs; *i.e.*, autotomy of six limbs synchronized the molt-cycle. These patterns were consistent with the hypotheses that LAF_{an} exerts an additive effect, *i.e.*, more anecdysial crabs enter proecdysis as more limbs are autotomized and that a delay of proecdysis occurs above a threshold (>4) of autotomized limbs. Multiple limb autotomy provides investigators with easy access to crabs in metecdysial molt-stages which is useful in studying interactions between rhizocephalan barnacles and their hosts. While 71% of metecdysial *C. sapidus* exposed to infective larvae of the sacculinid rhizocephalan, *Loxothylacus texanus*, developed the external stage of the parasite, no similarly-sized anecdysial crab was parasitized, suggesting that these crabs are not susceptible to infection during anecdysis. Size of *C. sapidus* at infection was inversely proportional to the number of ecdyses between infection and emergence of the parasite, but not correlated to final host size. These data suggest that there is a minimum threshold for host size; smaller hosts undergo more ecdyses before attaining the threshold. Rhizocephalans are being considered as biological control agents and there is a need to understand how they find and infect hosts. Because limb autotomy is such a useful research tool, D. M. Skinner's contributions to our understanding of how limb autotomy influences the crustacean molt cycle will continue to pay significant dividends in crustacean biology.**

INTRODUCTION

Zeleny (1905) reported that crayfish missing two chelipeds molted sooner than those lacking one. The significance of this observation was not realized by carcinologists until 50 years later when Dorothy Skinner, working as a graduate student with John Welsh at Harvard, observed that *Gecarcinus lateralis* that were missing many walking legs appeared to molt sooner than intact crabs. Dorothy Bliss (1956), who was studying limb regeneration of *G. lateralis* with John Welsh, also documented the phenomenon; but it has been the laboratory of D. M. Skinner that systematicaily investigated the effects limb loss or *autotomy* has upon the molt-cycle of decapods (Skinner and Graham, 1970, 1972; Holland and Skinner, 1976) and demonstrated its usefulness as a laboratory technique (Mykles and Skinner, 1982; Soumoff and Skinner, 1983; Stringfellow and Skinner, 1988; O'Brien *et al.*, 1991). This body of work showed that the timing of ecdysis can be accelerated, not affected, or even delayed depending upon the number of limbs removed and the molt-stage of the animal at autotomy. While autotomy of any number of limbs from crabs in terminal anecdysis (*i.e.*, adult majids) or loss of four or fewer limbs from anecdysial (intermolt) animals has no effect on the time to ecdysis, autotomy of ≥5 limbs during anecdysis initiates proecdysial (premolt) activities before similar events occur in controls. The acceleration of the molt-

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cycle (manifested as a shortening of instar duration of experimental relative to control animals) has been observed to occur during the subsequent instar as well (Hopkins, 1982; O'Brien and Skinner, 1990). In contrast, loss of even one regenerating limb (a regenerating limb or *limb bud* forms at the breakage plane during anecdysis) before a critical period in proecdysis will delay subsequent proecdysial events until the lost regenerate is replaced. Skinner (1985) proposed two factors to explain the above dichotomy: limb autotomy factor anecdysis (LAF_{an}), which initiates precocious molting and limb autotomy factor proecdysis (LAF_{pro}), which postpones proecdysis. Eyestalk ablation also initiates molting in decapods and does so more quickly than limb autotomy, but mortality is high (Skinner and Graham, 1972) making autotomy the method of choice for investigators who require large numbers of crabs in metecdysis (postmolt stages).

Limb autotomy is a useful technique for investigating the biology of rhizocephalans, an order of barnacles that parasitizes decapods and other crustaceans. Rhizocephalans, in the suborder Kentrogonida, release both male and female nauplius larvae. Following metamorphosis to the cypris stage, a female larva settles upon a vulnerable host and undergoes a metamorphic ecdysis to form a *kentrogon,* an immobile, stylet-possessing stage. Infection sites vary among rhizocephalans (Høeg and Lützen, 1995); kentrogons have been observed at the base of setal hairs (Delage, 1884; Veillet, 1941), gills (Ritchie and Høeg, 1981; Glenner and Høeg, 1995) and on intersegmental membranes (O'Brien, 1984*a*). The kentrogon remains attached to the exterior of the host for at least 60–72 hours, undergoing extensive tissue re-organization. An elongated, vermiform stage enters the host through the stylet, which has penetrated the exoskeleton (Glenner and Høeg, 1995). The rhizocephalan then grows into the *interna,* a mass of tissue with roots that permeate host organs. At this stage of development, the rhizocephalan is completely internal and the host continues to undergo ecdyses. Hosts of Kentrogonida in the family Sacculinidae eventually undergo a final ecdysis (Hosts of some other families continue to molt following emergence of the parasite; see O'Brien and van Wyk [1985] for review.) and acquire the wide abdomen characteristic of adult female crabs. Within a few days a small bud, the *virgin externa* (Høeg and Lützen, 1995), emerges from the host abdomen. Male cypris larvae settle upon virgin externae, metamorphose into the *trichogon* stage (Høeg, 1987), one or two of which successfully migrate into specialized receptacles in each externa where they undergo spermatogenesis. The externae of sacculinids and most other rhizocephalans occupy what would have been the brood pouch of unparasitized hosts.

Sacculinid rhizocephalans induce such effects as: 1) *parasitic castration* or *la castration parasitaire,* host gonads do not mature, (Giard, 1886) (The accepted term, *parasitic castration,* unfortunately implies that host gonads have been destroyed which is not necessarily the case [See Hartnoll, 1967, for discussion.]), 2) *precocious maturity*, adult hosts are usually significantly smaller than unparasitized hosts, (Hartnoll, 1967), 3) *feminization*, male hosts acquire the wide abdomen typical of female crabs (Hartnoll 1967), and 4) *parasitic anecdysis*, crabs bearing externae do not molt (O'Brien and Van Wyk, 1985). Because they are parasitic castrators, rhizocephalans offer ecologists and evolutionary biologists the opportunity to study two superficially identical (*i.e.*, same phenotype) organisms/species that possess two distinct genotypes (*i.e.*, unparasitized hosts whose offspring are crabs as well as parasitized hosts who look like crabs, but whose offspring are barnacles) in the same habitat (O'Brien, 1999).

Because it is the absence of, rather than the occurrence of an event, parasitic anecdysis has been difficult to demonstrate directly. Using autotomy, however, O'Brien and Skinner (1990) showed that not one host (*Rhithropanopeus harrisii*) carrying the sacculinid, *Loxothylacus panopaei*, that had had six limbs autotomized underwent an ecdysis during the 80 day period wherein all similar-sized, untreated, unparasitized controls molted at least once and all autotomized, unparasitized crabs underwent at

TABLE 1. *Effect of multiple limb autotomy on duration of the molt-cycle of* Callinectes sapidus.

Number of limbs autotomized	Number of crabs	Initial mean CW (mm)	Mean time from autotomy to ecdysis (days ± SD)	Range (days)
0	12	19.0	10.0 ± 5.7	2–22
2	13	18.2	11.0 ± 4.6	2–20
4	12	18.7	7.8 ± 4.3	1–15
6	12	18.6	9.7 ± 1.1	8–12

least two ecdyses. This report describes how autotomy provided enough metecdysial crabs to demonstrate that host molt-stage influences the ability of a sacculinid to infect a host species.

MATERIALS AND METHODS

Animals

Unparasitized, juvenile *Callinectes sapidus* (Brachyura: Portunidae) were collected by hand-held seine at Airport Marsh, Dauphin Island, Ala. Parasitized crabs were collected by otter trawl from Mississippi Sound, Miss. and Mobile Bay, Ala. All animals were maintained on a daily diet of beef liver at 21–22°C in either individually numbered plastic tackle box compartments or perforated freezer containers in a closed seawater (25–28‰) system at the University of South Alabama. Carapace width (CW)) was measured, using Vernier calipers, as the distance between the tips of the lateral spines (range 5.1 mm to 13.6 mm).

Autotomy

Autotomy was induced by pinching the merus of the walking legs (not chelipeds) with a narrow forceps. In the autotomy experiment, assignment to experimental (autotomized) and control (intact) groups was done on the day following capture of the small, hard-shelled crabs (that had not been molt-staged) possessing a full complement of limbs. A total of 49 crabs was divided into four groups of similar-size (mean CW = 18.6 mm) that had either 0, 2, 4 or 6 limbs autotomized (Table 1) and monitored daily for molting activity. Day of ecdysis was recorded as the day an exuvia was observed.

Infection

Callinectes sapidus parasitized by the sacculinid, *Loxothylacus texanus*, were isolated in aerated buckets containing approximately 20–30 liters of seawater when their externae became dark brown. Crabs were removed from buckets following release of nauplius larvae which usually occurred within 12–24 hours of isolation. Nauplii metamorphosed into infective cypris larvae on the third day following release and remained viable for seven to ten days. Crabs exposed to cypris larvae were molt-staged according to Drach's scheme (1939) based on the rigidity of the lateral spine and layers of the endoskeleton visible at the edge of the paddle (Freeman *et al.*, 1987). Anecdysial (Stage C_4) and metecdysial (Stages B_1–C_1) crabs were exposed individually to cypris larvae in glass finger bowls containing approximately 50 cypris larvae in 200 mls of seawater. Exposed and control (not exposed) crabs remained in finger bowls (with and without larvae, respectively) for one week before being returned to the maintenance seawater system. It is not known where settlement must occur for a kentrogon to be able to penetrate the host exoskeleton and presence/absence of kentrogon stages on experimental and control crabs was not determined. Crabs were identified as parasitized when a virgin externa extruded from the host. Virgin externae appeared within one week following the molt to maturity, the ecdysis at which crabs acquire the wide abdomen characteristic of adult females (Hartnoll, 1967). In the course of this investigation only parasitized crabs underwent the aforementioned morphometric molt.

RESULTS

Autotomy

Although mean time from limb autotomy to first ecdysis did not vary significantly among the four groups (Student's *t*-test; *P*

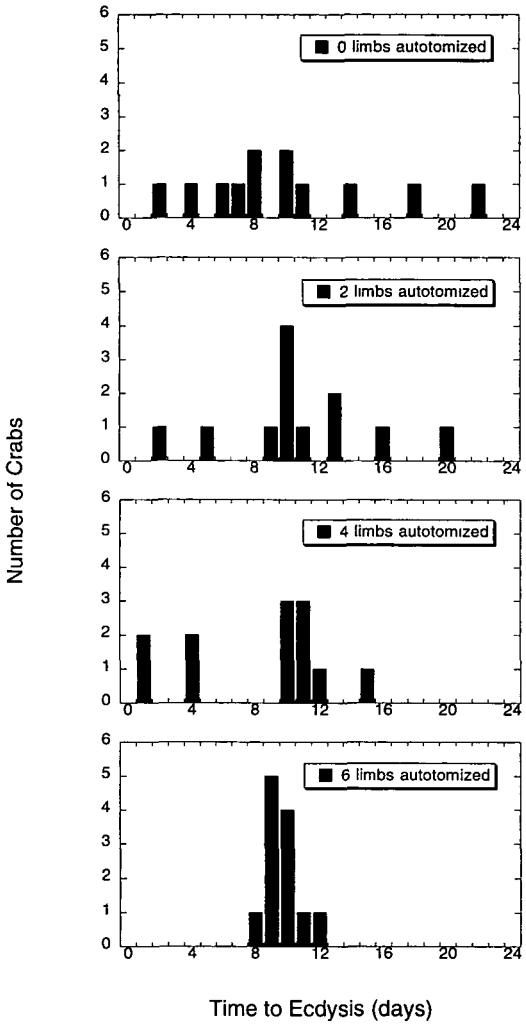

FIG. 1. Number of *Callinectes sapidus* undergoing ecdyses as a function of time (days) since Day 0 when multiple walking legs were autotomized.

= 0.05), variance was significantly less (Chi-square test, $P < 0.01$, $\chi^2 = 0.6836$) only for the group with six autotomized limbs relative to the combined total (Table 1). Reduction in range occurred for this group on both sides of the mean (Table 1, Fig. 1). While there were animals missing zero, two or four limbs that molted one or two days following autotomy, no crab missing six limbs molted before the eighth day. In this group, there was also an acceleration of molting by crabs that probably would have molted weeks after the date of autotomy. More than three weeks passed before some individuals in the groups with zero and two limbs autotomized underwent ecdyses. In the group missing four limbs, ecdysis occurred for one individual as late as two weeks after autotomy. In contrast, all individuals missing six legs had molted by the twelfth day.

Infection

Only metecdysial crabs (10 of 14; 71%) developed the external stage of the parasite following exposure to larvae; no anecdysial crab (n = 12) exposed to infective larvae (Comparison test for two proportions, $P < 0.001$, Z = 2.77) nor control crab (n = 12) exhibited signs of infection during the course of the experiment (Table 2). Data pertaining to the effect of host size at infection upon 1) number of ecdyses following infection 2) duration (in days) of internal development of the parasite, and 3) host sizes at the appearance of the externa are presented in Table 3. When means of parameters taken from infected crabs were subdivided into two groups: small (range CW = 5.6–9.9 mm, mean CW = 8.4 mm, n = 5) and large (CW = 11.1–18.6, mean CW = 13.9 mm, n = 5) and compared,

TABLE 2. Loxothylacus texanus *infection success as a function of host molt-stage.*

Host molt-stages	Number of crabs tested	Number of crabs infected[1]	%
Metecdysis (B_1-C_1)[2]	14	10	71
Anecdysis (C_4)[2]	12	0	0
Control[3]	12	0	0

[1] External stage of parasite appeared within 7 months of exposure.
[2] Molt-stage (Drach, 1939) of crabs when they were exposed to infective larvae of parasite.
[3] Collected with experimental crabs, but not exposed to parasitic larvae.

only the mean number of ecdyses (small = 7.8, large = 5.4, $P < 0.02$, Student's *t*-test) differed significantly between the two groups. Neither duration from infection to appearance of the externa (small = 168.8 days, large = 124.4 days, $0.2 < P > 0.1$) nor host size at appearance of the externa (small = 49.5 mm, large = 44.4 mm, $P > 0.5$) differed significantly from each another.

DISCUSSION

The primary purpose of the autotomy experiment was to determine when autotomy should be performed and how many limbs needed to be removed to ensure that an adequate number of potential hosts would be available when parasite larvae became infective. Prior to conducting the experiment, it was judged that, although the sample sizes would be relatively small, 12 or 13 individuals in each experimental group would be sufficient to answer the aforementioned questions. The data demonstrated that autotomy of six limbs induced ecdyses within ten days in animals that would probably have undergone ecdysis after two weeks and, indeed, the requisite information is now available. *Loxothylacus texanus* releases broods approximately every seven days at room temperature and infective cypris larvae are present the third day following release (O'Brien, unpublished); thus, if six limbs are autotomized from recently collected, small (CW approximately 19.0 mm) crabs the day a parasite releases larvae, metecdysial crabs will be available for exposure when larvae from the next brood of that parasite become infective.

Following data analysis of the autotomy experiment, one interpretation suggested that depending upon the number of limbs removed and, perhaps, the molt stage of the animal, limb autotomy might have influenced the timing of ecdysis in more than one manner. Yet, because (1) crabs had not been definitively molt-staged (other than being "hard-" or "soft-shelled") and (2) sample sizes were relatively small, no conclusions can be drawn. There was a significant decrease in variance of the time to first ecdysis for those *Callinectes sapidus* that had lost six limbs relative to the other groups. In other words, autotomy of six limbs synchronized the molt-cycle (Fig. 1). However, no significant effect of autotomizing different numbers of limbs was observed on mean time to ecdysis of *C. sapidus* at the sizes tested. Since autotomy of larger specimens of *C. sapidus* has been shown to accelerate the molt cycle (Skinner and Graham, 1972), one could argue that the absence of an acceleration of the molt-cycle observed was due to the small size of the crabs examined. For example, if LAF_{an} propels crabs into proecdysis by reducing the duration of the C_4 stage of anecdysis and if small, intact juveniles do not spend a significant portion of the molt-cycle in C_4, then autotomy would not shorten the molt-cycle. McConaugha and Costlow (1980, 1987) found that autotomy of megalopae and early post-larvae of *Rhithropanopeus harrisii* (which molt every few days) actually lengthened the anecdysial period if regenerating limbs were formed. Since O'Brien and Skinner (1990) found that autotomy of six limbs of larger *R. harrisii* accelerated molting; it is conceivable that there exists an intermediate size at which autotomy would have no effect upon timing of ecdysis. This argument, however, is not supported by the evidence. No crab missing six limbs underwent ecdysis after the 12th day; rather as more limbs were removed, the upper range (*i.e.*, the last crab to molt) in each group was closer to the mean time of ecdysis for that group: 0 limbs, 22nd day; 2 limbs, 20th day; 4 limbs, 15th day; and 6 limbs, 12th day (Fig. 1). Fingerman and Fingerman (1974) may have observed a similar phenomenon; the rate of ecdysis of *Uca pugilator* missing one to eight autotomized limbs increased directly with the number of limbs that had been autotomized. In the investigation reported here, at least two crabs in every treatment except those that had lost six limbs underwent ecdysis in the first week and one interpretation of these data is that ecdyses were not delayed if four or fewer limbs had been removed. Certainly those crabs that molted within two days following autotomy were undoubtedly already in proecdysis when limbs were removed and it is not unreasonable that, by chance, proecdysial crabs were not

included in the group from which six limbs had been autotomized. Yet these results are also consistent with the hypotheses that (1) the putative LAF_{an} exerts an additive effect, *i.e.*, more anecdysial crabs enter proecdysis as more limbs are autotomized and (2) a threshold (>4) of autotomized limbs is required before proecdysis is delayed. Still the data only suggest something interesting may have happened when six rather than four limbs were autotomized. Future efforts to elucidate mechanisms by which limb autotomy induces its' effects should not only utilize sample sizes larger than used here and determine the molt-stage of every crab at autotomy. In addition, the experimental protocol should incorporate the concept of limb regeneration as the first ecdysis following autotomy has been shown to occur later for crabs that regenerate limbs prior to that ecdysis compared to similar-sized crabs that molt quickly and then regenerate autotomized limbs (Kuris and Mager, 1975).

Others have anecdotally reported that sacculinid cypris larvae preferentially settle upon metecdysial crabs (Delage, 1884; Veillet, 1945; O'Brien, 1984a; Walker *et al.*, 1992). Even though larval settlement was not examined in this investigation, this is the first report to quantify the degree to which host molt-stage at exposure to infective larvae affects the relative number of crabs that will eventually carry the external stage of the parasite. The inability of *Loxothylacus texanus* to infect anecdysial *Callinectes sapidus* (Table 2) suggests that this host possesses a defense mechanism. The time from settlement of a cypris larva, through metamorphosis into the invasive vermiform larva, requires at least 60–72 hours for both *L. panopaei* (Glenner and Høeg, 1995) and *L. texanus* (O'Brien and Hed, unpublished), a period during which kentrogons could be dislodged by grooming behavior of the host. Pertinent to this discussion is a scene in a copyrighted video by the author in which the setose walking leg of a blue crab can be seen to vigorously rotate immediately after a *L. texanus* cypris larva "walks" on the intersegmental membrane joining the coxa to the thorax (O'Brien, 1998). Ritchie and Høeg (1981) reported that the anomuran, *Petrolisthes cabrilloi* removed kentrogons of *Lemaeodiscus porcellanae* (Lernaeodiscidae) with its cleaning appendages. A series of exposures to infective larvae of intact *P. cabrilloi* (controls) and crabs whose cleaning appendages had been removed resulted in prevalences ranging from 4.8–15.8% for the controls to 63.3–86.7% for the experimental animals. In Mobile Bay and Mississippi Sound, blue crabs bearing rhizocephalan externae are collected using benthic trawls or traps; in eight years no crab bearing an externa was collected using hand-held equipment in shallow water (O'Brien, unpublished). Where and when blue crabs become infected is unknown; but evidence is accumulating to suggest that infection does not occur in shallow water. None of the control animals (which had been collected in shallow water) in this study, for example, developed externae. Evidence from another study indicates that male cypris larvae are restricted to the deeper waters of inland waterways as only virgin externae on crabs placed on the bottom (3 to 4 m depth) of Mississippi Sound developed into egg-producing adults (Cej *et al.*, 1997). Recently, using the polymerase chain reaction (PCR) technique, it was possible to identify parasitized crabs as parasitized within days following infection in the laboratory (Woodard *et al.*, 1998) and the technique should shed light on the season and location within inland waters that blue crabs are being infected by the rhizocephalan.

Final host size was not significantly related to size at infection (Table 3). On the other hand, the number of ecdyses from infection until emergence of the externa was not fixed, but was inversely correlated with size at infection; animals that were smaller (<10.0 mm CW) at infection underwent significantly more ecdyses than larger (>10.0 mm CW) ones (Table 3). Although not highly significant ($0.2 < P > 0.1$), there was a tendency for *L. texanus* to develop for a longer period inside hosts that were small at infection than in those that were relatively larger (Table 3). Both these parameters could reflect the existence of a host size-threshold below which the parasite does not mature; hosts that are smaller at infection pass through more instars and

TABLE 3. *Duration of internal development of* Loxothylacus texanus *in* Callinectes sapidus.

Host size at exposure (mm)	Host ecdyses from exposure to appearance of externa[1]	Days from exposure to appearance of externa[2]	Host size at appearance of externa (mm)[3]
5.6	9|	167	49.6
7.9	7|	138	39.4
9.0	8|	143	38.3
9.8	7|	180	49.9
9.9	8|	216	70.5
11.1	5||	94	36.3
12.8	5||	130	43.1
12.9	6||	153	46.1
14.0	5||	128	38.0
18.6	6||	117	58 5

[1] Single bar (small crabs): mean = 7.8; double bar (large crabs): mean = 5.2 $P = 0.01$, Student's t- test.
[2] Small crabs, mean = 168.8 days; large crabs, mean = 124.4 days $0.2 < P > 0.1$, Student's t-test.
[3] Small crabs, mean = 49.5 mm CW; large crabs, mean = 42.7 mm CW $P = 0.5$, Student's t-test

require more time to attain the threshold than do larger ones. Such an interpretation is supported by Lützen (1984) who found a minimum size at first oviposition (*i.e.*, maturity) for all *Sacculina carcini* even though externae on large hosts would eventually attain a larger size than those on small hosts. The existence of a size-threshold for crustacean maturation was addressed by Born (1970) and Kuris (1971). O'Brien (1984b) discussed the Born-Kuris size-threshold model to explain size differences observed between unparasitized adult majids (*Pugettia producta*) and those parasitized by the sacculinid, *Heterosaccus californicus*. In that association, although mean size of unparasitized crabs was greater than that of parasitized crabs, the smallest unparasitized adults were as small as the smallest parasitized crabs. In the association examined here, however, there is typically no overlap in size between parasitized and unparasitized crabs; the smallest unparasitized adult *Callinectes sapidus* are usually much larger than parasitized crabs although occasional ovigerous adults are found in the size range of parasitized crabs (Overstreet *et al.*, 1983).

In recent decades, huge ships have transported great volumes of ballast water (and accompanying marine invertebrate larvae) across oceans resulting in massive introductions of marine invertebrates to novel habitats throughout the world (Carlton, 1989; Cohen and Carlton, 1998). Arriving as larvae, introduced species no longer encounter many of the pathogens and predators that helped control their numbers at their point of origin (Lafferty and Kuris, 1996). For some reason, many brachyurans, such as the European green crab, are particularly likely to become pests in their new habitats. Rhizocephalans, because they castrate hosts and exhibit some degree of host specificity, have been suggested as possible biological control agents against brachyuran pests (Lafferty and Kuris, 1996). The proposal is controversial because of the environmental repercussions should the rhizocephalans infect native as well as introduced crabs. There is a need, therefore, for more information concerning how rhizocephalan larvae detect and infect vulnerable hosts. Autotomy should be an invaluable tool in these investigations; routinely providing researchers with numerous similarly-sized, proecdysial, ecdysial, and metecdysial native and non-native crabs. Beyond a doubt, with her original observation concerning how limb loss accelerates molting of Bermuda land crabs, and her subsequent research on this phenomenon, Dorothy Skinner leaves a legacy to crustacean biology that will pay research dividends into the distant future.

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