

# Ontogenetic shift in host tolerance controls initiation of a cleaning symbiosis

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When the interests of mutualists are not perfectly aligned, control mechanisms that modulate interactions can maintain mutually beneficial outcomes and stabilize mutualisms over evolutionary time. However, the costs and benefits of symbiosis often change with ontogeny and whether control mechanisms are adjusted to reflect ontogenetic changes is largely unknown. We examined the recently described cleaning symbiosis between crayfish *Cambarus chaugaensis* and ectosymbiotic annelids (*Xironodrilus appalachius*) for evidence of ontogenetic changes in symbiont control. *Xironodrilus appalachius* provide a beneficial cleaning service to *C. chaugaensis* by removing epibiotic accumulations from the gills, but crayfish also incur costs via density-dependent facultative parasitism of gill tissue. A series of laboratory experiments using crayfish from three size (age) – classes demonstrated that crayfish use grooming to limit cleaner density and grooming effects on cleaners varied with crayfish age. Small crayfish quickly removed essentially all of their cleaners. Intermediate crayfish removed most of their cleaners, but some cleaners persisted at a location apparently inaccessible to grooming and far from the gill chamber. Large crayfish removed a smaller proportion of cleaners and cleaners were allowed access to the gill chamber, thus initiating the cleaning symbiosis. Cleaner removal was not dependent on cleaner density, suggesting that crayfish do not regulate cleaners to a specific density. Experimental results were corroborated by patterns observed during a field survey. We argue decreased cleaner removal and relaxed control of cleaner attachment sites corresponds to ontogenetic changes in the costs and benefits of symbiosis. This study integrates two major theoretical perspectives from ecological literature; control mechanisms and ontogenetic shifts, and illustrates how changes in control mechanisms with ontogeny may favor life-long positive outcomes of symbiosis. Ontogenetic shifts in the costs and benefits of symbioses may be common; therefore future theoretical and empirical studies of symbioses should incorporate both perspectives.

Mutualisms are pervasive, affecting most species either directly or indirectly (Herre et al. 1999). Although prevalent in nature, the evolution and long-term maintenance of mutualistic interactions may seem paradoxical because natural selection should favor individuals that cheat or overexploit their partners to gain increased benefits at the expense of their partners and non-cheating conspecifics (Trivers 1971, Axelrod and Hamilton 1981). Adaptive response that limit cheating and overexploitation and/or favor beneficial interactions ('control mechanisms') offer an explanation. Control mechanisms such as punishment, rewards, selectively choosing partners or dictating interaction duration allow a wide diversity of disparate species to maintain mutually beneficial interactions (Johnstone and Bshary 2002, Bshary and Bronstein 2011). Thus, the evolutionary stability of mutualisms can be preserved by adaptive responses between partners that prevent overexploitation and a resultant transition towards parasitism (Bull and Rice 1991, Pellmyr and Huth 1994, Johnstone and Bshary 2008, Bshary and Bronstein 2011, Kiers et al. 2011).

Changes in the age-specific biology of an organism add another layer of complexity to symbiotic interactions. An interaction may be more or less advantageous at one life-history stage and inconsequential or even disadvantageous at another (Palmer et al. 2010, Yang and Rudolf 2010, Yule et al. 2013). Consequently, the life-long effects of symbiosis on fitness may integrate variable effects experienced throughout ontogeny (Yule et al. 2013). It follows that species could maximize the life-long benefit of an interaction by adjusting control mechanisms to reflect ontogenetic changes in the costs and benefits of an interaction, but this potentially crucial facet of symbioses has yet to be thoroughly evaluated. We examined the recently described cleaning symbiosis between the crayfish *Cambarus chaugaensis* and an ectosymbiotic annelid (Branchiobdellida; *Xironodrilus appalachius*) (Brown et al. 2012) for evidence of ontogenetic changes in a control mechanism that limits overexploitation.

Branchiobdellidan annelids are ectosymbionts of freshwater crustaceans, and almost exclusively of crayfish (Gelder 2010). They graze on bacteria, algae, fungi and other

organisms and materials that collect on the exoskeleton and gills of their hosts (Weigl 1994, Brown et al. 2002, Gelder 2010). Branchiobdellidans can have positive effects on host growth rate and survivorship by grazing potentially harmful epibiotic material from the surfaces of their host, especially the gills (Brown et al. 2002, 2012, Lee et al. 2009). However, crayfish do not always benefit from interactions with branchiobdellidans. Some branchiobdellidan species may actually be obligate gill parasites (Hobbs et al. 1967, Grabda and Wierzbicka 1969, Quaglio et al. 2006, Rosewarne et al. 2012), whereas other branchiobdellidan species, including known cleaners, are facultative parasites (Brown et al. 2012). Parasitic attacks on crayfish gills increase with branchiobdellidan density (Brown et al. 2012, Rosewarne et al. 2012). Consequently, the negative effects of parasitic attacks under very high cleaner density can outweigh the positive effects of cleaning, resulting in a density-dependent shift from mutualism to parasitism (Brown et al. 2012).

The relationship between crayfish and their cleaners may also change as crayfish age and concomitantly the potential benefit of being cleaned increases. We hypothesized that crayfish regulate cleaner density to yield a net positive outcome, and that the density to which cleaners are maintained changes with crayfish age. Crayfish rid their respiratory surfaces of epibiotic material by molting (Bauer 1998), time spent between molts (inter-molt) increases with age, and therefore accumulations of epibiotic material also increases with age (St John 1976, Bauer 1998). Increasing epibiotic accumulations presumably results in a greater potential benefit from cleaning services and a density-dependent shift from net positive to net negative effects of branchiobdellidans likely occurs at higher densities on older hosts, whereas young crayfish likely experience little or no benefit at any cleaner density. Crayfish use the opposing finger and dactyl of their walking legs to groom debris and epibionts from their exoskeleton (Jones and Lester 1996, Farrell et al. 2013), and on several occasions we (JS and BLB) have observed crayfish removing and subsequently consuming individual branchiobdellidans from their carapaces. We conducted a series of laboratory experiments and a field survey to assess the following questions: 1) does crayfish grooming significantly influence cleaner density? 2) If crayfish influence cleaner density by grooming, are cleaners regulated to a particular density and does that density increase with crayfish age?

Understanding the ecological and evolutionary forces that maintain mutualisms has been a core objective of organismal biology since Darwin (1859). Our work builds on long-standing frameworks that explain the maintenance of mutualisms through control mechanisms. We show that incorporating ontogenetic behavioral changes can expand our understanding of how control mechanisms promote mutually beneficial outcomes of symbiosis. The perspective of controls in symbioses has an established history in ecological literature (Bull and Rice 1991, Pellmyr and Huth 1994), but the importance of ontogeny in dictating the costs and benefits of symbiosis is a newly emerging perspective (Palmer et al. 2010, Yang and Rudolf 2010, Yule et al. 2013). We have synthesized the long-standing controls perspective with the emerging ontogeny perspective to show how changing controls with ontogeny may promote

life-long benefits of symbiosis and therefore our work provides a new understanding of how mutualisms are maintained.

## Methods

### Study site

All crayfish and branchiobdellidans used in this study were collected from Walldrop Stone Creek (WSC), near Clemson, SC, USA. WSC is a low order perennial tributary ( $\approx 2$  m wetted width) of the Savannah River system that runs 1.2 km through a mostly closed hardwood canopy to its confluence with the Lake Hartwell impoundment. Although at least two species of branchiobdellidans occur in WSC, we only used the cleaner *X. appalachius* in grooming experiments because this species was previously demonstrated to have measurable positive and negative density-dependent effects on crayfish growth in WSC (Brown et al. 2012).

### Handling and aquarium setup

Crayfish were collected by hand, measured by carapace length (CL) in the field, and transported back to the laboratory contained individually in plastic bags filled with stream water packed into plastic coolers. Large *X. appalachius* ( $> 3$  mm) were carefully removed from the crayfish using fine-tipped forceps under a dissecting microscope and kept in a small dish of aerated stream water for later use. All crayfish were submerged in a 10%  $MgCl_2$  hexahydrate solution for 5 min, a procedure that effectively kills any remaining branchiobdellidans and their eggs, but causes no noticeable harm to the crayfish (Brown et al. 2002). Crayfish were then placed individually into 24 glass aquaria (38 l) filled to one third of total volume with aged and aerated tap water. Aquaria contained natural substrate from WSC, which consisted primarily of sand and fine gravel, and several small cobbles. Aeration was supplied by a single airstone, temperature was maintained at 18–20°C to reflect temperatures of WSC, and standard fluorescent ceiling fixtures provided light for 14 h per day. We replaced 50% of the water in each aquarium weekly and crayfish were fed 2–3 commercial shrimp pellets twice weekly. Crayfish were given one week to acclimate to aquaria and recover from dactyl ablation before cleaner treatments were applied. After acclimation, cleaners were placed on the dorsal aspect of the hosts' carapaces and successful attachment of all cleaners was confirmed before crayfish were returned to their aquaria.

### Grooming experiments

We conducted a series of laboratory experiments using hosts from three size-classes to assess grooming as an age-specific control in the cleaning symbiosis between crayfish *C. chaugaensis* and branchiobdellidans *X. appalachius*. Treatment groups in which the dactyls of the crayfish walking legs (pereopods 1 and 2) were ablated were compared to control groups which had their dactyls intact and we interpreted positive effects of dactyl ablation on branchiobdellidan

density (all else being equal) as an indication of a negative effect of grooming on branchiobdellidan density. To determine if crayfish regulate their cleaners to a targeted density and if the targeted density changes with ontogeny, we compared grooming effects of three crayfish size-classes at multiple initial cleaner densities. We supposed that if cleaner density is actively regulated by the host, then densities observed under natural conditions should reflect the targeted density of each host. Furthermore, if crayfish regulate cleaner density then crayfish exposed to densities higher than the targeted density should reduce cleaner loads to the targeted densities. Therefore, cleaner density treatments were scaled for host size-class to reflect normal and higher than normal densities observed in the field. For each size-class, the 'normal density' treatment was defined as the mean number of mature *X. appalachius* found on crayfish of that size-class at the time of collection. Mean field densities were determined for each size-class by counting the number of cleaners present on crayfish used in each experiment at the time of collection. High density was defined as the normal density plus two standard deviations, and very high was defined as the normal density plus four standard deviations. Small and large size-classes were exposed to 'normal' and 'high' cleaner density. The intermediate size-class received an additional 'very high' density treatment. The decision to include a third density treatment level in this size-class but not the others was based on the availability of appropriately sized *C. chaugaensis* and *X. appalachius*. This yielded 2 and 7 cleaners for the smallest size-class; 5, 10 and 15 cleaners for the intermediate size-class; and 20 and 40 cleaners for the largest size-class. Our first experiment began 11 May 2011 and included 24 crayfish from the smallest cohort present in WSC at that time (15–18 mm CL), hereafter referred to as 'small' crayfish. The second experiment began 6 June 2011 and included 24 crayfish that were 18–23 mm CL and are referred to hereafter as 'medium' crayfish. Our final experiment began 4 July 2011 and included 16 'large' crayfish (27–35 mm CL). The number of experimental units in the large size-class was limited by the number of *X. appalachius* that could be collected from WSC in a timely manner.

Each experiment lasted approximately 3 weeks (21 d, 21 d, and 18 d for small, intermediate and large hosts respectively), during which we periodically counted the number of cleaners remaining and their locations on their hosts. Cleaners were located by placing crayfish in a small beaker of water and systematically examining all visible surfaces with a 5× magnification hand lens. This approach permitted careful inspection of all the external aspects of the crayfish while minimizing disturbance to the crayfish and their cleaners. Cleaners were occasionally dislodged from their host while being retrieved from the experimental chambers and were counted as present on the host, but their location was recorded as unknown and not included in analyses of attachment sites. The large size-class experiment was terminated on day 18 due to a failure of the laboratory climate control system.

## Data analysis

For each crayfish size-class experiment, we tested for effects of dactyl ablation and initial cleaner density on the proportion of cleaners remaining on their hosts through time

(persistence) and all possible interaction terms using repeated measures analysis of variance (RMANOVA, `aov()` function in R base package). Additionally, we used a two-way ANOVA (`aov()` function in R base package) to test for the effects of dactyl ablation and initial cleaner density on the proportion of cleaners remaining at each day of data collection, for each experiment. All proportional data were normalized prior to analysis using an arcsine square root transformation. We also tested for effects of host size-class, dactyl ablation, and initial cleaner density on the proportion of cleaners present at all locations on the hosts' bodies using permutational multivariate ANOVA conducted on a Euclidean distance matrix (PERMANOVA, `adonis()` function in `vegan` package [2·0-0], R; Oksanen et al. 2011).

## Field survey

Crayfish of all sizes were collected from the entire length of WSC throughout June and July of 2010 to determine relationships between crayfish size and cleaner density and attachment sites. Crayfish were collected by hand and by dip net and transported individually in plastic bags filled with stream water. After sex and species identification, all crayfish were inspected live under low-magnification dissecting microscopes for the presence and location of branchiobdellidans. Due to the difficulty in determining the identity of branchiobdellidans still on their host, we did not distinguish branchiobdellidan species in these surveys. A subsequent collection of 493 branchiobdellidans from WSC (collected November 2012) recovered two sympatric species, *Cambarincola philadelphicus* in addition to *X. appalachius*. The smaller *Cambarincola philadelphicus* constituted an average of 84% of the branchiobdellidans on each crayfish and *X. appalachius* constituted 16% ( $\pm 12.8\%$  SD). Species identifications were made using a combination of live examination under high power magnification (125–400×) and differential interference contrast (DIC), as well as examinations of cleared and permanently mounted individuals (for description of methods see Gelder 2010). Identifications follow the keys and descriptions of Ellis (1919), Goodnight (1943), Hoffman (1963) and Holt and Opell (1993).

## Results

### Grooming experiments

Cleaners declined rapidly from small crayfish control groups and by day 7 almost no cleaners remained on un-ablated hosts (Fig. 1a). In contrast, ablated crayfish retained nearly half of their cleaners through the duration of the experiment resulting in a significant effect of ablation on the transformed proportion of cleaners remaining (Table 1). Controls and ablated crayfish were significantly different by day 7, and remained so until the conclusion of the experiment (Supplementary material Appendix 1 Table A1). Initial cleaner density did not have a significant main effect, but there was a significant interaction between ablation and initial cleaner density, particularly on day 1, but this interactive effect disappeared by day 3.

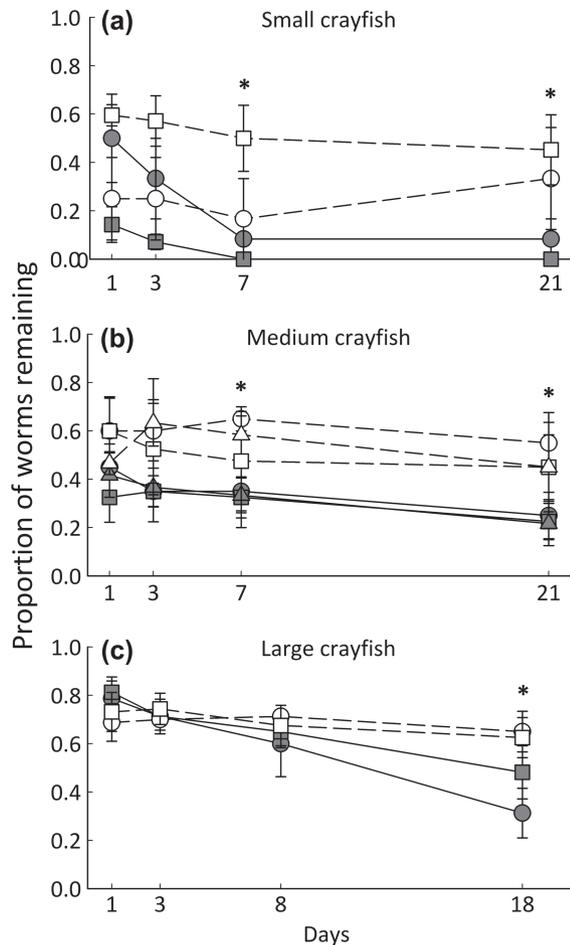


Figure 1. Persistence of the branchiobdellidan *Xironodrilus appalachius* on the crayfish *Cambarus chaugaensis* in laboratory experiments conducted using three size-classes of crayfish; (a) small at 15–18 mm carapace length (CL), (b) medium at 18–22 mm CL, and (c) large crayfish at 27–35 mm CL. Two treatments were crossed in a full factorial design to assess the removal of *X. appalachius* by the host under varying *X. appalachius* density. Hosts in ‘ablated’ groups (open symbols) had their dactyls removed from the first and second walking legs whereas ‘control’ groups (shaded symbols) were left with dactyls intact. Density levels refer to the initial abundance of *X. appalachius* at the beginning of each experiment and are relative to observed field density for each size-class; (○ = normal, □ = high, and △ = very high). Symbols represent mean proportion of *X. appalachius* remaining on each host within each treatment combination  $\pm 1$  SE. Asterisk (\*) indicates significant effect ( $p \leq 0.05$ ) of dactyl ablation for each day (two-way ANOVA, ablation and initial cleaner density as factors and normalized proportion of cleaners remaining as the response). There were no significant effects of initial cleaner density for any day.

Similar to the small host experiment, dactyl ablation significantly increased the proportion of cleaners that remained on medium hosts over the course of the experiment (Fig. 1b, Table 1). In contrast to small hosts, un-ablated medium hosts retained a considerable proportion of their cleaners for the duration of the experiment and the majority of cleaner loss occurred during the first 24 h. However, ablation effects were not significant until day 7 because of high within-group variability during days 1 and 3. There was no effect of initial cleaner density on the

Table 1. Results of three repeated measures ANOVA's testing for effects of ablation, initial density, and sampling day on the proportion of *Xironodrilus appalachius* remaining on crayfish during three experiments using small, medium and large crayfish. Bold font indicates statistical significance ( $\alpha = 0.05$ ).

Host size-class	Source	DF	MS	F	p
Small crayfish	<b>Ablation</b>	<b>1</b>	<b>3.011</b>	<b>4.409</b>	<b>0.049</b>
	Density	1	0.151	0.221	0.643
	<b>Density <math>\times</math> Ablation</b>	<b>1</b>	<b>3.011</b>	<b>4.409</b>	<b>0.049</b>
	Error Between	20	0.683		
	<b>Day</b>	<b>1</b>	<b>0.568</b>	<b>5.694</b>	<b>0.020</b>
	Day $\times$ Ablation	1	0.532	5.335	0.024
	Day $\times$ Density	1	0.002	0.002	0.963
Medium crayfish	Day $\times$ Ablation $\times$ Density	1	0.341	3.424	0.069
	Error Within	68	0.100		
	<b>Ablation</b>	<b>1</b>	<b>1.874</b>	<b>5.994</b>	<b>0.024</b>
	Density	1	0.013	0.040	0.843
	Density $\times$ Ablation	1	0.027	0.085	0.774
	Error Between	20	0.315		
	<b>Day</b>	<b>1</b>	<b>0.366</b>	<b>13.162</b>	<b>&lt;0.001</b>
Large crayfish	Day $\times$ Ablation	1	0.053	1.917	0.171
	Day $\times$ Density	1	0.007	0.247	0.621
	Day $\times$ Ablation $\times$ Density	1	0.001	0.002	0.962
	Error Within	68	0.028		
	Ablation	1	0.041	0.428	0.525
	Density	1	0.015	0.155	0.701
	Density $\times$ Ablation	1	0.007	0.075	0.788
Error Between	12	0.096			
	<b>Day</b>	<b>1</b>	<b>0.691</b>	<b>48.576</b>	<b>&lt;0.001</b>
	<b>Day <math>\times</math> Ablation</b>	<b>1</b>	<b>0.311</b>	<b>21.834</b>	<b>&lt;0.001</b>
	Day $\times$ Density	1	0.009	0.645	0.426
	Day $\times$ Ablation $\times$ Density	1	0.053	3.706	0.061
	Error Within	44	0.014		

proportion of cleaners that remained on medium hosts at any day.

In contrast to the small and medium size-classes, large crayfish showed no significant main effects of dactyl ablation on cleaner persistence over the duration of the experiment. There was however, a significant interaction between ablation and time, indicating that cleaners on controls were declining through time at a rate greater than amputees, resulting in a significant effect of ablation on the proportion of cleaners remaining at the final day of observation (two-way ANOVA;  $F_{1,12} = 6.222$ ,  $p = 0.028$ ), but on no previous days. This slow decline resulted in a significant effect of ablation on the final day of observation only, according to a two-way ANOVA (Fig 1, Supplementary material Appendix 1 Table A1). Similar to other size-classes, there were no effects of initial density on cleaner persistence on large hosts (Table 1).

Cleaner attachment sites were largely restricted to one location on small and medium hosts. In both cases cleaners were observed almost exclusively on the dorsal aspect of the host's carapace (Fig. 2a–b). In contrast, cleaners placed on large hosts occupied a greater variety of locations with the highest proportions occurring at the bases of the walking legs and relatively few occupying the dorsal aspect of the carapace (Fig. 2c). PERMANOVA (9999 permutations) showed a highly significant effect of host size-class ( $pseudo-F_{(2,168)} = 89.95$ ,  $r^2 = 0.49$ ,  $p = 0.0001$ ) on the distribution of cleaners. There was also a significant, but weak effect of initial density ( $pseudo-F_{(2,168)} = 5.082$ ,  $r^2 = 0.03$ ,  $p = 0.0015$ ). Our initial

model found no significant effects of dactyl ablation ( $pseudo-F_{(1,156)} = 2.894$ ,  $r^2 = 0.008$ ,  $p = 0.06$ ) or day ( $pseudo-F_{(5,156)} = 1.862$ ,  $r^2 = 0.03$ ,  $p = 0.06$ ) and therefore these predictors were excluded from the final analysis.

## Survey

*Cambarus chaugaensis* was the dominant crayfish species observed in WSC. A few *Cambarus asperimanus*, *Cambarus bartonii* and *Procambarus clarkii* were also collected but not included in the survey. We examined a total of 213 *C. chaugaensis* (99 males, 114 females) that ranged in size from 14.24 to 44.10 mm CL (1.07–28.06 g BW) and observed a total of 747 branchiobdellidans. There was a strong positive linear relationship between crayfish mass and the total number of branchiobdellidans on each crayfish ( $F = 164.1$ ,  $p < 0.001$ ,  $adj. r^2 = 0.43$ ; Fig. 3). Host

sex was not a significant predictor of branchiobdellidan density and was therefore excluded from the linear model ( $t = 0.926$ ,  $p = 0.36$ ). An analysis of a subsequent sample in which branchiobdellidans were identified to species revealed that *X. appalachius* and *C. philadelphicus* showed similar positive linear relationships with host mass ( $F = 4.768$ ,  $p < 0.05$ ,  $adj. r^2 = 0.22$  and  $F = 15.34$ ,  $p < 0.005$ ,  $adj. r^2 = 0.52$ , respectively). Also, the relative abundance of the two symbiont species did not change with host mass ( $F = 0.847$ ,  $p = 0.38$ ,  $r^2 = 0.066$ ; Supplementary material Appendix 1 Fig. A1). There were obvious differences in the most frequently used attachment sites of branchiobdellidans among host size-classes observed from the field (Fig. 3). Similar to experimental results, the largest proportion of branchiobdellidans inhabiting small and medium crayfish was found on the carapace. Branchiobdellidans attached to large crayfish occupied more locations overall.

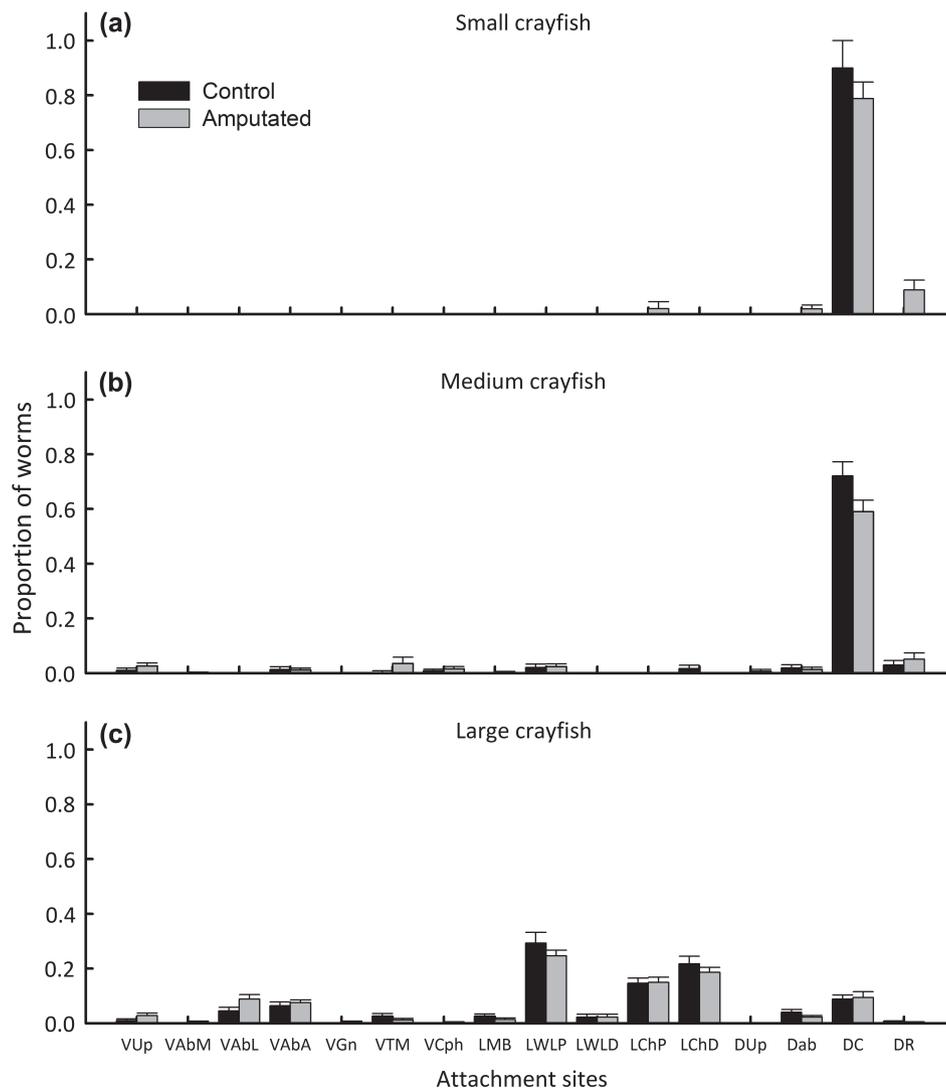


Figure 2. Proportional distribution of *Xironodrilus appalachius* on different attachment sites of the crayfish body (see Table 2 for attachment site descriptions) from all three experiments; small, medium, and large crayfish. Bars indicate the mean ( $\pm 1$  SE) proportion of *X. appalachius* found on each host at each attachment site and include observations from all sampling dates. Hosts which had no *X. appalachius* remaining were excluded. *X. appalachius* on small (a) and medium (b) hosts were largely restricted to the dorsal aspect of the host's carapace (shown as DC), but frequently occupied several locations on large hosts (c). We detected no significant effect of ablation on the distribution of *X. appalachius* on any size-class of host (PERMANOVA).

Table 2. Descriptions and abbreviations of attachment sites occupied by *Xironodrillus appalachius* on the host *Cambarus chaugaensis* during grooming experiments.

Attachment site	Description
Ventral	
VUp	ventral aspect of the uropods and telson
VAbM	ventral aspect of the abdomen, attached mesial to the swimmerets
VAbL	ventral aspect of the abdomen, attached to lateral margins of abdominal tergites or swimmerets not including VABA and VGn
VAbA	ventral aspect of abdomen, attached to or near lateral margins of first abdominal tergite
VGn	genital opening of females or the gonopods of males
Lateral	
LMB	branchiostegite within one cleaner body length of the lateral margin
LWLP	coxa, basis, and ischium of walking legs
LWLD	walking legs not including LWLP
LChP	coxa, basis, ischium and merus of chelipeds
LChD	chelipeds and chelae not including LChP
Dorsal	
DUUp	dorsal aspect of uropods and telson
Dab	dorsal aspect of abdomen
DC	dorsal aspect of carapace, not including LMB and DR
DR	rostrum

Approximately one fifth (20%) of branchiobdellidans observed on large crayfish in the field were found at the opening of the gill chamber, attached to the proximal portions of the walking legs. Conversely, no branchiobdellidans were observed attached to the walking legs of small or medium crayfish.

## Discussion

The results of our experiments demonstrate that crayfish effectively use the dactyls of their walking legs to remove ectosymbiotic cleaners through grooming. Contrary to our predictions, we did not find evidence of a density-dependent grooming response. Instead, young crayfish were entirely intolerant towards cleaners, whereas older crayfish were more tolerant, irrespective of cleaner density. Additionally we found that cleaners have host-size specific behavioral responses which allow them to occupy young and intolerant hosts by occupying an area apparently inaccessible to grooming. Since branchiobdellidans are thought to benefit their hosts by cleaning the respiratory surfaces, the restricted distribution of cleaners on intolerant hosts precludes any benefit to the host. Therefore, host tolerance dictates the ontogenetic stage at which a mutualistic association occurs. Our view is supported by several lines of evidence from our experiments and survey data.

In all three experiments, more cleaners persisted on crayfish with ablated dactyls than on controls indicating that the dactyls are used to remove cleaners. This result supports previous speculation that crayfish can detect branchiobdellidans by way of mechanoreceptors spread across their exoskeletons (Gelder 2010), and corroborate the findings of previous work on another group of crayfish ectosymbionts,

the temnocephalidans (Platyhelminthes), which replace branchiobdellidans as crayfish ectosymbionts throughout Southeast Asia and Australia (Gelder 1999). In an experiment that was methodologically similar to the one presented in this paper, Jones and Lester (1996) demonstrated that the crayfish *Charex quadricarnatus* uses its chelipeds and pereopods to remove temnocephalidans which results in a large and significant reduction of overall temnocephalidan densities. Clearly, crayfish are capable of detecting and removing their ectosymbiotic cleaners by grooming, thus the possibility that crayfish control cleaner density by removing excess cleaners is plausible.

Crayfish showed age-specific responses to the introduction of cleaners that indicates an ontogenetic shift towards tolerance of cleaners. Small and medium crayfish removed most or all cleaners at all experimental cleaner densities. After seven days, only a single cleaner remained on small crayfish with intact dactyls, whereas small crayfish with ablated dactyls retained ~40% of their cleaners to the conclusion of the experiment. Therefore, we concluded that small crayfish use the dactyls of the walking legs to remove cleaners and can effectively remove all cleaners. Dactyl ablation of medium crayfish also significantly increased cleaner persistence, though interestingly ~25% of cleaners persisted on controls for the duration of the experiment, regardless of initial cleaner density. We also observed some cleaner removal by large hosts, as evidenced by a slow decline in cleaners on control animals, but not on ablated animals. However, the difference between controls and ablated crayfish was only significant on the final day of the experiment. These results suggest that even large hosts remove some cleaners, but this response is weaker than in small and medium crayfish which are wholly intolerant of cleaners and quickly removed all or most cleaners introduced to them. One possible explanation is that slow and continual removal of cleaners offsets cleaner reproduction and colonization to maintain cleaner densities at beneficial levels (i.e. Brown et al. 2012), however this speculation requires experimental verification.

One explanation for increased cleaner persistence on large crayfish is that large crayfish are simply unable to detect cleaners or lack the dexterity to remove them. Experimental work on another crayfish species suggests that this explanation is unlikely. The crayfish *Orconectes cristavarius* occurs in sympatry with the New River crayfish *Cambarus chasmodactylus* and its ectosymbiotic cleaner *Cambarincola ingens* in the New River, near Boone, NC (Brown and Creed 2004). While adult New River crayfish often host dozens of cleaners, *O. cristavarius* typically host few if any (Brown and Creed 2004). Recent work demonstrated that even large *O. cristavarius* respond to the introduction of a single branchiobdellidan by quickly removing it, in contrast to similarly sized *C. chasmodactylus* (Farrell et al. 2013). Instead, adult *O. cristavarius* may rely on antimicrobial properties of their hemolymph to resist epibiotic accumulations (Farrell et al. 2013). This result demonstrates that large crayfish can both detect and remove branchiobdellidans, and strongly suggests host tolerance as an explanation for the proliferation of cleaners on the adults of some, but not all crayfish species (but see Brown and Creed 2004 for another possibility).

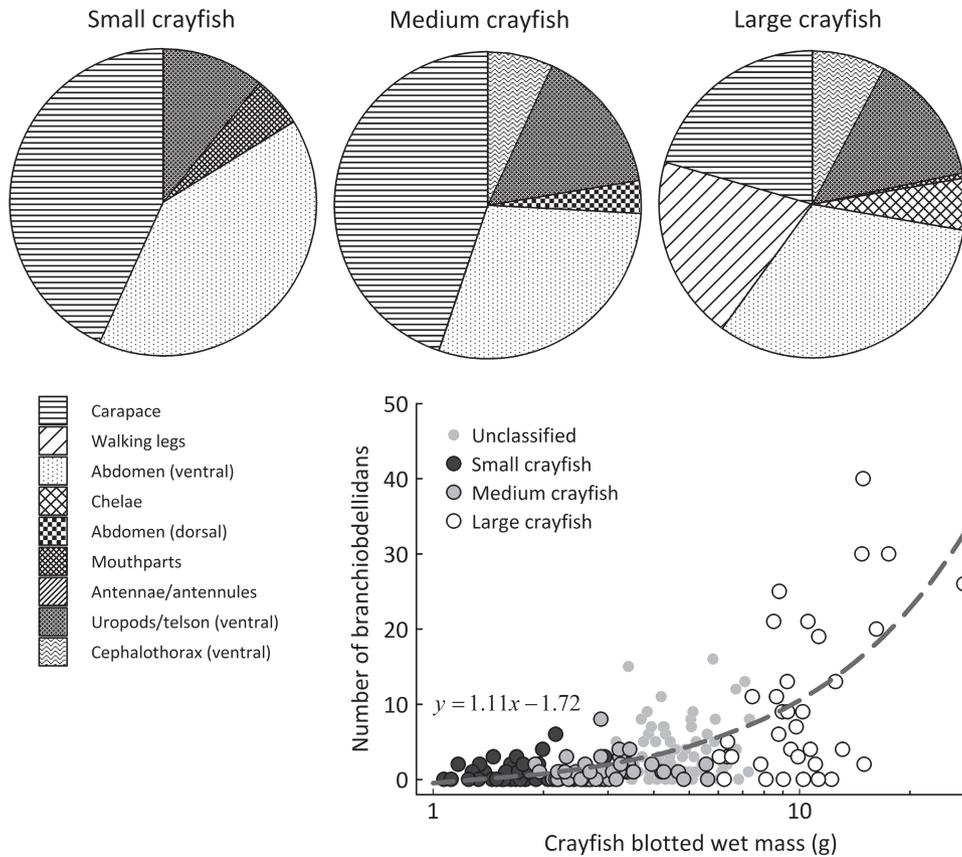


Figure 3. Distribution and density of branchiobdellidans on *Cambarus chaugaensis* in Walldrop Stone Creek from field survey data collected during June and July of 2010. (Upper) Pie chart slices represent the proportion of total branchiobdellidans observed within each size-class at each attachment site on the host. Divisions among size-classes are based on size (CL) and same as divisions made for the grooming experiments to aid in comparisons. The largest portion of branchiobdellidans occupied the carapace on small and medium hosts. (Lower) Number of branchiobdellidans observed versus host blotted wet mass (g). Symbols are color-coded to reflect size-classes. 'Unclassified' represents crayfish that did not fall into any of the size-classes used in grooming experiments. Dotted line represents best fit linear model. Note that x-axis is log scaled for clarity.

Additionally, the possibility that the observed change in host response was driven by temporal changes in environmental covariates unrelated to ontogeny requires discussion. Size-class experiments were conducted in sequence in order to capture the ontogenetic change of host response in a single cohort (small and intermediate size-classes). We are confident that our results reflect an ontogenetic shift in host tolerance rather than temporal environmental covariates for the following reasons: 1) all experiments were conducted within the summer months of 2011 to minimize temporal variation in environmental covariates, 2) experiments were conducted under constant controlled laboratory conditions (similar temperature, water conditions, photoperiod, etc.) and crayfish were given a week to acclimate to laboratory conditions, and 3) our experimental data are supported by field observations from a smaller time period (June and July) in the previous year (2010).

Based on the hypothesis that crayfish regulate cleaners to some targeted density, we predicted that they would remove cleaners only when supplied with cleaners in excess of size-specific densities observed in the field, and that the final density of all un-ablated hosts would converge on field densities. Contrary to our predictions, initial cleaner density did not affect the proportion of cleaners that persisted

on hosts of any size-class. Thus our results do not support the hypothesis that grooming is used to maintain a targeted density of cleaners, at least for the species examined here. Rather young crayfish are intolerant of cleaners at any density, and become more tolerant with age. However, we must address two points that appear to be inconsistent with this interpretation: 1) that ~25% cleaners remained on medium crayfish with intact dactyls for the entire experiment, and 2) that large crayfish with intact dactyls removed some of their cleaners. These inconsistencies can be reconciled with our interpretation of an ontogenetic switch in tolerance towards cleaners by considering host-age specific behavioral responses of the cleaners.

Just as crayfish showed an ontogenetic shift in their tolerance towards cleaners, the cleaners too displayed behavioral responses specific to host age. The large majority of cleaners placed on small and medium hosts remained attached to the dorsal-most aspect of the hosts' carapaces. Dactyl ablation had no detectable effect on the distributions of cleaners across their hosts' bodies and therefore the restricted distributions of cleaners on small and medium hosts did not arise simply as the result of the crayfish removing any cleaners that strayed from the dorsal aspect of the carapace. Instead, the restricted distribution of clean-

ers on small and medium hosts indicates a strong affinity for a particular attachment site. Unlike small and medium hosts, cleaners occupied a variety of locations on large hosts. Perhaps the strong affinity of cleaners for dorsal carapace attachment sites on small and medium hosts is an adaptive response to strong selective forces imposed by young crayfish that rapidly remove branchiobdellidans from easily accessible areas. However, our experimental data suggest that this strategy may only be effective once crayfish reach a particular size. Cleaners were unable to persist on small hosts with intact dactyls, indicating that small crayfish can effectively groom cleaners from the dorsal portion of their carapace. Conversely, ~25% of cleaners persisted on medium crayfish with intact dactyls for the duration of the experiment. Furthermore, cleaner removal on medium hosts occurred almost exclusively during the first 24 h of the experiment, indicating that the initial colonization time is critical to cleaner persistence on medium hosts, but once cleaners have attached to the dorsal refuge they are safe from removal. Combined, the results from the small and medium crayfish experiments suggest that cleaners seek refuge from grooming on young hosts by attaching to the dorsal aspect of the carapace, but this strategy may be ineffective on the smallest crayfish.

Host age-specific distributional patterns of cleaners during our experiments were generally consistent with patterns observed in the field. Similar to experimental results, the largest fraction of cleaners observed from the field were found on the dorsal carapace, and cleaners occupied a greater diversity of locations on larger hosts. Therefore the distributional patterns observed during laboratory experiments were not an artifact of experimental conditions. Thus, at some point during host ontogeny, cleaners leave the sanctuary of their refuge to occupy preferable locations at the continued, yet reduced peril of being removed. By selecting an attachment site that is inaccessible to grooming on young hosts, cleaners may be able to colonize crayfish earlier yielding an advantage over conspecifics competing for limited space on hosts.

Changing cleaner attachment sites through host ontogeny have important implications for the outcome of the association for the host. Branchiobdellidans are thought to benefit their hosts by cleaning potentially harmful epibiotic material from crayfish respiratory surfaces (Brown et al. 2002, 2012, Lee et al. 2009). Furthermore, previously reported negative effects of cleaners on crayfish were associated with damage inflicted to the gills under high cleaner densities (Brown et al. 2012). In our experiments, cleaner distribution was almost exclusively limited to the dorsal-most aspect of the carapace on small and medium crayfish, making the gills inaccessible to cleaners and precluding both known costs and benefits of symbiosis. In contrast, cleaners on large crayfish were found frequently in areas proximate to the gill chamber (e.g. proximal portions of the walking legs) under both field and experimental conditions. Therefore, crayfish may dictate the initiation of the cleaning symbiosis not only by removing cleaners, but also by permitting cleaners access to vital areas.

Can the observed ontogenetic shift in host tolerance to cleaners be related to changing potential costs and benefits of symbiosis? Although crayfish possess structures known to effectively relieve gills of large fouling particles (seto-

branches), molting of the gill cuticle is the only known means by which crayfish rid themselves of gill fouling micro-epibionts (Bauer 1998, but see Farrell et al. 2013 for another possibility). Molt frequency declines with crayfish age and small juvenile crayfish may undergo many closely spaced molts during their first year, whereas adults may only molt once or twice per year (St John 1976). Therefore, the potential for accumulation of epibiotic microorganisms is greater on adults than juveniles and the potential benefit of cleaning is presumably greater for older hosts. Additionally, low availability of epibiotic material on young crayfish may increase facultative parasitism of cleaners as they switch to feeding on host tissue in response to decreases in resource availability, as has been observed with increased cleaner densities (Brown et al. 2012) and decreased epibiotic resources (Cheney and Côté 2005).

We argue that the ontogenetic shift from intolerance to tolerance corresponds with a threshold stage in ontogeny at which the benefits of symbiosis outweigh the costs. Ontogenetic change in a control mechanism is not entirely without precedent. Previous work on cleaning symbioses among fishes of the tropical coral reefs provides some evidence of behavioral controls that are modulated with age-specific changes in costs and benefits of symbiosis. Like branchiobdellidans, cleaner fish can have a positive effect on clients by removing and consuming harmful epibionts (Limbaugh 1961, Grutter 1999, Clague et al. 2011) but often parasitize their clients by feeding on client tissues and mucus (Gorlick 1980, Grutter and Bshary 2003). Client fish use multiple controls to obtain the benefits of being cleaned while limiting parasitic attacks (Bshary and Grutter 2002, 2005, Bshary and Schäffer 2002, Johnstone and Bshary 2002, 2008, Bshary and Noë 2003). For instance, client fish reduce parasitism by controlling cleaning duration, as the likelihood of parasitism by cleaner fish increases with cleaning duration (Johnstone and Bshary 2002). Additionally, cleaner fish consume more client tissue when ectoparasites are scarce, suggesting increased parasitism in response to decreasing resources (Cheney and Côté 2005). Client fish extend the duration of encounters as they grow because larger clients carry higher parasite loads and thus the incurred benefit of being cleaned persists longer (Johnstone and Bshary 2002). We argue that the crayfish/branchiobdellidan cleaning symbiosis is similar insofar as young crayfish receive little to no benefit from being cleaned and therefore avoid or greatly reduce their contact with cleaners by simply removing them or restricting their distribution to areas inaccessible to grooming. However, when increased inter-molt periods lead to increased benefits of cleaning, resulting in a net positive outcome of symbiosis, cleaners are allowed to remain and permitted access to vital areas such as the openings of the gill chamber.

Strong positive relationships between branchiobdellidan density and host size have been reported here and elsewhere for several species of crayfish and branchiobdellidans (Mc Manus 1960, Young 1966, Bishop 1968, Keller 1992, Brown and Creed 2004). This seemingly ubiquitous pattern may be at least partly explained by age-specific differences in host control. Although other explanations for this pattern have been proposed, including resource limitation and disturbance associated with host molts (Mc Manus

1960, Young 1966, Bishop 1968), these speculations have not been experimentally verified. In our experiments, small and medium crayfish removed most of their cleaners within the first 24 h and small crayfish had removed essentially all cleaners within one week. The rapid removal of cleaners placed on small crayfish suggests that host control is a stronger driver than molting or resource limitation, at least on very young crayfish. Conversely, a recent study of the crayfish *Astacus leptodactylus* and their branchiobdellidan associates *Branchiobdella kozarovi* concluded that temperature was a stronger driver of branchiobdellidan density than host size (DeWitt et al. 2013). In our survey, all samples were collected within a short time period (~2 months) and therefore we cannot address the potential influence of varying environmental temperature. However, we have provided very strong evidence for a relationship between host size and branchiobdellidan density and demonstrated age-specific grooming behaviors as a likely contributing force for the relationship with *X. appalachius*. Additionally, we found similar relationships between host size and cleaner abundance for both species of cleaners found in WSC and the relative abundance of these species did not change over host ontogeny. This finding suggests that both *X. appalachius* and *C. philadelphicus* are under similar age-specific host control. There remains the enticing possibility that environmental factors such as temperature and host characteristics, such as age-specific tolerances, interact to create complex temporal and spatial patterns in branchiobdellidan abundance.

Our understanding of positive species interactions lags behind negative interactions such as predation and competition, despite being of similar importance to community structure and ecological processes (Bertness and Callaway 1994, Callaway 1995, Bertness and Leonard 1997, Bruno et al. 2003). This lag may be attributed to the inherent complexity and variability of such interactions. Because the net outcomes of potentially positive interactions often vary from positive to negative with changes in context and ontogeny, mutualisms and other positive interactions are often a moving target for researchers studying the outcomes and implications of species interactions. Improving our understanding of the role of mutualisms in community processes requires a better conceptualization of the internal and external factors that erode and promote reciprocally positive interactions among organisms. The work presented here suggests that organisms engaged in mutualisms can maintain reciprocally beneficial outcomes by exerting controls that change with ontogeny and concomitant changes in potential costs and benefits. This example highlights the potential for integrating ontogeny and control mechanisms to achieve a more complete picture of mutualisms and how they are maintained. Future efforts to specifically characterize and quantify age-specific costs and benefits of symbiosis are needed and will contribute greatly to our understanding of the role ontogeny plays in the evolution of stabilizing control mechanisms between mutualists.

*Acknowledgements* – We extended our sincerest appreciation to Adam Speake, Ryan Hassler, Travis Knott, Chelsea Weithman, Patty Whitener, John Brunt, Jon Burroughs and Tim Torongeau for help during field collections and survey work. Special thanks go to Ryan Hassler and Lucy Balthrop for help constructing and

maintaining experimental chambers. Funding for this research was provided by the National Science Foundation (DEB-0949780 to BLB and DEB-0949823 to RPC).

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Supplementary material (available online as Appendix oik-00963 at <www.oikosoffice.lu.se/appendix>). Appendix 1