# Epizoic acoelomorph flatworms impair zooplankton feeding by the scleractinian coral *Galaxea fascicularis*

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*Biology Open* 2, 10–17
doi: 10.1242/bio.20122741
Received 3rd August 2012
Accepted 17th September 2012

## Summary

**Many scleractinian coral species host epizoic acoelomorph flatworms, both in aquaculture and *in situ*. These symbiotic flatworms may impair coral growth and health through light-shading, mucus removal and disruption of heterotrophic feeding. To quantify the effect of epizoic flatworms on zooplankton feeding, we conducted video analyses of single polyps of *Galaxea fascicularis* (Linnaeus 1767) grazing on *Artemia* nauplii in the presence and absence of symbiotic flatworms. 18S DNA analysis revealed that flatworms inhabiting *G. fascicularis* belonged to the genus *Waminoa* (Convolutidae), which were hosted at a density of 3.6±0.4 individuals polyp$^{-1}$. Polyps hosting flatworms exhibited prey capture rates of 2.2±2.5, 3.4±4.5 and 2.7±3.4 nauplii polyp$^{-1}$ 30 min$^{-1}$ at prey concentrations of 250, 500 and 1,000 nauplii L$^{-1}$, respectively. Polyps that had their flatworms removed displayed prey capture rates of 2.7±1.6, 4.8±4.1 and 16.9±10.3 nauplii polyp$^{-1}$ 30 min$^{-1}$. Significant main and interactive effects of flatworm presence and ambient prey concentration were found, reflected by the fact that flatworms significantly impaired host feeding rates at the highest prey density of 1,000 nauplii L$^{-1}$. In addition, flatworms displayed kleptoparasitism, removing between 0.1±0.3 and 0.6±1.1 nauplii 30 min$^{-1}$ from the oral disc of their host, or 5.3±3.3 to 50.0±2.1% of prey acquired by the coral. We suggest classifying the coral-associated *Waminoa* sp. as an epizoic parasite, as its presence may negatively affect growth and health of the host.**



Key words: *Galaxea*, *fascicularis*, *Waminoa*, Flatworm, Symbiosis, Kleptoparasitism

## Introduction

It is well known that many coral species host epizoic acoelomorph flatworms, both *in situ* and in captivity. The presence of flatworms has potentially negative effects on the host, including light-shading and reduced resistance against environmental impacts and pathogens (Barneah et al., 2007b; Brown and Bythell, 2005; Naumann et al., 2010). Light-shading may be caused when acoelomorph flatworms move across polyps and coenenchyme of colonies, thereby reducing the amount of light reaching the zooxanthellae, thus impairing productivity of the holobiont (Barneah et al., 2007b). Reduced resistance may result from feeding on coral mucus by flatworms, thereby removing (part of) the layer that protects the coral against sedimentation, dehydration, UV-radiation and pathogens (Barneah et al., 2007b; Brown and Bythell, 2005; Naumann et al., 2010). Moreover, prey capture may be impaired as mucus serves as an effective adhesive layer for capturing prey (Sorokin, 1990; Wijgerde et al., 2011a).

Next to light-shading, reduction of the coral's defensive potential and possible impairment of mucociliary feeding, epizoic acoelomorph flatworms have been found to actively compete with their coral host for zooplankton (Wijgerde et al., 2011b), which could reduce prey acquisition by the host. Flatworms may also interfere with host feeding by physically blocking the coral's feeding apparatus, i.e. the oral disc and tentacles of the polyp. Finally, kleptoparasitism, the removal of acquired prey items from the coral polyp by flatworms, may further reduce coral feeding rates.

More insight into the effects of epizoic flatworms on coral feeding rates may elucidate the nature of the coral-flatworm symbiosis, which is at present unclear. In addition, a better understanding of how flatworms affect coral feeding is important as the amount of heterotrophically acquired nutrients is a limiting factor to coral growth, both in aquaculture as well as *in situ* (Houlbrèque and Ferrier-Pagès, 2009; Osinga et al., 2011). Based on the competitive and interfering nature of epizoic flatworms, we tested the hypothesis that flatworms impair the ability of their coral host to feed on zooplankton. In addition, we tested the hypothesis that impairment of host zooplankton feeding by flatworms is more pronounced at lower prey concentrations, as flatworms seem to be more efficient zooplanktivores when compared to their host (Wijgerde et al., 2011b). To this end, we conducted video analyses of the feeding behaviour of the scleractinian coral *Galaxea fascicularis* (Linnaeus 1767) with and without epizoic flatworms.

## Results

### Acoelomorph flatworms hosted by *G. fascicularis*

*Galaxea fascicularis* polyps hosted epizoic acoelomorph flatworms (Fig. 1) at a density of 3.6±0.4 flatworms polyp$^{-1}$. The size of the flatworms varied, with the anterior–posterior axes

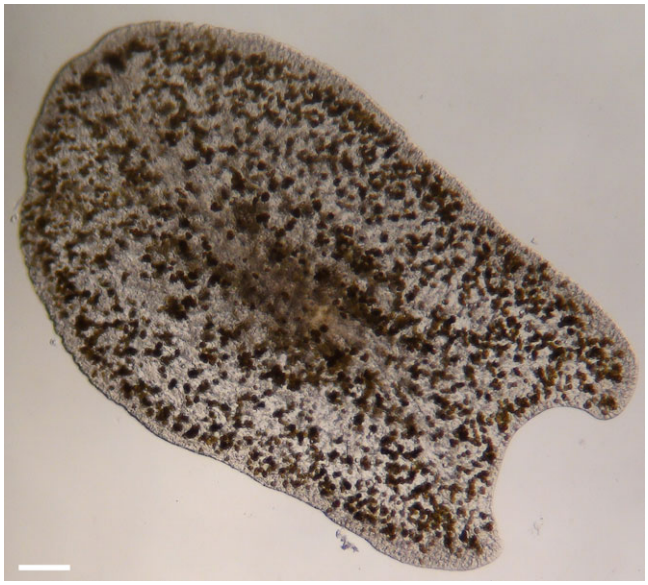

**Fig. 1. Photomicrograph of an epizoic acoelomorph flatworm (*Waminoa* sp.) isolated from *Galaxea fascicularis*.** Note the abundant symbiotic dinoflagellates in the worm's parenchyma. Scale bar: 100 µm.

between approximately 1 to 2 mm in length. Based on their 18S DNA sequence, the acoel flatworms were identified as *Waminoa* sp. (Winsor, 1990), family Convolutidae (von Graff, 1905), phylum Acoelomorpha (Ehlers, 1985). The parenchyma of the flatworms contained high densities of symbiotic algae, possibly *Symbiodinium* or *Amphidinium* sp.

### Zooplankton feeding by *G. fascicularis*

During all treatments, *G. fascicularis* polyps were active and well expanded. All single polyps captured, released and retained zooplankton prey (Fig. 2). Mucus excretion was apparent and resulted in clustering of captured nauplii in small mucus aggregates (not shown). Nauplii were either ingested or digested externally by mesenterial filaments, which were expelled through the actinopharynx and temporary openings in the ectoderm of the oral disc.

Prey capture rates of dewormed polyps were 2.7±1.6, 4.8±4.1 and 16.9±10.3 *Artemia* nauplii polyp$^{-1}$ 30 min$^{-1}$ at prey concentrations of 250, 500 and 1,000 nauplii L$^{-1}$, respectively (Fig. 2A). Polyps hosting epizoic acoelomorph flatworms exhibited prey capture rates of 2.2±2.5, 3.4±4.5 and 2.7±3.4 nauplii polyp$^{-1}$ 30 min$^{-1}$ at prey concentrations of 250, 500 and 1,000 nauplii L$^{-1}$, respectively (Fig. 2A). These capture rates were 81.5±1.3, 70.8±1.6 and 16.0±1.4% relative to dewormed polyps, respectively.

Prey release rates of dewormed polyps were 0.6±0.7, 1.4±1.6 and 7.8±5.3 nauplii polyp$^{-1}$ 30 min$^{-1}$ at prey concentrations of 250, 500 and 1,000 nauplii L$^{-1}$, respectively (Fig. 2B). Polyps hosting acoelomorph flatworms showed prey release rates of 0.4±0.9, 1.4±2.6 and 0.4±0.7 nauplii polyp$^{-1}$ 30 min$^{-1}$ at prey concentrations of 250, 500 and 1,000 nauplii L$^{-1}$, respectively (Fig. 2B). These release rates were 66.7±2.5, 100.0±2.2 and 5.1±1.9% relative to dewormed polyps, respectively.

Prey retention rates of dewormed polyps were 2.1±1.2, 3.3±3.6 and 9.1±8.0 nauplii polyp$^{-1}$ 30 min$^{-1}$ at prey concentrations of 250, 500 and 1,000 nauplii L$^{-1}$, respectively (Fig. 2C). Polyps

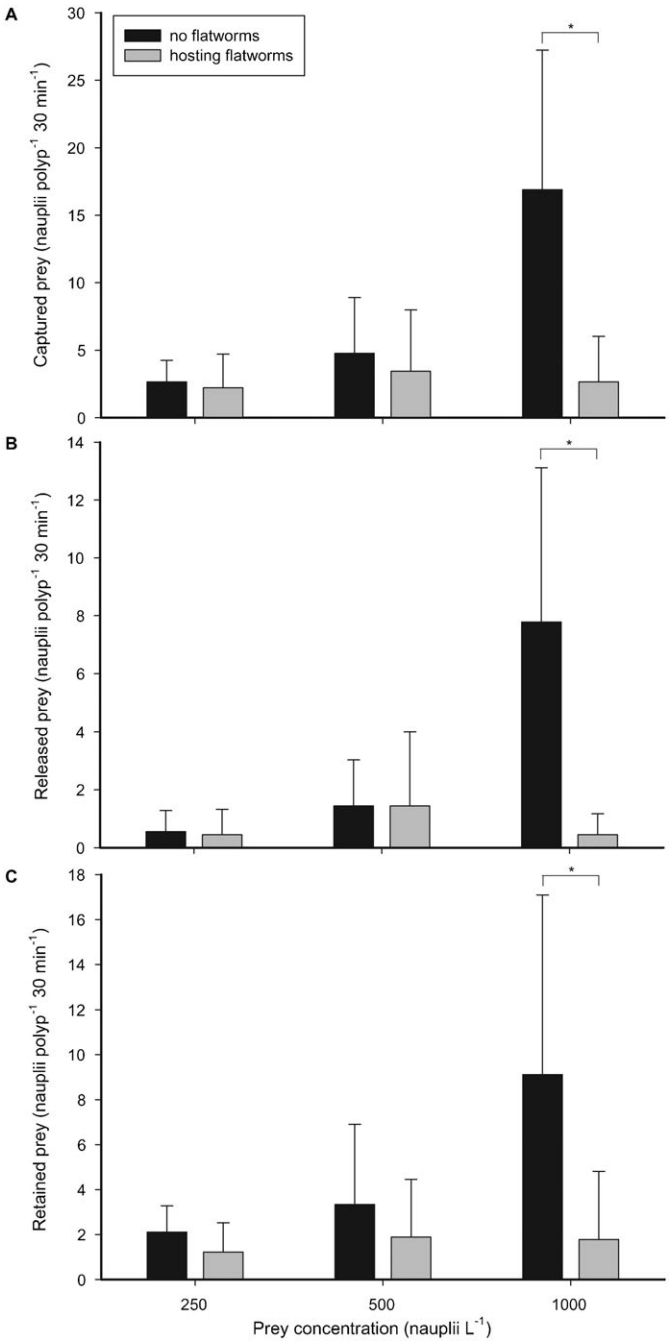


**Fig. 2. *Galaxea fascicularis* feeding rates with and without flatworms at different prey concentrations.** (**A**) Captured, (**B**) released and (**C**) retained prey by *G. fascicularis* single polyps, expressed as nauplii polyp$^{-1}$ 30 min$^{-1}$, at three different prey concentrations, 250, 500 and 1,000 nauplii L$^{-1}$, without (black bars) or hosting (grey bars) epizoic flatworms. Values are means + s.d. ($n$=9). *Indicates significant difference ($P$<0.050, simple effects analysis).

hosting acoelomorph flatworms exhibited prey retention rates of 1.2±1.3, 1.9±2.6 and 1.8±3.0 nauplii polyp$^{-1}$ 30 min$^{-1}$ at prey concentrations of 250, 500 and 1,000 nauplii L$^{-1}$, respectively (Fig. 2C). These retention rates were 57.1±1.2, 57.6±1.8 and 19.8±1.9% relative to dewormed polyps, respectively.

Significant main effects of flatworm presence and prey concentration on *G. fascicularis* prey capture were found

(Table 1). Overall prey capture was significantly higher for dewormed polyps when compared to individuals hosting flatworms. Overall prey capture was significantly higher at 1,000 nauplii $L^{-1}$ when compared to 250 nauplii $L^{-1}$ (Bonferroni, $P=0.011$). No overall differences in prey capture were found between 250 and 500 nauplii $L^{-1}$ (Bonferroni, $P=1.000$) and 500 and 1,000 nauplii (Bonferroni, $P=0.166$). A significant interactive effect between flatworm presence and prey concentration on prey capture was also found (Table 1). This was reflected by the fact that polyps without flatworms captured significantly more prey than their clonemates hosting flatworms at a prey concentration of 1,000 nauplii $L^{-1}$ only (simple effects, $F_{1,16}=18.750$, $P=0.001$). No significant difference in prey capture between polyps with and without flatworms was found at 250 and 500 nauplii $L^{-1}$ (simple effects, $F_{1,16}=0.680$, $P=0.421$ and $F_{1,16}=0.580$, $P=0.456$, respectively). Vice versa, the interaction was reflected by the fact that dewormed polyps exhibited higher prey capture rates with increasing prey concentration (simple effects, $F_{2,32}=10.880$, $P=0.000$), whereas polyps hosting flatworms did not (simple effects, $F_{2,32}=0.170$, $P=0.848$).

Similar main effects of flatworm presence and prey concentration were found for prey release (Table 1). Overall prey release was significantly higher for dewormed polyps when compared to individuals hosting flatworms. Overall prey release was significantly higher at 1,000 nauplii $L^{-1}$ when compared to 250 nauplii $L^{-1}$ (Bonferroni, $P=0.003$). No overall differences in prey release were found between 250 and 500 nauplii $L^{-1}$ (Bonferroni, $P=0.309$) and 500 and 1,000 nauplii (Bonferroni, $P=0.122$). A significant interactive effect between flatworm presence and prey concentration on prey release was also found (Table 1). This was reflected by the fact that polyps without flatworms released significantly more prey than their clonemates hosting flatworms at a prey concentration of 1,000 nauplii $L^{-1}$ only (simple effects, $F_{1,16}=22.190$, $P=0.000$). No significant difference in prey release between polyps with and without flatworms was found at 250 and 500 nauplii $L^{-1}$ (simple effects, $F_{1,16}=0.210$, $P=0.656$ and $F_{1,16}=0.060$, $P=0.813$, respectively). Vice versa, the interaction was reflected by the fact that dewormed polyps exhibited higher prey release rates with increasing prey concentration (simple effects, $F_{2,32}=17.460$, $P=0.000$), whereas polyps hosting flatworms did not ($F_{2,32}=0.810$, $P=0.454$).

Finally, a significant main effect of flatworm presence on prey retention was found (Table 1), where overall prey retention was significantly higher for dewormed polyps when compared to individuals hosting flatworms. Prey concentration had no significant main effect on prey retention (Table 1). No significant interactive effect between flatworm presence and prey concentration on prey retention was found (Table 1). Despite the apparent lack of interaction, polyps without flatworms retained significantly more prey than their clonemates hosting flatworms at a prey concentration of 1,000 nauplii $L^{-1}$ (simple effects, $F_{1,16}=8.110$, $P=0.012$). No significant difference in prey retention between polyps with and without flatworms was found at 250 and 500 nauplii $L^{-1}$ (simple effects, $F_{1,16}=2.580$, $P=0.128$ and $F_{1,16}=0.570$, $P=0.461$, respectively). Vice versa, dewormed polyps exhibited higher prey retention rates with increasing prey concentration (simple effects, $F_{2,32}=4.370$, $P=0.021$), whereas polyps hosting flatworms did not ($F_{2,32}=0.050$, $P=0.950$).

### Prey capture and kleptoparasitism by epizoic flatworms

From the incubations, it became clear that epizoic acoelomorph flatworms (*Waminoa* sp.) competed with their coral host for zooplankton under laboratory conditions. Flatworms captured nauplii by raising their anterior edge from the polyp surface, curling their lateral edges downward and encapsulating prey (Fig. 3). Subsequent paralysis of prey was observed, which was possibly followed by ingestion and digestion in the worm's

**Table 1. Effects of flatworm presence and prey concentration on coral feeding rates and flatworm behaviour.** Two-way mixed factorial ANOVA, showing main and interactive effects of flatworm presence and ambient prey concentration on prey capture, release and retention by *G. fascicularis* single polyps, and one-way repeated measures ANOVA demonstrating the effect of prey concentration on flatworm prey capture, prey stolen, flatworms observed and cumulative flatworm time ($n=9$).

| Factor | Variable | F | df | error | *P* |
|---|---|---|---|---|---|
| | Coral prey capture | | | | |
| Flatworm presence | | 10.881 | 1 | 16 | 0.005* |
| Prey concentration | | 5.314 | 2 | 32 | 0.010* |
| Flatworm presence * Prey concentration | | 5.733 | 2 | 32 | 0.007* |
| | Coral prey release | | | | |
| Flatworm presence | | 11.773 | 1 | 16 | 0.003* |
| Prey concentration | | 8.105 | 2 | 32 | 0.001* |
| Flatworm presence * Prey concentration | | 10.163 | 2 | 32 | 0.000* |
| | Coral prey retention | | | | |
| Flatworm presence | | 8.364 | 1 | 16 | 0.011* |
| Prey concentration | | 2.107 | 2 | 32 | 0.138 |
| Flatworm presence * Prey concentration | | 2.317 | 2 | 32 | 0.115 |
| | Flatworm prey capture | | | | |
| Prey concentration | | 0.914 | 2 | 16 | 0.421 |
| | Prey stolen by flatworms | | | | |
| Prey concentration | | 0.465 | 2 | 16 | 0.637 |
| | Flatworms observed | | | | |
| Prey concentration | | 0.157 | 2 | 16 | 0.856 |
| | Cumulative flatworm time | | | | |
| Prey concentration | | 1.954 | 2 | 16 | 0.174 |

*Indicates significant effect ($P<0.050$).

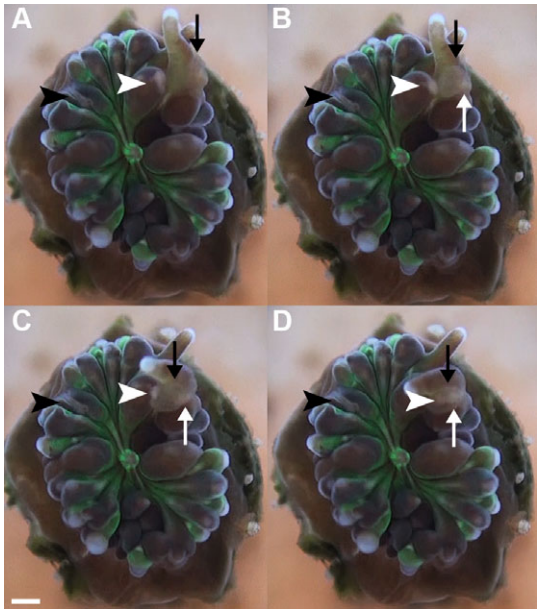


**Fig. 3. Overview of an epizoic flatworm capturing a single *Artemia* nauplius.** (**A**) Flatworm (*Waminoa* sp.) on the oral disc of its coral host (*G. fascicularis*), (**B**) raising its anterior edge from the polyp surface, (**C**) curling down over its prey (*Artemia* sp.) and (**D**) pressing its prey onto the oral disc. Black arrows indicate flatworm, white arrowheads indicate nauplius, black arrowheads indicate captured nauplius by the host coral, white arrows indicate previously captured nauplius by the flatworm. Scale bar: 500 µm.

syncytial digestive tract. Some flatworms captured additional prey whilst digesting previously captured prey, with a maximum of two prey items per worm (Fig. 3), although this behaviour was rare.

Epizoic flatworms inhabiting a single coral polyp captured a total of 1.4±1.5, 2.3±2.3 and 3.2±4.0 nauplii 30 $min^{-1}$ at prey concentrations of 250, 500 and 1,000 nauplii $L^{-1}$, respectively (Fig. 4A). Release of prey by flatworms was not observed. Flatworms also displayed kleptoparasitism, and stole prey previously captured by coral polyps, often within several minutes. Flatworms removed 0.6±1.1, 0.1±0.3 and 0.4±0.9 nauplii 30 $min^{-1}$ from the oral disc of the coral host at prey concentrations of 250, 500 and 1,000 nauplii $L^{-1}$, respectively (Fig. 4B). In relative terms, these removal rates were equal to 50.0±2.1, 5.3±3.3 and 22.2±2.8% of coral nauplii retention at the three prey concentrations, respectively. No translocation of nauplii or refractory organic material from the flatworms to the coral host was observed.

There was no significant effect of prey concentration on flatworm prey capture or number of prey stolen from the oral disc of the host coral (Table 1).

### Flatworm activity

Polyps that had their epizoic flatworms removed with an anthelminthic hosted 0±0 individuals $polyp^{-1}$ 30 $min^{-1}$ at all prey concentrations applied. For single polyps that did not have their epizoic flatworms removed, densities observed were 3.6±2.1, 3.2±2.6 and 4.1±4.4 individuals $polyp^{-1}$ 30 $min^{-1}$ at prey concentrations of 250, 500 and 1,000 nauplii $L^{-1}$, respectively (Fig. 5A). For the latter group, cumulative flatworm times spent on the oral disc were 38±35, 60±55 and 80±79 minutes 30 $min^{-1}$ at prey concentrations of 250, 500 and 1,000 nauplii $L^{-1}$, respectively (Fig. 5B).

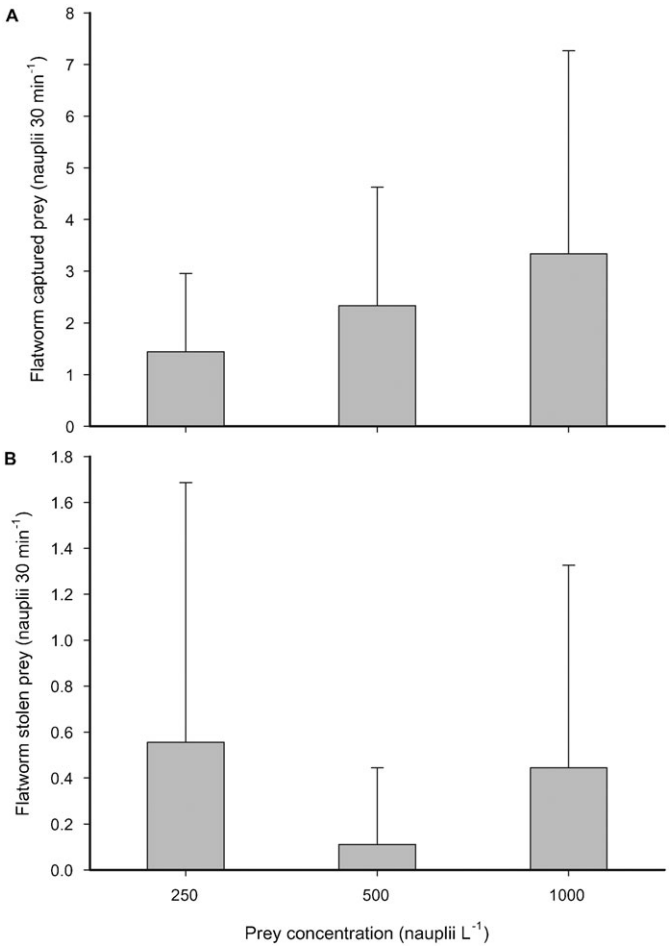


**Fig. 4. Prey capture and kleptoparasitism by epizoic flatworms.** (**A**) Total captured prey from the water column and (**B**) stolen prey from the host coral by epizoic flatworms inhabiting a single coral polyp, expressed as nauplii 30 $min^{-1}$, at three different prey concentrations: 250, 500 and 1,000 nauplii $L^{-1}$. Values are means + s.d. ($n$=9).

No significant effect of prey concentration on the number of flatworms observed and cumulative flatworm time (Table 1) was found. However, a significant positive relationship between cumulative flatworm time spent on the oral disc and total number of captured prey by flatworms was found (Spearman's rho, $r_s$=0.49, $P$=0.01, two-tailed) (Fig. 6).

## Discussion

### Flatworms hosted by *G. fascicularis*

Based on 18S DNA sequencing, it is evident that the flatworms hosted by *G. fascicularis* polyps are a hitherto undescribed species belonging to the genus *Waminoa*. This genus has been found to display low host specificity as it associates with many coral genera from several families (Barneah et al., 2007a; Barneah et al., 2007b; Haapkylä et al., 2009; Naumann et al., 2010). To our knowledge, there is only one record of *G. fascicularis* hosting *Waminoa* sp. (Wijgerde et al., 2011b). The symbiotic algae hosted by the *Waminoa* flatworms may be either *Symbiodinium* sp., *Amphidinium* sp., or both (Barneah et al., 2007b). We have not attempted to isolate and further identify these algae.

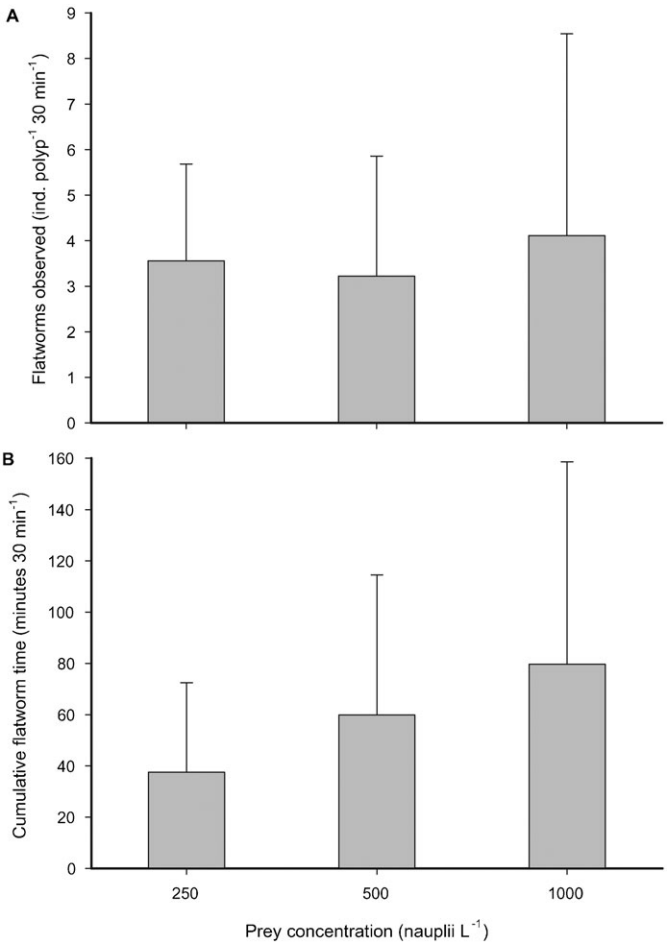


**Fig. 5. Flatworm density and activity on coral polyps.** (**A**) Flatworms observed on the oral disc of their host coral, expressed as individuals polyp$^{-1}$ 30 min$^{-1}$, and (**B**) cumulative flatworm time spent on the oral disc, expressed in minutes 30 min$^{-1}$, at three different prey concentrations: 250, 500 and 1,000 nauplii L$^{-1}$. Values are means + s.d. ($n$=9).

## Reduction of zooplankton feeding by flatworms

A significant main effect of flatworm presence on prey capture, release and retention by the coral host was found, where overall capture, release and retention rates were significantly higher for dewormed polyps when compared to individuals hosting acoelomorph flatworms. This is in line with our first hypothesis that epizoic acoelomorph flatworms impair the ability of their host coral to feed on zooplankton. However, this main effect was entirely caused by differences that occurred at the highest prey concentration applied. Thus, our second hypothesis that flatworms show a more pronounced impairment of coral feeding at lower prey concentrations is refuted. A limitation on zooplanktivory, rather than impairment, may be the most appropriate way to describe the effect of epizoic flatworms on their coral host, as feeding rates of polyps hosting flatworms did not increase with elevated prey concentrations. Several mechanisms may explain why the interfering effect of flatworms on coral feeding occurs at high prey concentrations only, which will be discussed below.

Flatworms may reduce feeding of the coral host due to several mechanisms: competition with the host coral for zooplankton prey (prey which come in close proximity to the coral polyp are regularly captured by epizoic flatworms instead of the coral); physical blocking of the oral disc of the host; mucus removal from the oral disc; and finally kleptoparasitism. At different prey concentrations, these four mechanisms may contribute to feeding impairment of the coral host to varying degrees. As flatworm feeding rates were moderate when compared to the worm-free coral host (3.2±4.0 *versus* 16.9±10.3 nauplii 30 min$^{-1}$ at 1,000 nauplii L$^{-1}$, i.e. 18.9±1.4% of prey capture by the corals), the competition effect did not account for the total reduction of host prey capture induced by flatworm presence, which was 84% (14.2±10.9 nauplii polyp$^{-1}$ 30 min$^{-1}$ at 1,000 nauplii L$^{-1}$). Hence, physical blocking of the oral disc, mucus removal from the disc and kleptoparasitism remain as the potential mechanisms by which flatworms impair the coral's ability to feed on zooplankton. Physical blocking of the oral disc by flatworms is likely to reduce feeding effectiveness as not all tentacles are able to respond to incoming prey. However, as flatworm presence and cumulative time spent on the oral disc did not differ between prey concentrations, this does not satisfactorily explain the absence of a flatworm effect at 250 and 500 nauplii L$^{-1}$. Grazing on coral mucus by flatworms, as demonstrated for *Waminoa* sp. (Barneah et al., 2007b; Naumann et al., 2010), could result in prey capture impairment due to reduced adhesive properties of the polyp. Indeed, at an ambient concentration of 1,000 nauplii L$^{-1}$, prey were observed to interact with flatworm-hosting coral polyps without adhering to the disc or tentacles on a number of occasions. Such lack of adherence was neither observed for polyps that had their symbiotic flatworms removed, nor for polyps supplied with lower concentrations of prey. This suggests that the observed impairment of prey capture and retention at 1,000 nauplii L$^{-1}$ was due to mucus grazing by flatworms, limiting the capacity of polyps to capture and retain more nauplii at higher prey concentrations. Indeed, Hii et al. and Wijgerde et al. found that, at high zooplankton concentrations in particular, *G. fascicularis* produces copious amounts of mucus, which is likely to facilitate the capture of higher amounts of prey (Hii et al., 2009; Wijgerde et al., 2011a). Finally, kleptoparasitism clearly contributed to a reduction of coral feeding by decreasing prey retention rates of the coral host (also see next section).

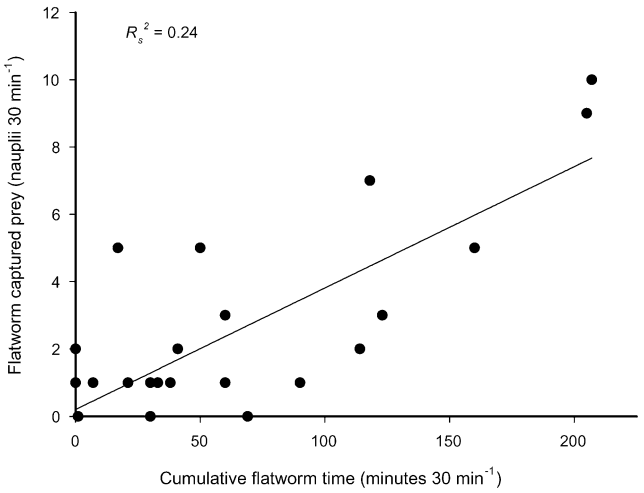


**Fig. 6. Correlation between activity and prey capture of epizoic flatworms.** Correlation between cumulative flatworm time, expressed as minutes 30 min$^{-1}$, and total captured prey, expressed as nauplii 30 min$^{-1}$, by epizoic flatworms (Spearman's rho, $r_s$=0.49, $R_s^2$=0.24, $P$=0.010, two-tailed). $n$=27.

Prey concentration had a significant main effect on prey capture and release by coral polyps, with approximate linear relationships, in accordance with previous studies on cnidarian feeding rates (Clayton and Lasker, 1982; Ferrier-Pagès et al., 1998; Ferrier-Pagès et al., 2003; Houlbrèque et al., 2004a; Lasker et al., 1982; Lewis, 1992). This main effect of prey concentration was reflected by the feeding behaviour of dewormed polyps. As stated above, polyps hosting flatworms did not exhibit enhanced prey capture, release or retention at higher prey concentrations. The positive linear effect of prey concentration was most likely due to the increased particle flux over the feeding polyp, which in turn increased prey encounter rate (Hunter, 1989). The fact that prey release rates also increased with higher prey concentrations was most likely a direct result of increased capture rates. This finding is in line with the study of Wijgerde et al. on the feeding dynamics of *G. fascicularis*, which showed that prey capture and release are coupled, and decrease over time concomitantly (Wijgerde et al., 2011a).

### Prey capture, kleptoparasitism and activity by epizoic flatworms

During this study, we found that *Waminoa* flatworms actively preyed on *Artemia* nauplii and thus competed with their coral host for zooplankton. Similar behaviour has been documented for this genus (Wijgerde et al., 2011b) and two other species: *Convolutriloba retrogemma* (Hendelberg and Åkesson, 1988) and *C. macropyga* (Shannon and Achatz, 2007). The fact that species from two different genera and families (Convolutidae and Sagittiferidae, respectively) display zooplanktivory suggests that this behaviour is generic for coral-associated acoels.

Prey concentration had no significant effect on prey capture and kleptoparasitism by epizoic flatworms, which did not differ significantly between treatments. The absence of a significant effect may be explained by satiation. During video analysis, it was observed that most flatworms retained only one zooplankter during the incubation period. As the number of flatworms observed on coral polyps was limited ($3.6\pm2.1$ to $4.1\pm4.4$ flatworms polyp$^{-1}$), this could explain why increased prey concentrations did not lead to higher flatworm feeding rates as many individuals may have become satiated during the time interval. However, a significant positive correlation was found between cumulative flatworm time spent on the oral disc and total number of captured nauplii by flatworms. This suggests that higher flatworm activity increases the impact of the worms on the feeding efficiency of their host.

As polyps lost a significant portion of their captured prey ($5.3\pm3.3$ to $50.0\pm2.1\%$) to their epizoic flatworms, the coral-flatworm symbiosis may impose a substantial loss of heterotrophically acquired nutrients on the coral host. This could lead to deficiencies in the acquisition of organic compounds such as amino acids and fatty acids, which are taken up through zooplankton predation (Houlbrèque and Ferrier-Pagès, 2009; and references therein). Amino acids are essential to organic matrix synthesis, which in turn is vital to coral calcification (Allemand et al., 1998; Ferrier-Pagès et al., 2003; Houlbrèque et al., 2004b). In addition, amino and fatty acids are important to soft tissue growth (reviewed by Houlbrèque and Ferrier-Pagès, 2009). Thus, flatworm-hosting corals may experience a significant growth retardation, both in aquaculture and *in situ*. Based on an average polyp density of 6.2 polyps cm$^{-2}$ for *G. fascicularis* (Wijgerde et al., 2011a), the rate of flatworm kleptoparasitism we found at the lowest prey concentration is equal to 0.6 prey cm$^{-2}$ coral tissue h$^{-1}$, which lies in the same order of magnitude as *in situ* coral feeding rates (Palardy et al., 2006; Sebens et al., 1996; Sebens et al., 1998). Moreover, flatworm presence, cumulative flatworm time, flatworm feeding and kleptoparasitism did not significantly decrease at lower prey concentrations, at least in the range we applied. Given these findings, it is plausible that *in situ*, corals hosting flatworms lose up to 100% of their daily acquired prey to epizoic flatworms. Given the fact that significant coral-associated flatworm populations have been found in the Red Sea and the Indo-Pacific (Barneah et al., 2007b; Haapkylä et al., 2009; Naumann et al., 2010), and the notion that their zooplanktivorous nature seems generic (Hendelberg and Åkesson, 1988; Shannon and Achatz, 2007; Wijgerde et al., 2011b), epizoic flatworms may limit coral growth by impairing both heterotrophic feeding and photosynthesis (Barneah et al., 2007b; Naumann et al., 2010). However, future experiments should determine to what extent epizoic flatworms affect coral zooplanktivory *in situ*.

Although it is evident that epizoic flatworms are able to impair zooplanktivory and thus nutrient acquisition by their host coral, we cannot exclude translocation of refractory organic material from the flatworm to the coral. In other words, remnants of partially digested zooplankton may be egested from the flatworm's syncytium, which in turn could be captured and digested by corals. However, even in such a case, this would very likely constitute a reduction in nutrient procurement for the coral as the flatworms will use at least part of ingested prey for their own respiratory and assimilatory processes.

No release of prey by flatworms was observed, which may be the result of the relatively short monitoring interval. It is likely that prey digestion by flatworms takes longer than 30 minutes, resulting in a lack of prey release or fragments thereof during the incubations. The fact that the coral host does release significant amounts of prey, and therefore has a lower relative prey retention when compared to its epizoic flatworms, underscores the efficient nature of flatworms as zooplanktivores.

### The coral–flatworm symbiosis defined

The behaviour of *Waminoa* flatworms hosted by *G. fascicularis* may be characterised as highly opportunistic, as these worms exploit and negatively affect their host in several ways: they may cause light-shading and thus reduce the primary productivity of the coral holobiont (Barneah et al., 2007b); they feed on coral mucus, possibly reducing the coral's resistance to pathogens and environmental stressors (Barneah et al., 2007b; Naumann et al., 2010) and limiting its capacity to feed on zooplankton (this paper); and finally, they steal prey acquired by their host (this paper). At this time, based on our findings, we suggest classifying the coral-associated *Waminoa* sp. as an epizoic parasite. Future studies should determine to what extent flatworms compromise the growth and health of *G. fascicularis* and other coral species, both in aquaculture and *in situ*. Recent field evidence suggests that *Waminoa* spp. indeed cause significant tissue loss in scleractinian corals, possibly through impairment of host respiration, feeding and sediment shedding capacities (Hoeksema and Farenzena, 2012).

## Materials and Methods

### Selected species and husbandry

For this study, we used the Indo-Pacific scleractinian species *Galaxea fascicularis* (Linnaeus 1767). Corals were kept in a closed system with a total volume of

approximately 3,000 L containing artificial seawater (AquaHolland BV, Dordrecht, The Netherlands). All individuals were placed on an epoxy-coated steel table at a water depth of approximately 20 cm. Filtration in each system was provided by a 200 L denitrification reactor (Dynamic Mineral Control or DyMiCo, US patent no. 6,830,681 B2, EcoDeco BV, Utrecht, The Netherlands). Water flow was provided by a 1 HP electrical outboard motor (Torqeedo GmbH, Starnberg, Germany). Extra surface flow was created with a Tunze Turbelle nanostream 6045 circulation pump (Tunze Aquarientechnik GmbH, Penzberg, Germany). Water parameters were maintained at the following levels: salinity 35.6±0.4 g $L^{-1}$, temperature 26.0±0.5℃, pH 8.2±0.1, $NH_4^+$-N 2.14±1.43 µmol $L^{-1}$, $NO_3^-$-N 1.43±0.71 µmol $L^{-1}$, $PO_4^{3-}$-P 0.32±0.32 µmol $L^{-1}$, $Ca^{2+}$ 10.0±0.3 mmol $L^{-1}$, $Mg^{2+}$ 58.1±0.2 mmol $L^{-1}$, alkalinity 3.51±0.05 mEq $L^{-1}$. Quantum irradiance was 200 µmol quanta $m^{-2}$ $s^{-1}$. Water flow around the corals was measured with a current velocity meter (Swoffer Model 2100, Swoffer Instruments, Inc., Seattle, USA) and ranged between 5 and 10 cm $s^{-1}$.

For all treatments, single polyp clones ($n$=18) were used. Single polyps were individually removed from a large parent colony by using pincers, and subsequently mounted onto 7×7 cm PVC plates (Wageningen UR, Wageningen, The Netherlands) with epoxy resin (Aqua Medic GmbH, Bissendorf, Germany). All single polyps were of the same genotype, since they all originated from a single parent colony.

### Removal of epizoic flatworms

Single polyps were either used for experiments together with their epizoic acoelomorph worms ($n$=9), or dewormed completely ($n$=9) with the anthelminthic levamisole hydrochloride (10 mg $mL^{-1}$, Beaphar Nederland BV, Hedel, The Netherlands). Levamisole is commonly used in the aquarium industry (Carl, 2008; Leewis et al., 2009) and induces spasms in flatworms, while corals seem unaffected. To deworm single polyps, each individual polyp was immersed in 1 L artificial seawater containing 25 mg $L^{-1}$ levamisole hydrochloride for 10 min at room temperature. Water flow was provided continuously with a magnetic stirrer (IKA Werke GmbH & Co. KG, Staufen, Germany) to allow the worms to detach from the coral. After the incubation, each polyp was shaken 10 times to remove flatworms that still attached to the coral, and subsequently washed twice in two separate beakers containing 1 L of artificial seawater to remove remaining worms and levamisole hydrochloride. Acoelomorph flatworms may produce eggs that are insensitive to chemical agents, therefore, the entire procedure was repeated one week after the first treatment in order to break the worm's reproductive cycle. The time between the two treatments was based on the life history of two acoels, *Convolutriloba macropyga* (Shannon and Achatz, 2007) and *Waminoa brickneri* (Barneah et al., 2007a). These species produce eggs that hatch after 3 to 4 days at a temperature comparable to this study, where *C. macropyga* reaches sexual maturity after 8 to 10 days. After the last levamisole treatment, all corals were allowed to recover for two weeks. No coral mortality or morbidity was observed after the levamisole treatments.

### Identification of epizoic flatworms

To identify the flatworms hosted by *Galaxea fasicularis*, 18S DNA sequencing was employed. Worms were isolated from a *G. fascicularis* colony with levamisole hydrochloride according to the protocol described above, after which approximately 100 specimens were transferred to a 15 mL tube with a Pasteur pipette. Subsequently, worms were washed three times and stored in 95% ethanol at 4℃ until analysis. Genomic DNA was extracted following the protocol of the DNeasy Mini Kit (Qiagen, Valencia, USA), QIAamp DNA Mini Kit, and DNA Blood Mini Kit (Qiagen, Hilden, Germany). DNA amplification was performed with illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK) in a 25 µL reaction mixture containing 21.5 µL ddH2O, 0.5 µL of each primer, and 2.5 µL DNA extract. The primers 30S/18S950R and 4FB/1806R were used to amplify the *Maja1* 18S rRNA gene. The cycling conditions used were as follows: 30S/18S950R: 95℃/5′ – 2× (94℃/30″ – 58℃/30″ – 72℃/30″) – 2× (94℃/30″ – 56℃/30″ – 72℃/30″) – 34× (94℃/30″ – 52℃/30″ – 72℃/30″) – 72℃/10′. 4FB/1806R: 95℃/5′ – 2× (94℃/30″ – 60℃/30″ – 72℃/30″) – 2× (94℃/30″ – 58℃/30″ – 72℃/30″) – 2× (94℃/30″ – 56℃/30″ – 72℃/30″) – 2× (94℃/30″ – 54℃/30″ – 72℃/30″) – 2× (94℃/30″ – 52℃/30″ – 72℃/30″) – 32× (94℃/30″ – 50℃/30″ – 72℃/30″) – 72℃/10′. The PCR product was purified using the Exonuclease I – Shrimp Alkaline Phosphatase (Fermentas, St. Leon-Rot, Germany) and the DyeEx 96 Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The purified gene fragment was directly sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer, Massachusetts, USA) and a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA). The obtained sequence was subsequently blasted (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and displayed 99% similarity to Genbank accession no. AB539806. At present, this is an undescribed *Waminoa* species.

### Feeding experiments and video analysis

To analyse the potential impairment of coral feeding by flatworms under different zooplankton concentrations, all *G. fascicularis* single polyps ($n$=18) were incubated individually in a respirometric flow cell (Wageningen UR, Wageningen, The Netherlands) with a volume of 3.5 L for 30 minutes. Water flow was created by a built-in paddle wheel driven by a Maxon DC motor, which was connected to a computer. Flow speed was controlled by EPOS user interface software (version 2.3.1), and was set at 200 rpm, equal to 5 cm $s^{-1}$. For more details about the flow cell see Schutter et al. (Schutter et al., 2010). Water from the holding tank was used for the incubations to rule out artefacts resulting from changes in water chemistry. Temperature in the flow cell was kept at 26±0.5℃ by means of a water jacket connected to a water bath equipped with a TC20 water cooler (Teco SRL, Ravenna, Italy). Photon flux density was set to holding tank intensity (200 µmol quanta $m^{-2}$ $s^{-1}$) with a T5 fluorescent lighting fixture containing four 24 W T5 fluorescent tubes with a colour temperature of 14,000 Kelvin (Elke Müller Aquarientechnik, Hamm, Germany). Each polyp was incubated in the flow cell with three different concentrations of *Artemia salina* nauplii (250, 500 and 1,000 nauplii $L^{-1}$) for 30 minutes. These concentrations were chosen as they reflect aquaculture conditions, and to ensure that sufficient feeding events would occur during the short incubations. *Artemia salina* nauplii were hatched from cysts (Great Salt Lake Artemia cysts, Artemia International LLC, Fairview, USA), at a salinity of 25 g $L^{-1}$ and a temperature of 28℃, and used immediately after hatching. Average nauplii size was 440 µm according to the manufacturer. Polyps were acclimated for 15 minutes before the start of every incubation. Each polyp was allowed to recover for one week after each experiment. To minimise the effect of time, treatments were randomised for each polyp. An HDR-CX505VE camera (Sony Corporation, Tokyo, Japan) was used for recording still and moving close-up images of incubated polyps in high definition. Several variables were scored during video analysis: capture, release and retention of prey by coral polyps; capture and release of prey by flatworms; prey stolen by flatworms; total number of flatworms present on the oral disc of the coral host; and cumulative flatworm time spent on the oral disc of the coral host. Nauplii capture by corals was defined as prey that attached to the polyp surface for at least 10 seconds. Nauplii release by corals was defined as prey that detached from the polyp surface and remained in suspension for longer than 10 seconds. Retention of nauplii by corals was defined as the number of nauplii that remained in contact with the polyp surface at the end of the incubation, where two or more clustered nauplii were considered to be an aggregate. Flatworm prey capture was defined as the total number of prey captured by flatworms inhabiting the oral disc of the host coral. Flatworm number was defined as the total number of flatworms observed on the oral disc. Cumulative flatworm time was defined as the sum of the time spent by all flatworms on the oral disc. Oral disc was defined as the structure containing the mouth, disc and tentacles of the polyp. Flatworms that did not inhabit the oral disc were systematically ignored, as it was assumed that these worms did not directly interfere with the coral feeding process.

### Data analysis

Normality of data was tested by plotting residuals of each dataset *versus* predicted values, and by performing a Shapiro–Wilk test. Homogeneity of variances and sphericity were determined using Levene's and Mauchly's test, respectively. As the data exhibited non-normality and heteroscedasticity ($P$<0.05), a 10 log transformation was employed. This resulted in normality, homogeneity of variances and sphericity ($P$>0.05) of the data. As our data contained one repeated measures or within subjects factor (ambient zooplankton concentration), we used a two-way mixed factorial ANOVA to test the main and interactive effects of flatworm presence and ambient zooplankton concentration on prey capture, release and retention by *Galaxea fascicularis* single polyps. We used a one-way repeated measures ANOVA to test the effect of ambient zooplankton concentration on flatworm prey capture, number of prey stolen from the oral disc of the host coral, number of flatworms observed and cumulative flatworm time. A Bonferroni *post hoc* was used for each dependent variable to determine differences between the different prey concentrations applied. Simple effects analysis was employed to infer capture, release and retention differences between polyps with and without flatworms at each prey concentration. To infer a correlation between cumulative flatworm time and prey captured by flatworms, we used Spearman's rho on untransformed data. A $P$<0.05 value was considered statistically significant. Statistical analysis was performed with SPSS Statistics 17.0 (IBM, Somers, USA). Graphs were plotted with SigmaPlot 11.0 (Systat software, San Jose, USA). All data presented are means ± s.d., unless stated otherwise.

## Acknowledgements

We are greatly indebted to professor Ulf Jondelius of the Naturhistoriska riksmuseet, Stockholm, Sweden for DNA analysis and flatworm identification, and the staff of Wageningen UR and Burgers' Zoo for technical support. We would also like to thank two anonymous reviewers for providing constructive comments on the draft manuscript. This work was funded by Wageningen UR.

## Competing Interests

The authors have no competing interests to declare.

## References


**Allemand, D., Tambutté, É., Girard, J.-P. and Jaubert, J.** (1998). Organic matrix synthesis in the scleractinian coral *Stylophora pistillata*: role in biomineralization and potential target of the organotin tributyltin. *J. Exp. Biol.* **201**, 2001-2009.

**Barneah, O., Brickner, I., Hooge, M., Weis, V. M. and Benayahu, Y.** (2007a). First evidence of maternal transmission of algal endosymbionts at an oocyte stage in a triploblastic host, with observations on reproduction in *Waminoa brickneri* (Acoelomorpha). *Invertebr. Biol.* **126**, 113-119.

**Barneah, O., Brickner, I., Hooge, M., Weis, V. M., LaJeunesse, T. C. and Benayahu, Y.** (2007b). Three party symbiosis: acoelomorph worms, corals and unicellular algal symbionts in Eilat (Red Sea). *Mar. Biol.* **151**, 1215-1223.

**Brown, B. E. and Bythell, J. C.** (2005). Perspectives on mucus secretion in reef corals. *Mar. Ecol. Prog. Ser.* **296**, 291-309.

**Carl, M.** (2008). Predators and pests of captive corals. In *Advances In Coral Husbandry In Public Aquariums* (*Public Aquarium Husbandry*), Vol. 2 (ed. R. J. Leewis and M. Janse), pp. 31-36. Arnhem: Burgers' Zoo.

**Clayton, W. S. and Jr and Lasker, H. R.** (1982). Effects of light and dark treatments on feeding by the reef coral *Pocillopora damicornis* (Linnaeus). *J. Exp. Mar. Biol. Ecol.* **63**, 269-279.

**Ehlers, U.** (1985). *Das Phylogenetische System Der Plathelminthes*. Stuttgart; New York: G. Fischer.

**Ferrier-Pagès, C., Allemand, D., Gattuso, J.-P., Jaubert, J. and Rassoulzadegan, F.** (1998). Microheterotrophy in the zooxanthellate coral *Stylophora pistillata*: effects of light and ciliate density. *Limnol. Oceanogr.* **43**, 1639-1648.

**Ferrier-Pagès, C., Witting, J., Tambutté, E. and Sebens, K. P.** (2003). Effect of natural zooplankton feeding on the tissue and skeletal growth of the scleractinian coral *Stylophora pistillata*. *Coral Reefs* **22**, 229-240.

**Haapkylä, J., Seymour, A. S., Barneah, O., Brickner, I., Hennige, S., Suggett, D. and Smith, D.** (2009). Association of *Waminoa* sp. (Acoela) with corals in the Wakatobi Marine Park, South-East Sulawesi, Indonesia. *Mar. Biol.* **156**, 1021-1027.

**Hendelberg, J. and Åkesson, B.** (1988). *Convolutriloba retrogemma* gen. et sp. n., a turbellarian (Acoela, Platyhelminthes) with reversed polarity of reproductive buds. *Fortschr. Zool.* **36**, 321-327.

**Hii, Y.-S., Soo, C.-L. and Liew, H.-C.** (2009). Feeding of scleractinian coral, *Galaxea fascicularis*, on *Artemia salina* nauplii in captivity. *Aquaculture International* **17**, 363-376.

**Hoeksema, B. W. and Farenzena, Z. T.** (2012). Tissue loss in corals infested by acoelomorph flatworms (*Waminoa* sp.). *Coral Reefs* **31**, 869.

**Houlbrèque, F. and Ferrier-Pagès, C.** (2009). Heterotrophy in tropical scleractinian corals. *Biol. Rev. Camb. Philos. Soc.* **84**, 1-17.

**Houlbrèque, F., Tambutté, E., Richard, C. and Ferrier-Pagès, C.** (2004a). Importance of a micro-diet for scleractinian corals. *Mar. Ecol. Prog. Ser.* **282**, 151-160.

**Houlbrèque, F., Tambutté, E., Allemand, D. and Ferrier-Pagès, C.** (2004b). Interactions between zooplankton feeding, photosynthesis and skeletal growth in the scleractinian coral *Stylophora pistillata*. *J. Exp. Biol.* **207**, 1461-1469.

**Hunter, T.** (1989). Suspension feeding in oscillating flow: the effect of colony morphology and flow regime on plankton capture by the hydroid *Obelia longissima*. *Biol. Bull.* **176**, 41-49.

**Lasker, H. R., Syron, J. A. and Clayton, W. S., Jr.** (1982). The feeding response of *Hydra viridis*: effects of prey density on capture rates. *Biol. Bull.* **162**, 290-298.

**Leewis, R. J., Wijgerde, T., Laterveer, M. and Osinga, R.** (2009). *Working With Aquarium Corals: A Book Of Protocols For The Breeding And Husbandry Of Scleractinian Corals*. Rotterdam: Rotterdam Zoo.

**Lewis, J. B.** (1992). Heterotrophy in corals: zooplankton predation by the hydrocoral *Millepora complanata*. *Mar. Ecol. Prog. Ser.* **90**, 251-256.

**Naumann, M. S., Mayr, C., Struck, U. and Wild, C.** (2010). Coral mucus stable isotope composition and labeling: experimental evidence for mucus uptake by epizoic acoelomorph worms. *Mar. Biol.* **157**, 2521-2531.

**Osinga, R., Schutter, M., Griffioen, B., Wijffels, R. H., Verreth, J. A. J., Shafir, S., Henard, S., Taruffi, M., Gili, C. and Lavorano, S.** (2011). The biology and economics of coral growth. *Mar. Biotechnol. (NY)* **13**, 658-671.

**Palardy, J. E., Grottoli, A. G. and Matthews, K. A.** (2006). Effect of naturally changing zooplankton concentrations on feeding rates of two coral species in the Eastern Pacific. *J. Exp. Mar. Biol. Ecol.* **331**, 99-107.

**Schutter, M., Crocker, J., Paijmans, A., Janse, M., Osinga, R., Verreth, A. J. and Wijffels, R. H.** (2010). The effect of different flow regimes on the growth and metabolic rates of the scleractinian coral *Galaxea fascicularis*. *Coral Reefs* **29**, 737-748.

**Sebens, K. P., Vandersall, K. S., Savina, L. A. and Graham, K. R.** (1996). Zooplankton capture by two scleractinian corals, *Madracis mirabilis* and *Montastrea cavernosa*, in a field enclosure. *Mar. Biol.* **127**, 303-317.

**Sebens, K. P., Grace, S. P., Helmuth, B., Maney, E. J. and Jr and Miles, J. S.** (1998). Water flow and prey capture by three scleractinian corals, *Madracis mirabilis, Montastrea cavernosa* and *Porites porites*, in a field enclosure. *Mar. Biol.* **131**, 347-360.

**Shannon, T., III and Achatz, J. G.** (2007). *Convolutriloba macropyga* sp. nov., an uncommonly fecund acoel (Acoelomorpha) discovered in tropical aquaria. *Zootaxa* **1525**, 1-17.

**Sorokin, Y. I.** (1990). *Ekosistemy Korallovykh Rifov [Ecosystems of Coral Reefs]*. Moscow: Nauka.

**von Graff, L.** (1905). Turbellaria. I. Acoela. In *Das Tierreich*, Vol. 23. Berlin.

**Wijgerde, T., Diantari, R., Lewaru, M. W., Verreth, J. A. J. and Osinga, R.** (2011a). Extracoelenteric zooplankton feeding is a key mechanism of nutrient acquisition for the scleractinian coral *Galaxea fascicularis*. *J. Exp. Biol.* **214**, 3351-3357.

**Wijgerde, T., Spijkers, P., Verreth, J. and Osinga, R.** (2011b). Epizoic acoelomorph flatworms compete with their coral host for zooplankton. *Coral Reefs* **30**, 665.

**Winsor, L.** (1990). Marine turbellaria (Acoela) from North Queensland. *Mem. Queensland Mus.* **28**, 785-800.