Heterotrophic feeding, growth and nutrient budget in the scleractinian coral *Galaxea fascicularis*

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Chapter 1

General introduction

Background

Coral reefs are of tremendous ecological and economic importance, and are currently in global decline (Hughes et al. 2003; Hoegh-Guldberg et al. 2007). This decline is due to global climate change and local anthropogenic disturbances, including pollution, coastal development and overfishing. Overfishing of herbivorous fish is a threat to coral reefs in Asia, mainly in Indonesia and the Philippines, where fish and corals are collected for the aquarium trade. According to estimates of the World Conservation Monitoring Centre of the United Nations Environment Programme (UNEP-WCMC), about two million people are active in the marine aquarium hobby today (Wabnitz et al. 2003). Together, they generate a high demand for tropical coral species as home ornamentals. Main markets are the US (73%) and Europe (14%), where 3.5 million coral colonies were imported between 1997 and 2003 (Wabnitz et al. 2003). This trade appears unsustainable, as wild collection of reef organisms has led to elimination of local populations and significant changes in age structure (Tissot et al. 2010 and references therein). Therefore, a major incentive exists to grow corals sustainably, so that local degradation of coral reef ecosystems may be reduced.

In order to optimise coral aquaculture, detailed knowledge of factors controlling the growth of corals is of high importance. Today, four major factors affecting the growth of zooxanthellate, scleractinian corals have been identified; light, water flow, water chemistry and finally nutrition through heterotrophy (reviewed by Osinga et al. 2011a). Heterotrophy, in particular feeding on zooplankton, is considered to be vital to coral nutrition, as it supplies the coral and its symbiotic algae with carbon, nitrogen and phosphorus (reviewed by Houlbrèque and Ferrier-Pagès 2009). Essential compounds provided through heterotrophic feeding include (un)saturated fatty acids and amino acids such as aspartic acid (Houlbrèque and Ferrier-Pagès 2009). The pronounced positive effects of zooplankton supplementation on

the skeletal and tissue growth of corals has led to the adage “fed corals are happy corals”.

Currently, feeding protocols for coral aquaculture are based on empirical studies (e.g. Lavorano et al. 2008). It is still unclear, however, to what extent scleractinian corals are capable of heterotrophic feeding, as the available research methodologies (Grottoli et al. 2006; Osinga et al. 2008; Purser et al. 2010) underestimate prey capture and do not take nutrient (i.e. organic compounds) depletion of plankton into account (Fabricius et al. 1995; Grottoli et al. 2006; Purser et al. 2010). Thus, a realistic quantification of heterotrophy in the carbon, nitrogen and phosphorous budget of zooxanthellate scleractinian corals is currently lacking. In addition, contrasting short- and long-term effects of heterotrophy on coral growth have been found, with immediate negative (Al-Horani et al. 2007; Colombo-Pallotta et al. 2010) and long-term positive effects (reviewed by Houlbrèque and Ferrier-Pagès 2009). To further complicate matters, (a)biotic factors, including water flow rate (Dai and Lin 1993; Fabricius et al. 1995; Lin et al. 2002), coral size (Hunter 1989; Helmuth and Sebens 1993; Sebens et al. 1997; Hii et al. 2009), the presence of episymbionts (Wijgerde et al. 2011b) and prey density (Osinga et al. 2011b) all affect zooplankton uptake, and thus heterotrophic nutrient input and growth.

The relevance of addressing the knowledge gaps presented above is twofold. First, it contributes to our fundamental understanding of the role of heterotrophic feeding in the nutrient budget and growth of corals, and how (a)biotic factors affect this role. Second, this knowledge will allow for optimisation of coral feeding efficiency, and thus aquaculture protocols. This, in turn, will benefit the sustainable trade in these endangered organisms.

In the following paragraphs, I will provide a brief overview of coral biology, discuss the knowledge gaps mentioned above in more detail, and present the research questions of the thesis.

Figure 1: Although coral aquaculture is an emerging practice, culture protocols require optimisation to improve efficiency.

Biology of scleractinian corals

The following paragraph provides a brief overview of the biology of scleractinian corals, to aid the reader in understanding coral growth and the importance of heterotrophic feeding.

Scleractinian corals (order Scleractinia, subclass Hexacorallia, class Anthozoa) are benthic invertebrates which are exclusively found in marine waters. Most species are colonial, with numerous polyps or zooids connected by common tissue called coenenchyme (Figure 2). The polyps of scleractinian

corals always possess six tentacles, or a multiple thereof. Corals are diploblastic animals with a relatively simple bauplan, with tissue consisting of only two cell layers; the ectoderm and gastroderm, connected by a predominantly acellular matrix referred to as the mesoglea (Brusca and Brusca 2003). The ectoderm and mesoglea are of ectodermal origin, whereas the gastroderm is of endodermal origin.

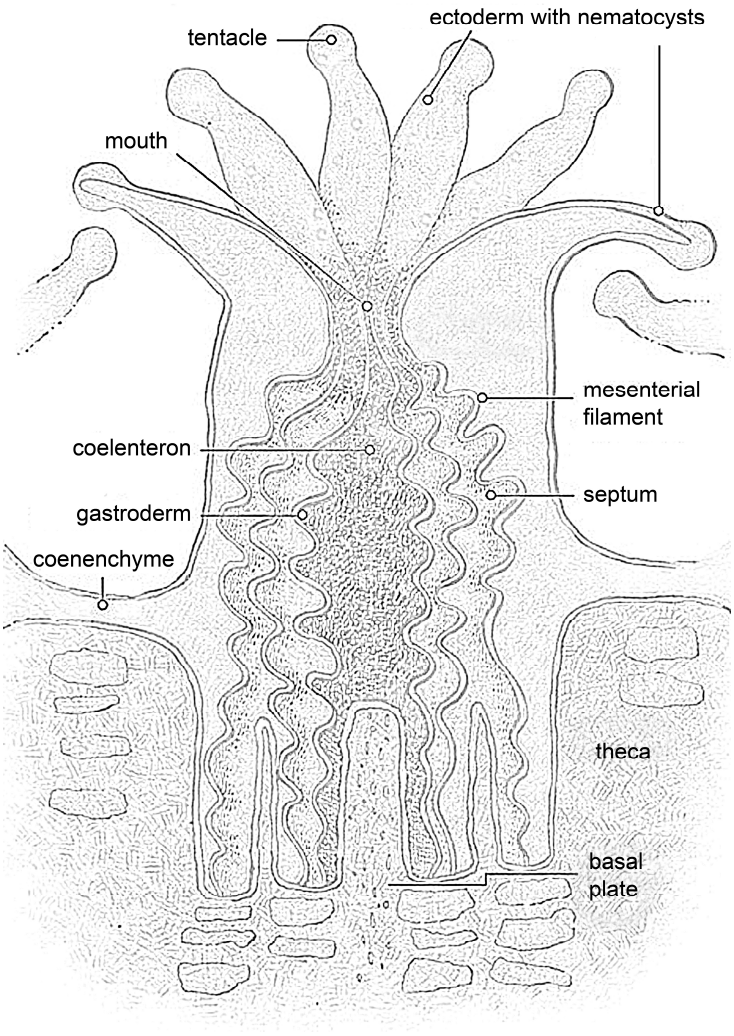

Figure 2: Basic anatomy of scleractinian corals. Modified from NOAA/Gini Kennedy (coralreef.noaa.gov).

Many scleractinian corals live in mutualistic symbiosis with unicellular algae (*Symbiodinium microadriaticum*) which reside in perisymbiotic membranes in the gastrodermal cells of the coral host (reviewed by Furla et al. 2005). By using light energy, these so-called zooxanthellae fix inorganic carbon in the form of bicarbonate (HCO_3^-) and carbon dioxide (CO_2) as organic compounds, including glycerol, glucose and amino acids, a process known as autotrophy or photosynthesis (Furla et al. 2005 and references therein). A significant part of these compounds is released to the gastrodermal cells of the coral host, providing both an energy and nutrient source (Muscatine 1990). The acquired carbon is respired, stored as lipid or exuded as carbohydrates and proteins in mucus (Crossland et al. 1980; Davies 1984; Brown and Bythell 2005; Wild et al. 2005). In addition to intracellular algae, endolithic algae inhabit the corallum (skeleton) of scleractinian corals, which also translocate organic carbon compounds to the coral host (Fine and Loya 2002). Furthermore, scleractinian corals may host nitrogen fixing bacteria (Lesser et al. 2004, 2007), which convert nitrogen gas (N_2) into ammonium (NH_4^+). The ammonium is used by zooxanthellae and converted into amino acids such as glutamic acid and glutamine (Lesser et al. 2007). This three-way symbiosis between corals, algae and cyanobacteria allows for tight nutrient recycling between the host and its symbiotic organisms, which allows corals to thrive in oligotrophic water (Muscatine 1990; Lesser et al. 2007).

Next to effectively exploiting the autotrophic capacity of their symbiotic zooxanthellae and bacteria, scleractinian corals are able to feed heterotrophically (reviewed by Houlbrèque and Ferrier-Pagès 2009; Ferrier-Pagès et al. 2011). Preferred food sources vary among species, and comprise bacteria, nanoeukaryotes, phytoplankton, zooplankton, particulate and dissolved organic matter, zooxanthellae and benthic algae (Houlbrèque and Ferrier-Pagès 2009; Marhaver 2011). The current view on heterotrophy is that it is the dominant source of organic nitrogen (and possibly phosphorous) for the coral host, as nutrients translocated by zooxanthellae exhibit a high C:N ratio and are mainly used by the coral host for respiration or mucus production

(Davies 1984; Falkowsky et al. 1984; Brown and Bythell 2005). This presumed highly energetic but low nutritional value of photosynthates is also known as the "junk food hypothesis" (Falkowsky et al. 1984). In addition to providing nitrogen and phosphorous, feeding on zooplankton may provide essential organic compounds such as aspartic acid, which are used to synthesize coral tissue and the organic matrix (Allemand et al. 1998; Houlbrèque et al. 2004a).

Another important feature of scleractinian corals is their ability to calcify, i.e. to produce an exoskeleton composed of calcium carbonate (aragonite). This allows individual polyps to retreat into depressions known as corallites as protection against predators (Brusca and Brusca 2003). The corallum is produced by the aboral ectoderm, which is able to secrete calcium ions to the growing corallum by means of Ca^{2+}/H^{+} ATP-ases (Ip et al. 1991; Furla et al. 2000; Al-Horani et al. 2003). The calcium ions end up in a fluid layer that lies between the calicoblastic ectoderm and the corallum, known as the calcifying medium (Figure 3). At the same time, protons are removed from the calcifying medium by the same Ca^{2+}/H^{+} ATP-ases. This antiport system requires ATP hydrolysis to generate the required energy for pumping calcium ions and protons against an electrochemical gradient (Ip et al. 1991). The required ATP is generated through respiration of organic compounds derived from photosynthesis or heterotrophic feeding. The antiport of calcium ions and protons generates a high calcium concentration and pH in the calcifying medium, resulting in a supersaturation of calcium carbonate. As a result, calcium carbonate precipitates as aragonite crystals and a skeleton is produced (Furla et al. 2000; Al-Horani et al. 2003). The ability of scleractinian corals to calcify has resulted in massive geological structures known as coral reefs, which have persisted since the Triassic period (206-251 Ma, Veron 2000).

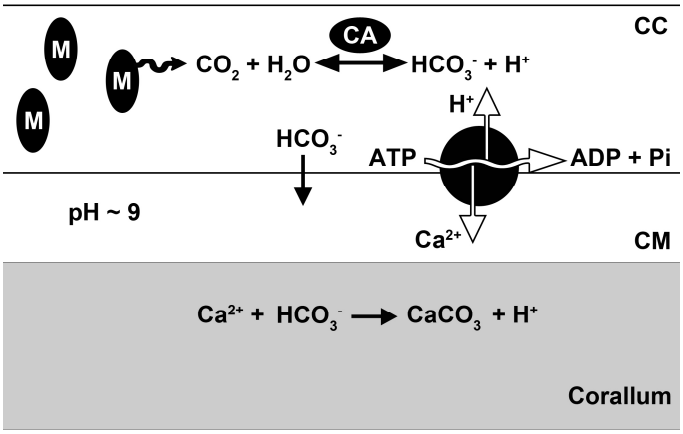

Figure 3: Schematic overview of the calcification process in scleractinian corals. Carbon dioxide produced by calicoblastic mitochondria is converted to bicarbonate by the enzyme carbonic anhydrase. Bicarbonate diffuses or is transported to the calcifying medium (CM). Calcium ions are transported to the calcifying medium by a Ca^{2+}/H^+ ATP-ase, which also removes protons from the calcifying medium. The antiport of calcium ions and protons generates a high calcium concentration and pH in the calcifying medium, resulting in a supersaturation of calcium carbonate. As a result, calcium carbonate precipitates as aragonite crystals and a corallum is produced. Based on Furla et al. (2000) and Al-Horani et al. (2003a,b). CC: calicoblastic cell. CM: calcifying medium. M: mitochondrion. CA: carbonic anhydrase.

The potentially underestimated role of coral heterotrophy in nutrient acquisition

Heterotrophic feeding is known to substantially contribute to the nutrient acquisition of scleractinian corals (reviewed by Houlbrèque and Ferrier-Pagès 2009; Ferrier-Pagès et al. 2011). For example, it is clear that uptake of organic carbon and nitrogen through ingestion of zooplankton can be considerable. For the well-studied species *Stylophora pistillata*, the minimal heterotrophic contribution to carbon input is 18 µg C cm^{-2} tissue day^{-1}, but can be as high as 56 µg C cm^{-2} tissue day^{-1} (Table 1). In relative terms, the contribution of heterotrophy represents 13 to 71% of the total carbon input for scleractinian

corals (Ferrier-Pagès et al. 2011). When corals are bleached, and photosynthesis is absent, this value can reach 100% (Grottoli et al. 2006). For nitrogen, the heterotrophic input is approximately 3 µg N cm^{-2} tissue day^{-1}, which is sufficient to meet the nitrogen-specific growth rate of *S. pistillata* (0.5 - 1.8 µg N cm^{-2} tissue day^{-1}, Ferrier-Pagès et al. 2003; Grover et al. 2008). Because it is still unclear how much nitrogen is translocated from the zooxanthellae to the coral host, the relative contribution of heterotrophy to nitrogen input is unknown. However, given the high C:N ratio of translocated organic compounds obtained through photosynthesis (Davies 1984; Falkowsky et al. 1984; Brown and Bythell 2005), this relative value is probably high.

Table 1: Overview of organic carbon and nitrogen inputs from auto- and heterotrophy for *Stylophora pistillata*.

	photo-synthesis	DOM	SPM	pico/ nanoplankton	zooplankton	% contribution heterotrophy
carbon (µg cm^{-2} day^{-1})	0-123	0-20	5	8	5-23	13-100
nitrogen (µg cm^{-2} day^{-1})	?	0.5	0.3	0.4	1.8	?

DOM: dissolved organic matter. SPM: suspended particulate matter. Data based on Anthony (1999); Ferrier-Pagès et al. (2003); Houlbrèque et al. (2004b); Palardy et al. (2005); Grover et al. (2008).

Although the view that scleractinian corals are polytrophic is well supported by the literature, the used methodologies may underestimate the importance of heterotrophy. This is because only internal digestion of prey is taken into account (Grottoli et al. 2006; Osinga et al. 2008; Purser et al. 2010), while it is known that corals may also digest prey externally with extruded mesenterial filaments (Duerden 1902; Carpenter 1910; Matthai 1918; Yonge 1930,1973; Abe 1938; Goreau et al. 1971; Lang 1973; Logan 1984; Lang and Chornesky 1990; Goldberg 2002). Externally digested prey may contribute significantly to

the daily carbon, nitrogen and phosphorus uptake of scleractinian corals, but this has never been quantified adequately. Until now, studies have resorted to particle analysis of the polyp coelenteron or prey clearance rate (Leversee 1976; Dai and Lin 1993; Webber and Roff, 1995; Sebens et al. 1996,1998; Witting 1999; Ferrier-Pagès et al. 2003; Houlbrèque et al. 2004; Palardy et al. 2005; Grottoli et al. 2006; Osinga et al. 2008; Hii et al. 2009; Purser et al. 2010). Both techniques have clear limitations. The first method only quantifies ingestion, excluding extracoelenteric digestion which may be an important process in terms of prey items digested and nutrients assimilated. The alternative, prey clearance rate, takes both ingested and externally retained particles into account, but fails to reveal the dynamics of prey capture, (extracoelenteric) digestion and release, possibly obscuring realistic estimates of nutrient input from zooplankton. This is because (partially) digested and subsequently released particles are re-counted in the water column, and therefore not quantified as captured and digested. In addition, previous studies have not quantified the fraction of organic matter utilised by corals after prey capture, but rather assumed various quantities of carbon assimilation from prey items (Fabricius et al. 1995; Grottoli et al. 2006; Purser et al. 2010), preventing realistic estimates of nutrient input through heterotrophy. Finally, little is known about the gain of phosphorous from heterotrophic feeding, which is an important element for coral growth (D'Elia 1977). Until now, only Sorokin (1973) determined phosphorous uptake from bacteria, which was estimated at 3 μg P day^{-1}, which is difficult to compare with other elements as it was not expressed per unit of biomass.

Heterotrophy and coral growth: conflicting results

Next to the important role of heterotrophy in the coral nutrient budget, feeding on organic matter has been demonstrated to have profound effects on coral growth (reviewed by Houlbrèque and Ferrier-Pagès 2009; Ferrier-Pagès et al. 2011). Both calcification and soft tissue synthesis are positively affected by

zooplankton supplementation (Houlbrèque and Ferrier-Pagès 2009; Ferrier-Pagès et al. 2011). At present, three important mechanisms have been identified that may explain the enhancement of coral calcification by heterotrophic feeding.

First of all, feeding enhances the synthesis of the organic matrix (Houlbrèque et al. 2004a), the protein structure that provides a framework for calcification by acting as a nucleation site for calcium carbonate crystals (reviewed by Allemand et al. 2004). This extracellular matrix is essential to biomineralisation (Falini 1996), and is composed of proteins, polysaccharides, glycosaminoglycans, lipids and chitin (Wainwright 1963; Young et al. 1971; Constanz and Weiner 1988). After 8 weeks of zooplankton supplementation, organic matrix synthesis rates of *Stylophora pistillata* microcolonies, measured by ^{14}C-aspartic acid incorporation, were 60 to 209% higher compared to unfed corals (Houlbrèque et al. 2004a). This was accompanied by an 85 to 113% increase in calcification rates of fed corals compared to starved ones, suggesting a link between organic matrix synthesis and calcification. In addition, pharmacological interference of organic matrix synthesis with emetin, cycloheximide or tunicamycin almost instantaneously impaired calcification (Allemand et al. 1998). The relationship between heterotrophic feeding, organic matrix synthesis and calcification may be explained by aspartic acid, an amino acid. Aspartic acid is a major component of the organic matrix (Young 1971; Cuif and Gautret 1995; Dauphin and Cuif 1997; Allemand et al. 1998), and no tissue pool of this amino acid is found in corals (Allemand et al. 1998), suggesting the need for a constant supply from an exogenous source (Houlbrèque et al. 2004). In this perspective, zooplankton feeding most likely enhances calcification indirectly by providing additional amino acids for organic matrix synthesis.

The same and other studies have also demonstrated that feeding increases zooxanthellae density, chlorophyll content and as a result, photosynthetic potential (Dubinsky et al. 1990; Titlyanov et al. 2000a,b; Titlyanov et al. 2001; Houlbrèque et al. 2003, 2004a). Photosynthesis, in turn,

is a major driver of calcification, as it provides the coral host with organic compounds and oxygen for generating metabolic energy to fuel calcium and proton transport. It also enhances calcium carbonate precipitation by increasing the pH of coral tissue and the calcification site (Furla et al. 2000; Al-Horani et al. 2003; Venn et al. 2011).

The third process which is thought to underlie the positive effects of heterotrophic feeding on calcification is increased supply of metabolically derived dissolved inorganic carbon (DIC, Ferrier-Pagès et al. 2003). DIC is used as a substrate for calcification, as carbon dioxide (CO_2), bicarbonate (HCO_3^-) or carbonate (CO_3^{2-}). Up to 75% of the DIC used for calcification is generated by the coral's own respiration, while only 25 to 30% is derived from the ambient seawater (Furla et al. 2000). Feeding corals results in thicker tissue, possibly providing more DIC as substrate for calcification due to increased respiration rates (Ferrier-Pagès et al. 2003).

Although the enhancement of coral calcification by heterotrophy has been demonstrated with long-term experiments, little is known about its short-term effects. Interestingly, heterotrophy has been shown to exert an inhibitory short-term effect on dark calcification rates (Al-Horani et al. 2007; Colombo-Pallotta et al. 2010). This discrepancy is puzzling, and has not been addressed to date. The current literature suggests that in darkness, inhibition of calcification resulting from heterotrophic feeding may be caused by a temporal reallocation of energy, for example to prey capture and nutrient uptake (Al-Horani et al. 2007; Colombo-Pallotta et al. 2010). This reallocation of energy in darkness may result in a temporal decrease in tissue oxygen concentrations during prey capture, without photosynthetic oxygen production to compensate for this. As oxygen is a prerequisite for ATP-synthesis through oxidative phosphorylation in calicoblastic mitochondria (Babcock and Wikström 1992), oxygen limitation may result in impaired ATP production and, hence, impaired calcification rates, as Ca^{2+}/H^+ ATP-ases require ATP or ADP for active transport of calcium ions and protons over the calicoblastic membrane (Ip et al. 1991). Indeed, Rinkevich and Loya (1984) and Colombo-Pallotta et al.

(2010) found that external oxygen supplementation enhances dark calcification rates of *Stylophora pistillata* and *Montastraea faveolata*, respectively, supporting the theory that oxygen limitation may indeed impair dark calcification of scleractinian corals during feeding. It is likely that in light, heterotrophic feeding does not exert an inhibitory effect on calcification, as zooxanthellae may compensate for the increased oxygen demand through photosynthesis. This, however, remains to be determined.

(A)biotic factors modulating coral heterotrophy

Several factors may affect feeding rates of benthic marine invertebrates, and therefore the role of heterotrophy in their growth and nutrient budget. These factors include bleaching status (Grottoli et al. 2006), water flow rate (Hunter 1989; Dai and Lin 1993; Helmuth and Sebens 1993; Fabricius et al. 1995; Heidelberg et al. 1997; Sebens et al. 1997, 1998; Lin et al. 2002), prey density (Osinga et al. 2011b) and symbiotic organisms (Wijgerde et al. 2011b).

Water flow is a key parameter in this respect, as sessile organisms including corals depend on water movement to provide them with prey items (Brusca and Brusca 2003). Increased flow rates will increase the encounter rate or flux of food particles (Best 1988; Hunter 1989; Fabricius et al. 1995; Sebens et al. 1998), but will also increase the kinetic energy of particles passing by. A higher kinetic energy of food particles may impose constraints on the capturing abilities of coral polyps, as has been documented for octocorals (Wainwright and Koehl 1976; Patterson 1984; McFadden 1986). Moreover, drag forces caused by water flow can result in deformed feeding structures, decreasing capture efficiency (Wainwright and Koehl 1976; Leonard et al. 1988; Sebens and Johnson 1991; Dai and Lin 1993; Fabricius et al. 1995; Anthony 1997; Sebens et al. 1997). Furthermore, corals may contract their tentacles if extension is no longer cost efficient (Dai and Lin 1993). These mechanisms explain why bell-shaped relationships between water flow rate and prey capture have been found for several coral species

(Dai and Lin 1993; Helmuth and Sebens 1993; Sebens et al. 1997; Lin et al. 2002).

In addition, the presence of neighbouring polyps (i.e. the polyp's context) may also influence individual polyp feeding rates, both in negative and positive ways. Negative effects may include local particle depletion and polyp shading, resulting in decreased prey capture by downstream polyps (Hunter 1989). At low water flow, and thus low particle flux, upstream polyps may reduce particle availability for their downstream clonemates, which as a result capture less prey. At high flow rates, upstream polyps may cover downstream polyps due to deformation, thereby shading the feeding structures of the latter. Positive effects may include the generation of intracolonial turbulence and mucus secretion by upstream polyps, enhancing prey capture by downstream polyps (Helmuth and Sebens 1993; Sebens et al. 1997; Hii et al. 2009).

Next to the factors listed above, prey concentration is known to affect coral feeding rates, with approximate linear or curvilinear relationships between prey availability and feeding rates (Clayton and Lasker 1982; Lasker 1982; Lewis 1992; Ferrier-Pagès et al. 1998a, 2003; Houlbrèque et al. 2004b). The positive linear relationship is likely due to increased particle fluxes over feeding polyps at higher prey concentrations, which in turn increase prey availability (Hunter 1989).

Finally, the presence of symbiotic epibionts may affect corals in many ways, including heterotrophic feeding. For example, 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, i.e. the removal of acquired prey items from the coral polyp by flatworms, may further reduce coral feeding rates.

Insight into the individual and interactive effects of the (a)biotic factors above will enhance our understanding of the relative importance of coral

heterotrophy under a wide range of conditions. This also has implications for aquaculture, as heterotrophic feeding is a limiting factor to coral growth (Houlbrèque and Ferrier-Pagès 2009; Osinga et al. 2011a).

General aim and research questions

Based on the important knowledge gaps outlined above, the aim of this thesis was to increase our understanding of the role of heterotrophic feeding in the nutrient budget and growth of the scleractinian coral *Galaxea fascicularis*, and how (a)biotic factors affect this role.

The research questions for this thesis were:

1. What is the potential role of heterotrophic feeding in the nutrient acquisition and budget for the scleractinian coral *Galaxea fascicularis*? (**Chapters 2 and 6**)
2. What mechanism explains the inhibitory short-term effect of zooplankton feeding on skeletal growth of *G. fascicularis*? (**Chapter 3**)
3. How does water flow rate affect zooplankton feeding by solitary polyps and colonies of *G. fascicularis*? (**Chapter 4**)
4. What is the effect of epizoic acoelomorph flatworms on zooplankton feeding by *G. fascicularis*, and is this effect dependent on prey availability? (**Chapter 5**)

Thesis outline

This thesis is composed of a general introduction (**Chapter 1**), four research chapters (**Chapter 2, 3, 4** and **5**) and a general discussion (**Chapter 6**). **Chapter 2** presents analyses of zooplankton feeding by the scleractinian coral *Galaxea fascicularis*. For this study, corals were individually incubated and fed in a flow cell, and their feeding activity was recorded with a video camera. In

addition, nutrient depletion of digested zooplankton was measured, and the data were used to calculate daily nutrient inputs from heterotrophic feeding. **Chapter 3** describes the short-term effects of zooplankton feeding on calcification rates of *G. fascicularis* under different light and oxygen conditions. Calcification rates were determined using the alkalinity anomaly technique, which allows for accurate measurement of skeletal growth. **Chapter 4** investigates the interactive effects of water flow rate and colony size on zooplankton feeding by *G. fascicularis*. For this study, both solitary polyps and whole colonies were incubated in a flow cell at water flow rates ranging from 1.25 to 40 cm s^{-1}. **Chapter 5** focuses on the interactive effects of epizoic acoelomorph flatworms and ambient prey concentration on zooplankton feeding by *G. fascicularis*. Solitary polyps were either incubated together with their symbiotic flatworms, or dewormed using an anthelminthic, and exposed to prey concentrations of 250 to 1,000 *Artemia* nauplii L^{-1}. Finally, **Chapter 6** discusses and integrates the obtained results in the context of existing literature, and provides recommendations for future research.

Chapter 2

Extracoelenteric zooplankton feeding is a key mechanism of nutrient acquisition for the scleractinian coral *Galaxea fascicularis*

This chapter was published as:

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Abstract

Internal and external feeding on zooplankton may provide scleractinian corals with important nutrients. The latter process has however never been properly quantified. To quantify the dynamics of zooplankton capture, digestion and release for a scleractinian coral, we performed detailed video analyses of *Galaxea fascicularis* feeding on *Artemia* nauplii. A highly dynamic process of prey capture, digestion and release was observed. A single *G. fascicularis* polyp captured 558±67 nauplii, and released 383±75 *Artemia* nauplii (*N*=3) over a 6 hour interval. On average, 98.6% of prey captured was not ingested. Instead, prey items were clustered in aggregates that were digested externally by mesenterial filaments.

In addition, we employed carbon, nitrogen and phosphorus analysis of zooplankton before and after digestion by *G. fascicularis* colonies (*N*=6). For total organic carbon (TOC), 43.1% (0.298±0.148 µg *Artemia*$^{-1}$) was lost after 6 hours of digestion. For total organic nitrogen (TON), total organic phosphorus (TOP) and orthophosphate (PO_4^{3-}), these values were 51.3% (0.059±0.028 µg *Artemia*$^{-1}$), 50.9% (0.009±0.004 µg *Artemia*$^{-1}$) and 84.6% (0.0019±0.0008 µg *Artemia*$^{-1}$), respectively. For extracoelenteric zooplankton feeding alone, total estimated nutrient inputs for *G. fascicularis* colonies were 76.5±0.0 µg organic carbon, 15.2±0.0 µg organic nitrogen, 2.3±0.2 µg organic phosphorus and 0.5±0.8 µg inorganic phosphorus per cm^2 coral tissue per day. These values exceed calculations based on intracoelenteric feeding by up to two orders of magnitude. Our results demonstrate that extracoelenteric zooplankton feeding is a key mechanism of nutrient acquisition for a scleractinian coral. These results are of importance to coral aquaculture and our understanding of benthic-pelagic coupling on coral reefs.

Introduction

Heterotrophy is vital to coral nutrition, as it supplies the coral and its symbiotic algae with essential elements such as carbon, nitrogen and phosphorus. For the scleractinian *Stylophora pistillata* (Esper 1797), it has been found that heterotrophy increases tissue protein concentration, stimulates growth directly by enhancing calcification and organic matrix synthesis, and indirectly by increasing photosynthetic rates (Houlbrèque and Ferrier-Pagès 2009 and references therein). Furthermore, heterotrophy is an important source of nutrients during coral bleaching episodes, when autotrophy is virtually absent due to loss of symbiotic dinoflagellates (Grottoli et al. 2006). Heterotrophic sources for corals consist of dissolved organic matter (DOM), and detrital and live particulate organic matter (POM) including bacteria, protozoa, phytoplankton and zooplankton. Of these sources, zooplankton constitutes a significant proportion of the daily carbon and nitrogen input for scleractinians, up to 100% of the total organic carbon input (Grottoli et al. 2006) and approximately 49% of the total organic nitrogen input when high prey concentrations are used (Ferrier-Pagès et al. 2003; Houlbrèque and Ferrier-Pagès 2009).

Scleractinian corals employ intricate mechanisms of zooplankton capture, which encompass tentacle movement combined with cnidocyte firing and subsequent mucociliary feeding to ingest immobilised prey (Sorokin 1990). It has long been known that scleractinian corals may also digest prey externally, by expulsion of mesenterial filaments as a response to prey detection (Duerden 1902; Carpenter 1910; Matthai 1918; Yonge 1930,1973; Abe 1938; Goreau et al. 1971; Lang 1973; Logan 1984; Lang and Chornesky 1990; Goldberg 2002). Mesenterial filaments may be extruded through any part of the polyp epithelium, after which prey is either ingested (Goldberg 2002) or digested externally (Lang 1973). Externally digested prey may contribute significantly to the daily carbon, nitrogen and phosphorus input to the diet of scleractinian corals, but this has never been quantified adequately.

Until now, studies have resorted to particle analysis of the polyp coelenteron or prey clearance rate (Leversee 1976; Dai and Lin 1993; Webber and Roff 1995; Sebens et al. 1996,1998; Witting 1999; Ferrier-Pagès et al. 2003; Houlbrèque et al. 2004b; Palardy et al. 2005; Grottoli et al. 2006; Osinga et al. 2008; Hii et al. 2009; Purser et al. 2010), with clear limitations. The first method only quantifies ingestion, excluding extracoelenteric digestion which may be an important process in terms of number of prey items digested and nutrients assimilated. The alternative, prey clearance rate, takes both ingested and externally retained particles into account, but fails to reveal the dynamics of prey capture, (extracoelenteric) digestion and release, possibly obscuring realistic estimates of nutrient input from zooplankton. This is because (partially) digested and subsequently released particles are re-counted in the water column, and therefore not quantified as captured and digested.

To quantify the dynamics of zooplankton prey capture, digestion and release for a scleractinian coral, we performed detailed video analyses of single polyps of the Oculinid scleractinian *Galaxea fascicularis* (Linnaeus 1767) feeding on nauplii of the brine shrimp *Artemia*. In addition, we employed carbon, nitrogen and phosphorus analysis of *Artemia* nauplii before and after capture by *G. fascicularis* colonies to estimate the quantitative role of (extracoelenteric) zooplankton feeding in the diet of a common Indo-Pacific scleractinian coral.

Materials and methods

Selected species and husbandry

For this study, the Indo-Pacific scleractinian species *Galaxea fascicularis* (Linnaeus 1767) was used, bearing corallites which are usually less than 10 mm in diameter (Veron 2000). All colonies were genetically identical to rule out genotype-specific effects. Corals were kept in a closed system of 400 L, with the following parameters (± indicates min-max deviations) salinity 35±0.5

g L^{-1}, temperature 26±0.5 °C, pH 8.2±0.3, photon flux density 368 µmol m^{-2} s^{-1} (12/12h light regime), nitrate 2±1 mg L^{-1}, phosphate 0.03±0.01 mg L^{-1}, calcium 400±20 mg L^{-1}, magnesium 1300±50 mg L^{-1}. Water flow was provided by four Turbelle nanostream 6045 circulation pumps (Tunze Aquarientechnik GmbH, Penzberg, Germany) and an Eheim 1260 return pump (Eheim GmbH Co. KG, Deizisau, Germany), providing a total flow rate of 20,000 L h^{-1} or 5-10 cm s^{-1}. Single polyp clones were used for the photographic and video analysis. Single polyps were individually removed from a large parent colony by using pincers, and subsequently glued onto 7x7 cm PVC plates with epoxy resin (Aqua Medic GmbH, Bissendorf, Germany). Whole colonies were used for the carbon, nitrogen and phosphorus depletion studies. All single polyps and colonies were of the same genotype, since they all originated from a single parent colony.

Analysis of colony surface area, polyp number and polyp density

To determine average colony surface area, polyp number and polyp density for *G. fascicularis*, we photographed colonies (*N*=4) and analyzed images using ImageTool 3.0. Surface area was determined by using a ruler as a reference. Polyp numbers were scored and polyp densities were calculated from colony surface areas and polyp numbers.

Determination of aggregate density

To determine the average aggregate density on *G. fascicularis* colonies, we incubated colonies (*N*=4) in a respirometric flow cell together with *Artemia* nauplii at a concentration of 4,100 *Artemia* nauplii L^{-1}. Colonies were photographed at 6 hours of incubation and images were analyzed using Adobe Photoshop 11.0.1. Aggregate numbers were scored and aggregate densities were calculated from colony surface areas and aggregate numbers.

Video analysis

For photographic and video analysis, single polyp clones of *G. fascicularis* (*N*=3) were incubated in a respirometric flow cell (Wageningen UR, Wageningen, The Netherlands) with a volume of 3.5 L for 6 hours. Water flow was created by a built-in model boat propeller, driven by a Maxon DC motor which was connected to a computer. Flow speed was set at 200 RPM, equal to 5 cm s^{-1}, controlled by EPOS user interface software (version 2.3.1). For more details see Schutter et al. (2010). Water from the holding tank was used for the experiments to rule out artifacts resulting from changes in water chemistry. Temperature was kept at 26±0.5°C by means of a water jacket connected to a TC20 water cooler (Teco SRL, Ravenna, Italy). Photon flux density was set to holding tank intensity, 368 µmol m^{-2} s^{-1}, with a T5 fluorescent lighting fixture containing four 24W T5 fluorescent tubes with a color temperature of 14,000 Kelvin (Elke Müller Aquarientechnik, Hamm, Germany). An HDR-CX505VE handy cam (Sony Corporation, Tokyo, Japan) was used for recording still and moving images in high resolution formats. *Artemia* 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°C, and used immediately after hatching. Average nauplii size was 440 µm according to the manufacturer. A concentration of 10,000 *Artemia* nauplii L^{-1} was used for all experiments (*N*=3). Polyps were acclimated for 15 minutes before the start of every incubation. Each polyp was analyzed once. Capture and release of *Artemia* nauplii by the coral polyps was scored by analyzing videos after all experiments. Captured nauplii were defined as prey that attached to the polyp surface for at least 10 seconds. Released nauplii were defined as prey that detached from the polyp surface and remained in suspension for longer than 10 seconds. Aggregate formation was defined as a cluster of two or more nauplii attached to the polyp surface.

Carbon, nitrogen and phosphorus depletion

For the carbon, nitrogen and phosphorus depletion studies, the same setup was used as described above. *G. fascicularis* colonies (*N*=6) with an average

of 449±22 polyps were used and incubated for 6 hours in a respirometric flow cell. A concentration of 10,000 *Artemia* nauplii L^{-1} was used for all experiments. Colonies were acclimated for 15 minutes before the start of every incubation. Each colony was analyzed once. As a negative control, nauplii from each experiment's stock were incubated in a water bath at 26±0.5°C for 6 hours, to determine their inherent metabolism (mainly yolk sac consumption). Data on lost carbon, nitrogen and phosphorus, due to this inherent metabolism, was used to calculate net loss of nutrients after digestion. To determine the nutrient content of nauplii at the start of each experiment, nauplii were collected from the stock population, washed on a 150 µm filter mesh, quantified by multiplying the collected volume (500 µL) with that day's determined stock concentration and frozen shortly after hatching. The same procedure was carried out for the control samples, after 6 hours of incubation in a water bath at 26°C. About 2,000 nauplii were collected during each experiment for both the start and control samples. After 6 hours of incubation, nauplii from the digestion experiment were collected with plastic Pasteur pipettes. As *G. fascicularis* polyps retain most of their prey externally, aggregates of *Artemia* nauplii could easily be collected from the polyp surface. After collection, nauplii were transferred onto a 150 µm filter mesh and washed thoroughly with demineralised water. After washing, nauplii were quantified by counting all individuals under an M8 stereomicroscope (Wild Heerbrugg, Heerbrugg, Switzerland). All samples were transferred to 50 ml tubes (Greiner Bio One GmbH, Frickenhausen, Germany), resuspended in 50 ml demineralised water, and frozen at -20°C until further analysis.

Carbon, nitrogen and phosphorus content analysis

To determine carbon, nitrogen and phosphorus depletion, several methods were used. All samples were thawed in water baths at 25°C and subsequently centrifuged at 3,000 RPM and 4°C for 3 minutes. 40 ml of each supernatant was removed. Samples were homogenized with an Ultratorax X1030 homogenizer (Ingenieurbüro M. Zipperer GmbH, Dottingen, Germany) for 5

minutes in 10 ml remaining volume at room temperature. For each sample, the Ultratorax was washed with demineralised water after homogenization to collect remaining *Artemia* tissue. Next, samples were centrifuged at 3,000 RPM and 4°C and were adjusted to 20 ml final volume with demineralised water. Total carbon (TC) was measured by high temperature catalytic oxidation on a TOC-5050A auto analyzer (Shimadzu Scientific Instruments, Shimadzu Corporation, Kyoto, Japan) followed by detection of CO_2 with a non-dispersive infrared sensor (NDIR). Inorganic carbon (IC) was measured by acidifying subsamples to a 25% phosphoric acid solution followed by NDIR detection of purged CO_2. Total nitrogen (N_{tot}), total phosphorus (P_{tot}), inorganic nitrogen (ammonium, nitrite and nitrate) and inorganic phosphorus (orthophosphate), were analyzed with a San^{++} continuous flow analyzer (Skalar Analytical BV, Breda, The Netherlands). All measured concentrations were converted to µg per *Artemia* nauplius, by taking volume (20 ml) and number of *Artemia* nauplii in each sample into account. Total organic carbon (TOC), total organic nitrogen (TON) and total organic phosphorus (TOP) were calculated by subtracting the inorganic from the total fractions.

Data analysis

Normality of data was tested by plotting the residuals of each dataset versus predicted values, and by performing a Shapiro-Wilk test. Homogeneity of variances was determined using Levene's test. Residuals of TOC, TON, TOP and PO_4^{3-} depletion and N:P ratios were normally distributed ($P>0.05$), whereas those of C:N and C:P ratios were not ($P<0.05$). All depletion data showed homogeneity of variances ($P>0.05$), except those for PO_4^{3-} and C:P ratios ($P<0.05$). For TOC, TON and TOP we used one-way ANOVA followed by Bonferroni's test. For PO_4^{3-} we used one-way ANOVA followed by Games-Howell. For the C:N, C:P and N:P ratios we used Kruskal-Wallis followed by Mann-Whitney. A value of $P<0.05$ was considered statistically significant, except for Mann-Whitney where we used a critical value of $P<0.025$, based on a Bonferroni correction factor of 2. Statistical analysis was performed with

SPSS Statistics 17.0. Graphs were plotted with SigmaPlot 11.0. All data presented are means ± s.d.

Results

Throughout all of the 6 hour incubations, tentacles of *G. fascicularis* polyps were active and well expanded. Tentacles moved towards *Artemia* nauplii which came in contact with the polyp, actively maneuvering nauplii onto the oral disc. During the last hour of incubation, a slight retraction of tentacles was visible (Figure 1 and supplemental video*). Mucus excretion was apparent, which seemed to aid in prey capture. Several flatworms, possibly *Waminoa* sp., were also observed, slowly moving across the oral disc. At approximately 20 minutes, expulsion of mesenterial filaments through several areas of the polyp ectoderm and oral pore was clearly visible (Figure 1 and supplemental video*). Within the first 30 minutes of the incubations, aggregates of *Artemia* nauplii started to appear on the polyp surface. These aggregates increased in size over time (Figure 1, Figure 2 and supplemental video*). One to three aggregates per polyp were observed.

G. fascicularis polyps captured and released significant amounts of *Artemia* nauplii during the incubations (Figure 2). On average, a single polyp captured 558±67 nauplii, and released 383±75 nauplii over the entire 6 hour period (Figure 2). Ingestion of nauplii was observed for only one of the three single polyps tested. In total, 27 nauplii were ingested, which was only 4.1% of the total number of captured nauplii at 360 minutes (659) for that polyp. During the first 300 minutes, more nauplii were captured than released (Figure 2A). This was reflected in the size of the aggregates that formed, which increased considerably to an average size of 165 nauplii (93.8% of maximum aggregate size) during the first 210 minutes (Figure 2B and supplemental video*). As time progressed, the dynamics of prey capture and release leveled off. This was indicated by the decreasing amounts of nauplii captured and released per time interval (Figure 2A), as well as the cumulatives for *Artemia* captured and released (Figure 2B). Maximum average aggregate size was

176 nauplii, which was reached at 300 minutes. After 300 minutes, capture and release rates became similar and as consequence, aggregate size did not increase further (Figure 2B and supplemental video*). After 6 hours, polyps slowly released aggregates, possibly by increasing mucus production (not shown).

The concentration of *Artemia* nauplii decreased from 10,000 prey L^{-1} to a minimum of approximately 9,950 prey L^{-1}, at 300 minutes, calculated by a maximum average aggregate size of 176 nauplii. This equaled a concentration decrease of 0.5%.

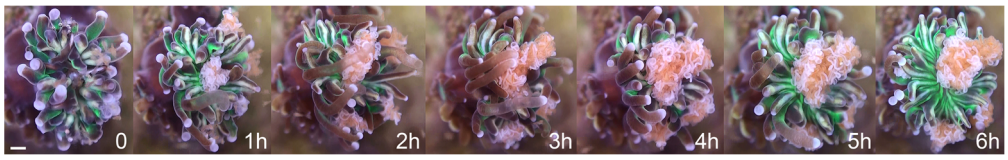

Figure 1: Photographic time lapse series of *Artemia* nauplii aggregate formation during a six-hour incubation, representative for all individual polyps tested (*N*=3). Polyps displayed tentacle expansion and extrusion of mesenterial filaments (white strands). Aggregates increased significantly during the first 210 minutes, after which they stabilized. h = hour, scale bar = 500 µm.

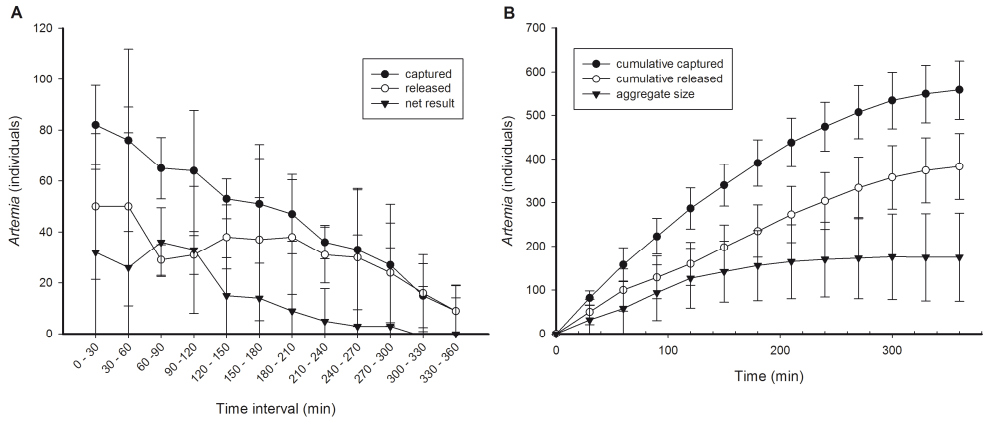

Figure 2: Overview of *Artemia* nauplii capture and release dynamics of single polyps of *G. fascicularis*. A. Numbers of captured and released nauplii, and their net result, shown in 30

minute intervals. B. Cumulative capture, release and accumulation (aggregate size). Values are means ± s.d. (*N*=3).

Multiple-polyp colonies of *G. fascicularis* also retained aggregates of *Artemia* nauplii at the polyp surface during six-hour incubations, at a density of 0.08±0.03 aggregates polyp^{-1}. *Artemia* aggregates remained in intimate contact with protruded mesenterial filaments for several hours, suggesting extensive digestive processes (supplemental video*). Captured *Artemia* nauplii appeared fragmented and heavily depigmentated at the end of the incubations. Subsequent elemental analysis showed that the putatively digested *Artemia* nauplii were significantly depleted of total organic carbon (TOC), total organic nitrogen (TON), total organic phosphorus (TOP) and inorganic phosphorus (PO_4^{3-}) (Figure 3) when compared to nauplii that had not been captured. After 6 hours of incubation with *G. fascicularis* colonies, captured *Artemia* nauplii were found to have a TOC content of 0.381±0.114 µg *Artemia*$^{-1}$, a TON content of 0.056±0.023 µg *Artemia*$^{-1}$, a TOP content of 0.009±0.004 µg *Artemia*$^{-1}$ and a PO_4^{3-} content of 0.0007±0.0002 µg *Artemia*$^{-1}$ (Figure 3). These values were significantly lower ($P \leq 0.03$, Bonferroni for TOC, TON, TOP and $P < 0.01$, Games-Howell for PO_4^{3-}) than the values found for the negative controls (i.e. *Artemia* nauplii that had been incubated in seawater for 6 hours), which were 0.678±0.206 µg *Artemia*$^{-1}$ for TOC, 0.115±0.037 µg *Artemia*$^{-1}$ for TON, 0.018±0.006 µg *Artemia*$^{-1}$ for TOP and 0.0025±0.0008 µg *Artemia*$^{-1}$ for PO_4^{3-}, respectively (Figure 4). No significant differences between the negative controls and freshly hatched *Artemia* nauplii (t=0) were found ($P > 0.05$, Bonferroni for TOC, TON and TOP; $P > 0.05$, Games-Howell for PO_4^{3-}). Inorganic nitrogen species (ammonium, nitrite and nitrate) could not be measured accurately due to very low concentrations and are therefore not shown. When taking the nutrient content of *Artemia* nauplii at the start of every experiment into account, and correcting for all negative controls, 43.1% (0.298±0.148 µg *Artemia*$^{-1}$) of TOC was lost after 6 hours of incubation with *G. fascicularis* colonies (Figure 4). For total organic nitrogen (TON), total

organic phosphorus (TOP) and orthophosphate (PO_4^{3-}), these values were 51.3% (0.059±0.028 µg *Artemia*$^{-1}$), 50.9% (0.009±0.004 µg *Artemia*$^{-1}$) and 84.6% (0.0019±0.0008 µg *Artemia*$^{-1}$), respectively (Figure 4).

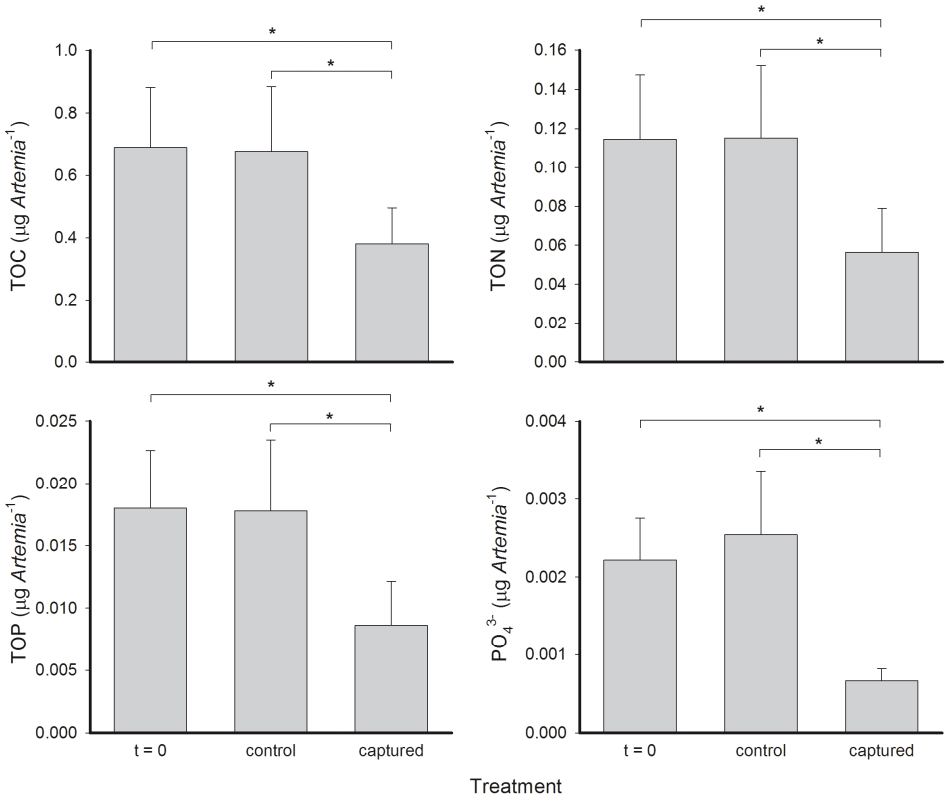

Figure 3: Total organic carbon (TOC), total organic nitrogen (TON), total organic phosphorus (TOP) and inorganic phosphorus (orthophosphate, PO_4^{3-}) content expressed in µg per *Artemia* nauplius for three different treatments. t = 0 indicates *Artemia* nauplii shortly after hatching. Control indicates 6 hour incubations of *Artemia* nauplii in a water bath at 26ºC. Captured indicates captured *Artemia* nauplii after 6 hours of incubation together with *G. fascicularis* colonies. Values are means + s.d. (*N*=6). Asterisks indicate significant differences between treatments ($P \leq 0.03$, Bonferroni for TOC, TON and TOP; $P < 0.01$, Games-Howell for PO_4^{3-}).

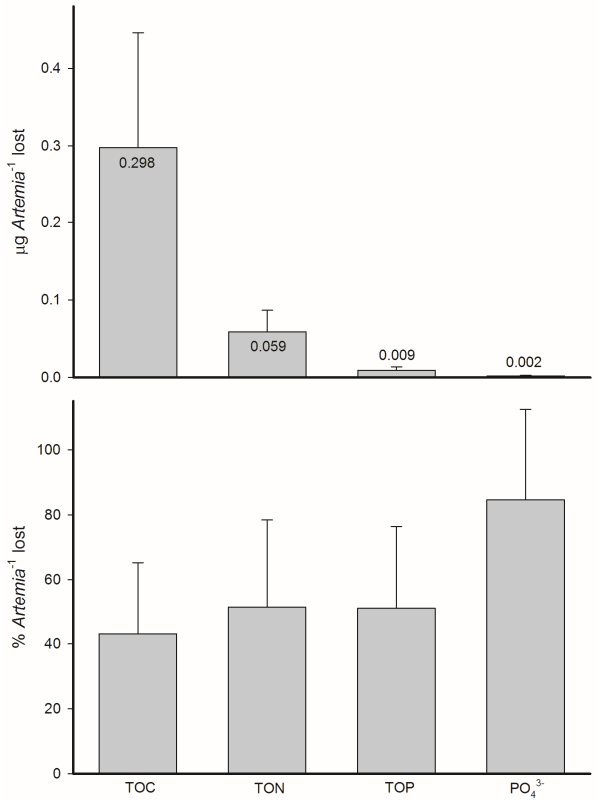

Figure 4: Loss of TOC, TON, TOP and PO_4^{3-} content expressed in absolute (µg *Artemia*$^{-1}$) and relative (% *Artemia*$^{-1}$) values of captured *Artemia* nauplii after 6 hours of incubation together with *G. fascicularis* colonies. All values were corrected for negative controls. Values are means + s.d. (*N*=6).

The carbon to nitrogen (C:N) ratios were 6.1±0.3 at t=0, 6.0±0.4 for the negative control and 7.5±2.1 for captured nauplii after 6 hour incubation with *G. fascicularis* colonies (Table 1). For the C:P ratios, these values were 38.1±1.9, 38.6±2.8 and 51.2±20.1, respectively (Table 1). For the N:P ratios we found values of 6.3±0.3, 6.5±0.3 and 6.7±0.7, respectively (Table 1). The carbon to nitrogen (C:N) ratio was not significantly different among the three groups of *Artemia* nauplii (captured, control and t=0, *P*>0.025, Mann-Whitney). The carbon to phosphorus (C:P) ratio of captured nauplii did not differ significantly from the negative control (*P*>0.025, Mann-Whitney), but was significantly different from t=0 (*P*<0.025, Mann-Whitney) after 6 hour

incubation with *G. fascicularis* polyps. The nitrogen to phosphorus ratio (N:P) did not differ significantly among the three groups of nauplii (P>0.025, Mann-Whitney).

Table 1: Nutrient ratios of *Artemia* nauplii.

	C:N ratio	C:P ratio	N:P ratio
t = 0	6.1±0.3	38.1±1.9	6.3±0.3
control	6.0±0.4	38.6±2.8	6.5±0.3
captured	7.5±2.1	51.2±20.1	6.7±0.7

Carbon to nitrogen (C:N), carbon to phosphorus (C:P) and nitrogen to phosphorus (N:P) ratios for *Artemia* nauplii at the start of the experiments (t=0), after 6 hour incubation in a water bath (control) and after 6 hour incubation with *G. fascicularis* colonies (captured). Values are means ± s.d. (N=6).

Table 2: Aggregate densities of *Artemia* nauplii on *G. fascicularis* colonies.

colony	aggregate density (aggregate polyp^{-1})
1	0.11
2	0.08
3	0.05
4	0.06
mean	0.08±0.03

Aggregate densities of *Artemia* nauplii on *G. fascicularis* colonies at 6 hour incubation, expressed in aggregate per polyp. We used a concentration of 4,100 nauplii L^{-1} and 5 cm s^{-1} as the water flow rate. Corals were allowed to feed for 6 hours. Mean is ± s.d. (N=4).

Discussion

Our results show that the scleractinian coral *Galaxea fascicularis* is capable of capturing large amounts of zooplankton prey within a time frame of several hours. The observed extrusion of mesenterial filaments and the clearly fragmented and depigmentated outer appearance of captured *Artemia* nauplii at the end of the incubations strongly suggest effective extracoelenteric

digestion of zooplankton. This assumption is supported by frequent reports of cnidarian mesenterial filaments as digestive structures (Duerden 1902; Carpenter 1910; Matthai 1918; Yonge 1930,1973; Abe 1938; Goreau et al. 1971; Lang 1973; Logan 1984; Lang and Chornesky 1990; Goldberg 2002), which may contain both digestive zymogen cells and absorptive cells (Yonge 1930; Abe 1938; Van Praët 1980). The observed decrease in prey capture and release over time (Figure 2), and the slow release of aggregates after 6 hours of incubation indicate satiation. This phenomenon, also found for the coral species *Acanthogorgia vegae* (Lin et al. 2002), is what would be expected if feeding were indeed the process at hand. As the concentration of *Artemia* nauplii, calculated by the total average nauplii aggregate size, only dropped slightly (with 0.5%), changes in capture rate due to a concentration effect can be ruled out.

On average, our *G. fascicularis* polyps captured 93±0.12 nauplii per hour, which is substantially higher compared to a similar study by Hii et al. (2009), who found a lower capture rate for *G. fascicularis* (50±30 nauplii polyp^{-1} hour^{-1}) under similar conditions by using prey clearance rate. This indicates that prey clearance rate studies may indeed underestimate the amount of prey captured and digested. Intracoelenteric prey analysis is another commonly used method to quantify zooplankton capture, and is highly precise. However, all externally digested prey items are not quantified, which may represent a major fraction of nutrient input. Grottoli et al. (2006) found that *Montipora capitata* polyps increased their feeding rate whilst in a bleached status, in contrast to two other species which displayed no increased capture of zooplankton (*Porites compressa* and *P. lobata*). According to the authors, this may lead to shifts in coral species composition on the reef within several decades from now, due to increased bleaching events and the heterotrophic advantage of species such as *M. capitata*. Taking extracoelenteric zooplankton feeding into account however could place this theory in a completely new perspective, as many species may be able to utilize this feeding mechanism. Although it could be argued that our observations are

genotype or species-specific, we found similar digestive behavior for a different genotype of *G. fascicularis* and a species with much smaller polyps, *Stylophora pistillata* (about 1 mm in diameter) by using video analysis (unpublished results). Extracoelenteric feeding has now been reported for many scleractinian coral species from various families including the Mussidae, Faviidae, Fungiidae, Meandrinidae, Astrocoeniidae, Pocilloporidae, Agariciidae, Siderastreidae, Poritidae and Oculinidae (Duerden 1902; Carpenter 1910; Matthai 1918; Yonge 1930,1973; Abe 1938; Goreau et al. 1971; Lang 1973; Logan 1984; Lang and Chornesky 1990; Goldberg 2002). This shows that extracoelenteric zooplankton feeding is a common feeding mechanism amongst scleractinian corals bearing a wide variety of polyp sizes.

The assumption of significant extracoelenteric feeding is supported by analysis of organic carbon, nitrogen, phosphorus and inorganic phosphorus of *Artemia* nauplii captured and processed by *G. fascicularis* polyps, which showed clear depletion of the aforementioned nutrients (Figs. 3,4). The carbon to nitrogen (C:N), carbon to phosphorus (C:P) and nitrogen to phosphorus (N:P) ratios of captured nauplii did not differ significantly from the negative controls after 6 hour incubation together with *G. fascicularis* colonies, indicating that all elements were taken up in equal proportions. Proportional uptake of carbon and nitrogen may have been due to the fact that *Artemia* nauplii are rich in proteins, with an average content of 52.2±8.8% (Léger et al. 1987), possibly resulting in concomitant uptake of both carbon and nitrogen from proteins. This theory is supported by the findings of Piniak and Lipschultz (2004), who found for *Oculina arbuscula* and *O. diffusa* that approximately 90% of ingested ^{15}N from labeled *Artemia* nauplii comprised proteins, amino acids and nucleic acids. Proportional uptake of carbon and phosphorus may have been the result of phospholipid and nucleic acid removal from prey: the lipid content of *Artemia* nauplii is on average 18.9±4.5% (Léger et al. 1987), of which 19.1±0.2% are phospholipids (Navarro et al. 1991). Indeed, both Al-Moghrabi et al. (1995) and Treignier et al. (2008) found an increase in the lipid content of scleractinian coral tissue after feeding with zooplankton, although

they did not specifically address phospholipids. Substantial assimilation of organic nitrogen from zooplankton is supported by the literature, as this element is considered an important building block for organic matrix synthesis and tissue growth (reviewed by Houlbrèque and Ferrier-Pagès 2009). Assimilation of organic phosphorus may be important for maintenance and growth of coral tissue (Sorokin 1973; D'Elia 1977). Sorokin (1973) demonstrated that corals are able to consume organic phosphorus in the form of planktonic bacteria (approximately 3 mg day^{-1}), although it is difficult to compare this value with our data as it is not expressed per unit of tissue surface area. The significant depletion of inorganic phosphorus (PO_4^{3-}) of captured *Artemia* nauplii (Figures 3,4) could have been due to uptake by symbiotic zooxanthellae. It is known that zooxanthellae reside in the coral gastroderm (reviewed by Furla et al. 2005; Stat et al. 2006), allowing efficient uptake of nutrients from digested prey by these symbiotic dinoflagellates. Moreover, it has been proposed that zooxanthellae may take up inorganic nitrogen from zooplankton directly, in the form of ammonium (NH_4^+) (Piniak and Lipschultz 2004). As phosphate is an important inorganic nutrient for zooxanthellae (Deane and O' Brien 1981; Jackson and Yellowlees 1990; Belda et al. 1993), it is possible that the observed phosphate depletion of *Artemia* nauplii was due to assimilation by zooxanthellae.

When estimating heterotrophic nutrient input from feeding on zooplankton, it is important to take digestive efficiency into account. Previous studies have assumed a 100% assimilation of available carbon from zooplankton during intracoelenteric digestion (Fabricius et al. 1995; Sebens et al. 1996, 1998; Houlbrèque et al. 2004a; Grottoli et al. 2006; Purser et al. 2010), which may not be accurate. In this study, depletion of total organic carbon, nitrogen and phosphorous was only 43.1%, 51.3% and 50.9% respectively. On the other hand, as polyps of *G. fascicularis* continue to capture prey throughout the observed period, and taking a digestion time of 3 to 6 hours into account (Lewis 1982; Fabricius et al. 1995; Hii et al. 2009), collected *Artemia* aggregates may have represented a heterogeneous pool in

terms of digestive status. Therefore, our measured nutrient depletions may reflect an average extracoelenteric feeding efficiency of *G. fascicularis* for *Artemia* nauplii. Another possible shortcoming of nutrient depletion measurements is that this method cannot distinguish between nutrients assimilated and those leaked into the surrounding environment. Therefore, tracer studies with stable isotopes, such as ^{13}C and ^{15}N, may provide even more detailed information about the efficiency of coral prey digestion and assimilation. Nevertheless, taking a certain proportion of refractory (i.e. resistant to digestion) organic material into account when estimating nutrient input from zooplankton seems important.

When assuming an average capture rate based on video analysis, average residence time of *Artemia* nauplii, and coupled to that an average digestive efficiency under environmental conditions as described above, nutrient input for *G. fascicularis* from zooplankton feeding can be calculated with the following formula:

$$X_H \text{ acquired} = ((X_{Artemia\ t=0} - X_{Artemia\ captured}) - (X_{Artemia\ t=0} - X_{Artemia\ control})) * 0.08P$$

where X_H is the amount of heterotrophically acquired carbon, nitrogen, phosphorus or orthophosphate expressed in µg per cm^2 of coral tissue per day, $X_{Artemia}$ is the average amount of TOC, TON, TOP or orthophosphate expressed in µg per *Artemia* nauplius, and P is the number of average prey items (*Artemia* nauplii) captured per cm^2 of coral tissue per day. The factor 0.08 corrects for intracolonial polyp competition, as incubations with colonies revealed that not all polyps in the context of a colony form aggregates (Table 2). The subscript *t=0* indicates freshly hatched nauplii, the subscript *control* indicates an incubation for 6 hours at 26°C without a *G. fascicularis* colony, and the subscript *captured* indicates captured nauplii during 6 hours of incubation with a *G. fascicularis* colony. The assumption is made that all nutrients lost are assimilated. Based on our observations and by using the above formula, we calculate that for *G. fascicularis* colonies, extracoelenteric

zooplankton feeding can provide 76.5±0.0 µg organic carbon, 15.2±0.0 µg organic nitrogen, 2.3±0.2 µg organic phosphorus and 0.5±0.8 µg inorganic phosphorus per cm^2 of tissue per day (Table 3). Following the same procedure, intracoelenteric feeding provides only 1.1±1.7 µg organic carbon, 0.2±1.7 µg organic nitrogen, 0.03±1.74 µg organic phosphorus and 0.01±1.91 µg inorganic phosphorus per cm^2 of tissue per day (Table 3). The obtained values for extracoelenteric feeding exceed calculations based on intracoelenteric feeding data for *G. fascicularis* by two orders of magnitude, and by one order of magnitude for *Stylophora pistillata* (Ferrier-Pagès et al. 2003), underscoring the vital importance of extracoelenteric zooplankton feeding. Interestingly, a recent study by Hii et al. (2009) revealed that *G. fascicularis* acquires 279±27.9 µg carbon per polyp per day under similar conditions as this study. This lies in the same order of magnitude as calculated for our study (166.3±0.5 µg C $polyp^{-1}$ day^{-1}), although they used a higher *Artemia* carbon content (0.93 µg C ind^{-1}) and did not correct for refractory organic material which is a significant factor to take into account. In addition, Purser et al. (2010) demonstrated that the azooxanthellate cold-water scleractinian coral *Lophelia pertusa* (Linnaeus 1758) is able to take up a high theoretical maximum of 350.9±46.1 µg carbon per polyp per day from zooplankton feeding, even though polyp number per cm^2 for *L. pertusa* is much lower than that of *G. fascicularis*. When taking a dark respiration rate of 19.2 µg carbon per cm^2 tissue per day and a daily net photosynthetic production of 68.4 µg carbon per cm^2 tissue per day (Schutter 2010) for *G. fascicularis* into account (based on a 12L:12D photoperiod), it becomes clear that when feeding high daily prey concentrations extracoelenteric zooplankton feeding is the major source of nutrient input and by itself easily meets the daily metabolic energy requirements (DME) for this species.

Table 3: Estimated nutrient inputs for *G. fascicularis* colonies from zooplankton feeding.

species	prey captured (ind cm^{-2} day^{-1})	nutrient input (µg cm^{-2} day^{-1})			
		TOC	TON	TOP	Pi
G. fascicularis, extracoelenteric	256±0	76.5±0.0	15.2±0.0	2.3±0.2	0.5±0.8
G. fascicularis, intracoelenteric	4±2	1.1±1.7	0.2±1.7	0.03±1.74	0.01±1.91
S. pistillata, intracoelenteric*	35	3.8	0.8		

Estimated nutrient inputs (total organic carbon, nitrogen, phosphorus and inorganic phosphorus) for *G. fascicularis* colonies from both intra- and extracoelenteric zooplankton feeding, compared to previous literature estimates and expressed in µg per cm^2 of coral tissue per day. We used a daily concentration of 10,000 nauplii L^{-1} and 5 cm s^{-1} as water flow rate. Corals were allowed to feed for 6 hours. We used an average of 6.2±0.9 polyps cm^{-2} and a conservative average aggregation density of 0.08±0.03 aggregates $polyp^{-1}$ (see Table 2) to estimate nutrient input for whole *G. fascicularis* colonies. Values are means ± s.d. (N=6). *Data based on Ferrier-Pagès et al. (2003) and recalculated for similar conditions.

Our results put an entirely new perspective on heterotrophic nutrient input from zooplankton, as extracoelenteric feeding may greatly exceed intracoelenteric feeding in terms of prey numbers digested and nutrients assimilated. Although external prey digestion may seem to have the disadvantage of nutrient leakage into the water column, it may be an energetically favorable process as coral polyps do not have to transport all prey items into the coelenteron by mucociliary feeding and muscle action. Even though we used high prey concentrations, which are four orders of magnitude higher than ambient *in situ* concentrations (Palardy et al. 2006), our results provide a well-founded estimation of maximum daily nutrient input from extracoelenteric zooplankton feeding for *G. fascicularis* under high prey concentrations. In the field, nutrient input from extracoelenteric feeding is likely to be much lower than found during this study, however this is equally true for internal feeding, as both processes depend on prey capture rates. Prey capture rates, in turn, are strongly influenced by zooplankton concentration,

and ingestion is indeed relatively low *in situ* (Johannes and Tepley 1974; Palardy et al. 2006). It is therefore possible that even in the field, extracoelenteric feeding contributes a relatively large part to the daily heterotrophic nutrient input for scleractinian corals, even though overall feeding rates are low. Furthermore, we have observed that when applying lower concentrations (1,000 *Artemia* nauplii L^{-1}), *Artemia* aggregates also form on *G. fascicularis* polyps. Future studies should focus on determining thresholds for extracoelenteric zooplankton feeding in terms of prey size and concentration, both in captivity and *in situ*. In addition, quantifying daily nutrient input from extracoelenteric feeding for coral species *in situ* would provide more realistic insights into benthic-pelagic coupling on coral reefs.

In conclusion, our results demonstrate that under high prey concentrations, extracoelenteric zooplankton feeding is a key mechanism of daily nutrient acquisition for a zooxanthellate scleractinian coral, which is of importance to aquaculture efforts. In addition, our findings provoke new thoughts about the nature and extent of benthic-pelagic coupling on coral reefs.

*Supplemental video available at
http://jeb.biologists.org/content/suppl/2011/09/24/214.20.3351.DC1/Movie1.mov

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Chapter 3

Oxygen and heterotrophy affect calcification of the scleractinian coral *Galaxea fascicularis*

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Abstract

Heterotrophy is known to stimulate calcification of scleractinian corals, possibly through enhanced organic matrix synthesis and photosynthesis, and increased supply of metabolic DIC. In contrast to the positive long-term effects of heterotrophy, inhibition of calcification has been observed during feeding, which may be explained by a temporal oxygen limitation in coral tissue. To test this hypothesis, we measured the short-term effects of zooplankton feeding on light and dark calcification rates of the scleractinian coral *Galaxea fascicularis* (*N*=4) at oxygen saturation levels ranging from 13 to 280%. Significant main and interactive effects of oxygen, heterotrophy and light on calcification rates were found. Light and dark calcification rates of unfed corals were severely affected by hypoxia and hyperoxia, with optimal rates at 110% saturation. Light calcification rates of fed corals exhibited a similar trend, with highest rates at 150% saturation. In contrast, dark calcification rates of fed corals were close to zero under all oxygen saturations. We conclude that oxygen exerts a strong control over light and dark calcification rates of corals, and propose that *in situ* calcification rates are highly dynamic. Nevertheless, the inhibitory effect of heterotrophy on dark calcification appears to be oxygen-independent. We hypothesise that dark calcification is impaired during zooplankton feeding by a temporal decrease of the pH and aragonite saturation state of the calcifying medium, caused by increased respiration rates. This may invoke a transient reallocation of metabolic energy to soft tissue growth and organic matrix synthesis. These insights enhance our understanding of how oxygen and heterotrophy affect coral calcification, both *in situ* as well as in aquaculture.

Introduction

It is well established that coral calcification, the precipitation of aragonite from calcium and carbonate ions by scleractinian corals, is stimulated by heterotrophy (Houlbrèque and Ferrier-Pagès 2009). The positive effect of heterotrophy on calcification is thought to be mediated through enhanced organic matrix synthesis (Allemand et al. 1998; Ferrier-Pagès et al. 2003; Houlbrèque et al. 2004), increased photosynthesis rates (Dubinsky et al. 1990; Titlyanov et al. 2000a,b, 2001; Houlbrèque et al. 2003, 2004) and increased supply of metabolic DIC (Furla et al. 2000; Ferrier-Pagès et al. 2003). Although the enhancement of coral calcification by heterotrophy has been demonstrated with long-term experiments (Houlbrèque and Ferrier-Pagès 2009), little is known about the short-term effects of feeding. In fact, heterotrophy has been shown to have a short-term inhibitory effect on dark calcification rates (Al-Horani et al. 2007; Colombo-Pallotta et al. 2010). This discrepancy is not well understood. Several authors have stated that in darkness, inhibition of calcification during zooplankton, glycerol or glucose supplementation may be caused by a temporal reallocation of energy, for example to prey capture and nutrient uptake (Al-Horani et al. 2007; Colombo-Pallotta et al. 2010). This reallocation of energy in darkness may involve a temporal decrease in tissue oxygen concentrations during prey capture and nutrient uptake, without photosynthetic oxygen production to compensate for this. As oxygen is a prerequisite for ATP-synthesis through oxidative phosphorylation in calicoblastic mitochondria (Babcock and Wikström 1992), oxygen limitation may result in impaired ATP production and, hence, impaired calcification rates, as Ca^{2+}/H^{+} ATP-ases require ATP or ADP for active transport of calcium ions and protons over the calicoblastic membrane (Ip et al. 1991). Indeed, Rinkevich and Loya (1984) and Colombo-Pallotta et al. (2010) found that external oxygen supplementation enhances dark calcification rates of *Stylophora pistillata* and *Montastraea faveolata*,

respectively, supporting the theory that oxygen limitation may indeed impair dark calcification of scleractinian corals during feeding.

In this study, we aimed to improve upon the current model of coral calcification by determining the combined effects of dissolved oxygen and heterotrophy on calcification. To this end, we measured light and dark calcification rates of the scleractinian coral *Galaxea fascicularis* with and without zooplankton supplementation under a range of ambient oxygen saturations. Profound interactive effects of oxygen, heterotrophy and light were found, demonstrating that these factors exert a strong control over coral calcification.

Materials and methods

Ethics statement

Captive bred corals (under CITES no. 52139) were provided by Burgers' Zoo BV (Arnhem, The Netherlands). All experiments were conducted at Wageningen University (Wageningen, The Netherlands). No approval from an ethics committee was required as scleractinian corals are exempted from legislation concerning the use of animals for scientific purposes in the European Union (Directive 2010/63/EU).

Selected species and husbandry

For this study, we used the Indo-Pacific scleractinian species *Galaxea fascicularis* (Linnaeus 1767). All colonies were genetically identical as they originated from the same parent colony. Corals were kept in a closed system of 400 L. Water flow was provided by three Turbelle nanostream 6045 circulation pumps (Tunze Aquarientechnik GmbH, Penzberg, Germany) providing a total flow rate of 13,500 L h^{-1}. Water parameters were maintained at the following levels: salinity 35.0±0.3 g L^{-1}, temperature 26±0.5°C, pH

8.2±0.3, a quantum irradiance (QI) of 170 µmol m^{-2} s^{-1} (12/12h light regime), ammonium 0.01±0.01 mg L^{-1}, nitrate 0.13±0.03 mg L^{-1}, phosphate 0.02±0.01 mg L^{-1}, calcium 400±25 mg L^{-1}, magnesium 1300±60 mg L^{-1}.

Analysis of colony surface area and polyp number

To determine projected surface area and polyp number, colonies (*N*=4) were removed from the aquarium and photographed directly from above, together with a ruler. A HDR-CX505VE digital camera (Sony Corporation, Tokyo, Japan) was used to record images. Projected surface area was determined by image analysis using ImageTool 3.0 every two weeks, during which the live circumference of the colonies was traced. Surface area was calculated by using the ruler as a reference and was expressed in cm^2. Polyp number was determined by marking individual polyps using the count function of the software. To prevent stress-induced artefacts, surface area and polyp number were never measured before treatments.

Analysis of colony volume

Water displacement was used to determine colony volume. Drip-dry corals were submerged in 500 mL seawater in 800 mL beakers after which the displaced water was transferred and measured in graduated cylinders and expressed in mL.

Calcification measurements

To measure calcification rates for *G. fascicularis*, we used the alkalinity anomaly technique (Chisholm and Gattuso 1991). Colonies with a starting size of 30.37±4.56 cm^2 and polyp count of 164±15 polyps (*N*=4) were incubated in cells with a gross volume of 1547±3 ml for 6 hours. Net water volumes were calculated by subtracting total volumes of all objects in the cells from the gross

cell volumes, including colony volumes. To determine the short-term effects of heterotrophy on light and dark calcification rates under a wide range of oxygen saturations, all 4 colonies were subjected to a total of 24 different treatments in a randomised factorial repeated measures design that were carried out over a four-month period. Treatments were light (QI of 250 µmol m^{-2} s^{-1}) or complete darkness (2 levels), with or without 150 *Artemia* nauplii per coral polyp (2 levels), at ambient oxygen saturations of 13, 50, 80, 110, 150 and 280% (or 0.87; 3.33; 5.33; 7.33; 10.00 and 18.67 mg L^{-1} O_2, respectively, 6 levels). The QI was chosen to saturate zooxanthellae photosynthesis, thereby preventing a possible light limitation which could obscure the (interactive) effect of light (Osinga et al. 2011a) The prey dosage was chosen in order to reflect aquaculture conditions, and to ensure that sufficient feeding events would occur during the short incubations. To maintain stable oxygen saturations during the entire incubations, five 5850S smart flow mass controllers (Brooks International, Hatfield, USA) were connected to two digital microprocessor units, models 0152/0154 (Brooks International, Hatfield, USA) which allowed for controlling volumetric flow rates of various gases in each cell. Nitrogen gas (N_2) was used for the 13, 50 and 80% oxygen saturation treatments. Compressed air was used for the 110% treatment. Pure oxygen (O_2) was used to for the 150 and 280% treatments. Oxygen concentrations were monitored throughout all experiments with IntelliCAL™ LDO101 luminescent dissolved oxygen probes (Hach-Lange GmbH, Düsseldorf, Germany). *Artemia* nauplii (average nauplii length was 440 µm) 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°C, and used immediately after hatching. The daily concentrations of *Artemia* cultures were determined by counting three seawater-diluted (1:99 mL) aliquots under an M8 stereomicroscope (Wild Heerbrugg, Heerbrugg, Switzerland), and used to calculate required volumes to obtain a dosage of 150 nauplii per polyp. Temperature was kept at 26±0.5°C by means of water jackets surrounding each incubation chamber, which were connected to a water bath equipped

with a TC20 water cooler (Teco SRL, Ravenna, Italy). Water flow was provided with magnetic stirring plates (IKA Werke GmbH & Co. KG, Staufen, Germany), and was estimated at approximately 5 cm s^{-1}. Water from the maintenance system was used to fill the incubation chambers, to minimise stress to the coral colonies. Calcium and alkalinity are known to influence calcification rates [35,36], and were always measured and adjusted when required to 400 mg L^{-1} and 2.50 mEq L^{-1}, respectively, before every experiment. Two water samples of 50 mL each were taken from every incubation chamber at t = 0 and t = 6 hours for determination of total alkalinity (A_T) and inorganic nutrients. This was taken into account during calculation of net cell volumes. During feeding treatments, water samples were filtered on a sterile filter mesh (150 µm pore size) to remove nauplii before measurement. To determine A_T, 50 ml samples were potentiometrically titrated on a Titralab 840 (Radiometer Analytical SAS, Lyon, France) with 0.02 M HCl to inflection point. Changes in A_T, expressed in mEq L^{-1}, were calculated for each cell. During each experiment, a control cell containing only the same seawater was used, except for feeding experiments where the average amount of *Artemia* nauplii dosed to the coral cells was included. Background alkalinity changes were used to correct all data.

Inorganic nutrients are known to influence alkalinity, and can therefore be a source of artifacts in the alkalinity anomaly technique (Brewer and Goldman 1976). To correct for changes in inorganic nutrient concentrations and therefore A_T, we measured ammonia (NH_3) and orthophosphate (PO_4^{3-}) concentrations during all experiments at t = 0 and t = 6 hours with a seawater calibrated DR 2800 spectrophotometer (Hach-Lange GmbH, Düsseldorf, Germany). Changes in NH_3 and PO_4^{3-} concentrations were converted to alkalinity changes in mEq L^{-1} and subsequently used to correct all data, including controls. We used mmol L^{-1} to mEq L^{-1} ratios of 1:1 and 1:3 for NH_3 and PO_4^{3-}, respectively. Total A_T depletions in mEq were calculated by taking net cell volumes into account. These were subsequently converted to mg calcium carbonate ($CaCO_3$) fixed, by using a mEq to mg $CaCO_3$ ratio of

1:50.04. Differences in coral biomass, and related to that the amount of *Artemia* nauplii fed, were taken into account by expressing all data as mg $CaCO_3$ per cm^2 coral tissue per hour. Between incubations, we incorporated resting periods lasting at least 48 hours to minimise artifacts due to stress caused by the experiments. All corals were acclimated to each experimental condition for 15 minutes before the start of every experiment, i.e. t = 0 was defined as the time point directly following the acclimation period. As calcification rates of *G. fascicularis* may vary during daytime (Al-Horani et al. 2007), experiments were conducted within the same time interval of 9:00 to 17:00 hrs.

Data analysis

Normality of data was evaluated by plotting residuals of each dataset versus predicted values, and by performing a Shapiro-Wilk test. All data were found to be normally distributed ($p > 0.050$). As sphericity for oxygen could not be calculated using Machly's test, we used a conservative Greenhouse-Geisser correction to adjust the degrees of freedom for oxygen and its interactions with light and feeding. We used a three-way factorial ANOVA for repeated measures, followed by Bonferroni's test for *post-hoc* analysis of oxygen treatments. Simple effects were used to elucidate interactive effects. Statistical analysis was performed with IBM SPSS Statistics 19 (IBM Corp., Armonk, USA). Graphs were plotted with SigmaPlot 11.0 (Systat Software, Inc., San Jose, USA). All data presented are means ± s.d.

Results

G. fascicularis colonies exhibited highly variable calcification rates between treatments, ranging from -0.006±0.006 to 0.113±0.012 mg $CaCO_3$ cm^{-2} h^{-1} (Figure 1). At the end of all feeding treatments, coral polyps exhibited a distinct feeding response, reflected by extrusion of mesenterial filaments which

enveloped *Artemia* aggregates (not shown). Light and dark calcification rates of unfed corals were clearly affected by oxygen. In light, calcification rates were negative at 13%, impaired at 50 and 80%, optimal at 110%, and inhibited at 150 to 280% saturation. Dark calcification rates exhibited a similar pattern, where calcification impairment was highly pronounced at 150 and 280% saturation.

Corals fed with zooplankton exhibited a different trend. Light calcification rates of fed corals were negative at 13% saturation, impaired at 50 and 80%, optimal at 150% oxygen saturation, and considerably inhibited at 280% saturation. In contrast, dark calcification rates of fed corals were close to zero under all oxygen saturations.

Statistical analysis revealed that oxygen, heterotrophy and light exerted main and/or interactive effects on calcification rates (Table 1). Oxygen had a significant effect on calcification ($F_{1.379,4.138}$=21.009, P=0.008, Table 1), where overall calcification rates were significantly higher at 80, 110 and 150% oxygen saturation compared to 13% (Bonferroni, P=0.039, P=0.020 and P=0.038, respectively), irrespective of light conditions and feeding. At 150% saturation, overall calcification was also significantly higher compared to 50% (Bonferroni, P=0.015).

A significant main effect of light on calcification rates was also found ($F_{1,3}$=38.597, P=0.008, Table 1), where overall light calcification rates were significantly higher compared to those in darkness, irrespective of oxygen saturation and feeding.

There was no significant main effect of heterotrophy on calcification rates ($F_{1,3}$=2.207, P=0.234, Table 1), hence in general, calcification rates of fed corals were not different from unfed corals, irrespective of oxygen saturation and light.

Light and heterotrophy exhibited a significant interactive effect on calcification rates ($F_{1,3}$=18.380, P=0.023, Table 1), irrespective of oxygen saturation. This was reflected by the fact that feeding inhibited calcification in

darkness but not in light (simple effect, $F_{1,3}$=26.510, P=0.014 and $F_{1,3}$=0.070, P=0.815, respectively), irrespective of oxygen saturation.

A significant interactive effect of oxygen and heterotrophy on calcification rates was also found $F_{2.014,6.043}$=10.386, P=0.011, Table 1). This was reflected by the fact that heterotrophy had no effect on calcification rates except at 150% oxygen saturation, at which calcification was enhanced (simple effect, $F_{1,3}$=12.800, P=0.037), irrespective of light conditions.

Oxygen and light exhibited a significant interactive effect on calcification rates ($F_{2.207,6.620}$=13.339, P=0.004, Table 1). This was reflected by the fact that light enhanced calcification at all oxygen saturations except at 13% (simple effect, $F_{1,3}$=0.020, P=0.887), irrespective of feeding.

Finally, there was a significant interactive effect of oxygen, light and heterotrophy on calcification rates ($F_{2.557,7.672}$=15.350, P=0.002, Table 1). This was reflected by a different interaction between oxygen and heterotrophy in light compared to darkness. More specifically, in light, feeding had no effect on calcification rates at 13% (simple effect, $F_{1,3}$=0.150, P=0.723), 80% ($F_{1,3}$=3.480, P=0.159) and 110% oxygen saturation ($F_{1,3}$=0.570, P=0.506), a positive effect at 50 and 150% ($F_{1,3}$=19.310, P=0.022 and $F_{1,3}$=55.290, P=0.005, respectively) and an inhibitory effect at 280% ($F_{1,3}$=34.940, P=0.010). In darkness, however, feeding had an inhibitory effect at oxygen saturations of 50% ($F_{1,3}$=30.940, P=0.011), 80% ($F_{1,3}$=104.207, P=0.002), 110% ($F_{1,3}$=27.080, P=0.014) and 150% ($F_{1,3}$= 103.83, P=0.002) and no effect at extreme saturations of 13 and 280% ($F_{1,3}$=0.780, P=0.441, $F_{1,3}$ = 2.650, P=0.202, respectively).

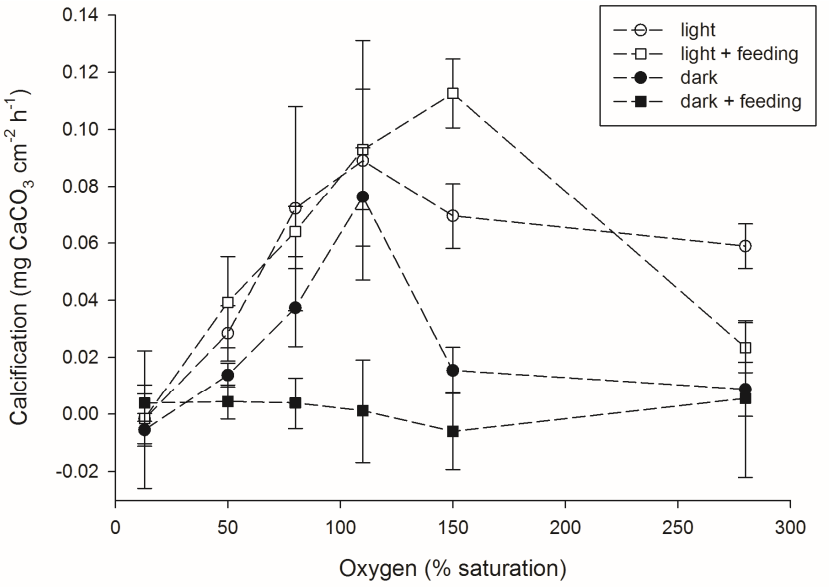

Figure 1: Effects of oxygen and heterotrophy on light and dark calcification of *Galaxea fascicularis*. Feeding quantity was 150 *Artemia* nauplii polyp^{-1}. QI in light was 250 µmol m^{-2} s^{-1}. Values are means ± s.d. (*N*=4).

Table 1: Three-way factorial ANOVA for repeated measures, demonstrating main and interactive effects of oxygen, heterotrophy and light on calcification rates of *G. fascicularis* colonies (*N*=4).

Factor	Variable	*F*	df	*P*
	calcification			
Oxygen		21.009	1.379	0.008*
Heterotrophy		2.207	1	0.234
Light		38.597	1	0.008*
Oxygen*Heterotrophy		10.386	2.014	0.011*
Oxygen*Light		13.339	2.207	0.004*
Light*Heterotrophy		18.380	1	0.023*
Oxygen*Heterotrophy*Light		15.350	2.557	0.002*

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

Discussion

G. fascicularis exhibited highly variable calcification rates between treatments, which lie in the same range as found for the scleractinian coral *Montastraea faveolata* (Colombo-Pallotta et al. 2010). This study revealed significant main and interactive effects of oxygen, heterotrophy and light on calcification rates of the scleractinian coral *Galaxea fascicularis*, demonstrating that these factors affect calcification in a complex manner.

First of all, significant main and interactive effects of oxygen were found. Overall calcification rates were highest at 80, 110 and 150% oxygen saturation, irrespective of light conditions and zooplankton feeding. At lower saturations of 13 and 50%, overall calcification rates were significantly impaired. This observation suggests a limiting role of oxygen in the calcification process. Not only did higher oxygen saturations initially promote overall calcification rates, at 110% saturation, dark calcification rates of unfed corals were not significantly different from those in light. This is in accordance with the findings of Rinkevich and Loya (1984) and Colombo-Pallotta et al. (2010), who found that oxygen enhances dark calcification rates of *Stylophora pistillata* and *Montastraea faveolata*, respectively. The causal mechanism behind the enhancement of light and dark calcification by oxygen may involve augmented ATP production through increased oxidative phosphorylation inside calicoblastic mitochondria, subsequently promoting Ca^{2+}/H^{+} ATP-ase activity (Chalker and Taylor 1975; Ip et al. 1991). Apparently, this oxygen effect is more important than other proposed mechanisms underlying light enhanced calcification, most notable regulation of tissue pH by photosynthesis (Furla et al. 2000; Al-Horani et al. 2003) Interestingly, light calcification rates of fed and unfed corals were also impaired by hypoxia, which may be explained by a significant efflux of oxygen to the surrounding water. At low ambient oxygen saturations, a high oxygen gradient between gastrodermal cells harbouring photoautotrophic zooxanthellae and the surrounding water (approx. 240 versus 13 to 80% saturation, Kühl et al. 1995) may have induced high oxygen efflux rates via the coelenteron, at the expense of the calicoblastic cells. As the incubation chambers were provided with ample water flow, this

phenomenon is likely to have been further enhanced as flow enhances oxygen efflux from coral tissue in light (Mass et al. 2010). This may have resulted in oxygen depletion of calicoblastic cells and a subsequent calcification impairment during the hypoxia treatments.

The inhibition of light and dark calcification rates at 150% saturation and beyond suggests oxygen intoxication. The toxic effect of hyperoxia on cells and organisms is well-known, and is caused by the formation of reactive superoxide radicals (O_2^-), i.e. oxygen molecules with one or more unpaired electrons (Fridovich 1977). In corals, such a hyperoxic environment is generated intracellularly by photosynthetic activity of zooxanthellae and xanthine oxidase (Shick and Dykens 1985; Kühl et al. 1995). Although the coral holobiont uses superoxide dismutases, catalase and ascorbate peroxidase to eliminate superoxide radicals (Shick and Dykens 1985; Lesser and Shick 1989; Matta and Trench 1991; Shashar and Stambler 1992), these antioxidants may become overwhelmed at high oxygen levels (Fridovich 1977). This may have occurred during light and dark incubations at 150 oxygen saturation and higher, resulting in (calicoblastic) cellular damage and a subsequent inhibition of calcification. In light, cellular damage may have impaired photosynthesis as well, and as photosynthesis is a major driver of calcification (Kawaguti and Sakumoto 1948; Chalker and Taylor 1975), this may have contributed to the observed reductions in light calcification.

The pronounced inhibition of dark calcification by hypoxia, and the impairment of light calcification by hyperoxia has implications for our understanding of *in situ* calcification rates. Corals inhabiting lagoons and reef flats regularly experience hypoxia and hyperoxia due to minimal water flow rate and exchange during low tide, resulting in oxygen saturations ranging from approximately 30 to 194% (Kinsey and Kinsey 1967; Kraines et al. 1996). In addition, the coral-water interface becomes anoxic (approximately 1% saturation) during night time (Shashar et al. 1993; Wangpraseurt et al. 2012) and hyperoxic (up to 373% saturation) during the day (Shashar et al. 1993). This suggests that corals may have highly variable calcification rates

throughout the day and night, especially on reefs that experience low tides accompanied by low water flow. These daily oxygen dynamics should be taken into consideration when measuring reef accretion.

A significant main effect of light was also found, in accordance with the hypothesis of light-enhanced calcification (Kawaguti and Sakumoto 1948; Chalker and Taylor 1975), as overall calcification rates were significantly higher in light compared to darkness, irrespective of oxygen saturation and zooplankton feeding. However, this main effect was in large part due to low dark calcification rates of fed corals. The enhancement of calcification by light was likely caused by intracellular oxygen production (Kühl et al. 1995) and elevated tissue pH (Furla et al. 2000; Al-Horani et al. 2003) resulting from photosynthesis. As oxygen supplementation significantly enhanced dark calcification rates of unfed corals, the former process may have been most relevant.

Next to oxygen and light, heterotrophy had a pronounced interactive effect on calcification. Zooplankton feeding inhibited calcification in darkness but not in light, irrespective of oxygen saturation. We initially hypothesised that under dark conditions, the causal inhibitory mechanism of heterotrophy involves temporal oxygen limitation of calcifying calicoblastic cells, which could result in depletion of the intracellular ATP pool and a subsequent reduction of Ca^{2+}/H^{+} ATP-ase activity (Ip et al. 1991; Babcock and Wikström 1992). The three-way interaction, however, reveals that oxygen only promoted calcification rates of fed corals under light conditions at 50 and 150% oxygen saturation, which may be explained by increased oxygen demand during feeding. In darkness, oxygen was unable to alleviate the inhibitory effect of feeding on calcification rates. This strongly suggests that oxygen limitation is not the causal mechanism underlying inhibition of dark calcification by heterotrophy, even though oxygen demand may be higher in darkness.

An alternative mechanism for the short-term inhibitory effect of heterotrophy on dark calcification may involve increased respiration rates, resulting in a temporary decrease of tissue pH levels through the conversion

of carbon dioxide and water to bicarbonate and protons by carbonic anhydrase. This would increase the proton gradient between the calicoblastic ectoderm and the calcifying medium (CM), the layer in which precipitation of new aragonite occurs (Furla et al. 2000; Al-Horani et al. 2003). If the Ca^{2+}/H^{+} ATP-ases on the calicoblastic membranes are not able to cope with this increased gradient in terms of proton removal from the CM, this would temporarily decrease its pH and aragonite saturation state, resulting in a reduction of calcification rates (Figure 2). To confirm this mechanism, pH micro sensor studies such as those described by Al-Horani et al. (2003) should be conducted during feeding experiments. This allows for measuring changes in the pH of the CM during feeding, which could be used to infer changes in its aragonite saturation state. The fact that Szmant-Froelich and Pilson (1984) found a pronounced increase (approximately 2.5-fold) in respiration rates of the coral *Astrangia danae* immediately after feeding on *Artemia* lends credence to this hypothesis. Tissue acidosis may induce a transient energy reallocation to processes other than calcification, including soft tissue growth and organic matrix synthesis, as this may be more energetically favourable.

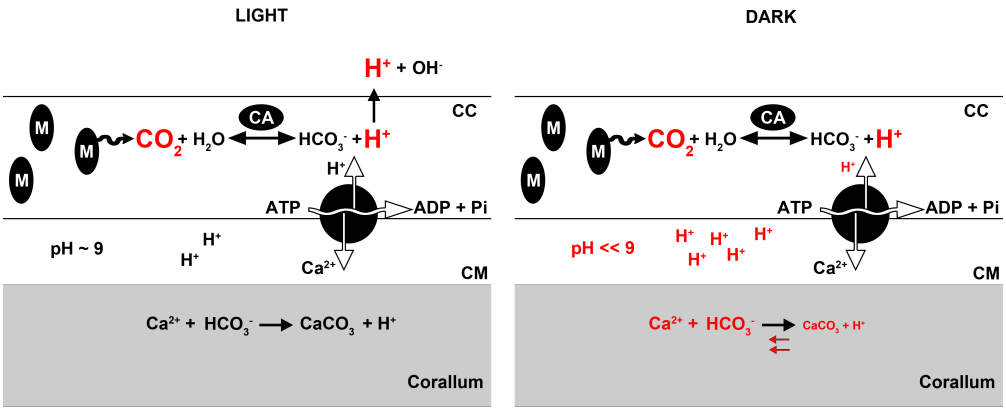

Figure 2: Conceptual model of dark calcification impairment by heterotrophy. Feeding increases metabolic rates, CO_2 production, and as a result proton production in calicoblastic cells. In light, these protons are titrated by photosynthetically generated hydroxide ions in the coelenteron. In darkness, protons accumulate in the calicoblastic ectoderm, increasing the proton gradient

between the calicoblastic ectoderm and the calcifying medium (CM). This causes a temporary decrease of the CM pH and aragonite saturation state, shifting the calcification reaction to the left. CC: calicoblastic cell. CM: calcifying medium. M: mitochondrion. CA: carbonic anhydrase. Model based on Furla et al. (2000) and Al-Horani et al. (2003).

Future studies may determine the threshold zooplankton concentration or zooplankton to coral biomass ratio below which no short-term reduction of dark calcification can be detected. Another issue which should be addressed is how long the inhibitory effect of heterotrophy lasts, which is likely to be only several hours when taking feeding and digestion rates of *G. fascicularis* into account (Hii et al. 2009; Wijgerde et al. 2011a). A temporal effect would explain the discrepancy between the inhibitory short-term (Al-Horani et al. 2007; Colombo-Pallotta et al. 2010; this paper) and enhancing long-term effects (Houlbrèque and Ferrier-Pagès 2009) of heterotrophy on coral calcification. Although dark calcification is temporarily inhibited during zooplankton feeding, in between feeding events, corals can benefit from enhanced organic matrix synthesis (Allemand et al. 1998; Ferrier-Pagès et al. 2003; Houlbrèque et al. 2004), photosynthesis rates Dubinsky et al. 1990; Titlyanov et al. 2000a,b, 2001; Houlbrèque et al. 2003, 2004) and metabolic DIC supply (Furla et al. 2000; Ferrier-Pagès et al. 2003) which promote calcification. In this perspective, the nocturnal feeding behaviour of corals, possibly an adaptation strategy to higher zooplankton availability (Houlbrèque and Ferrier-Pagès 2009 and references therein), may impose a significant physiological cost to corals in terms of impaired dark calcification rates. In addition, our results suggest that feeding scleractinian corals in aquaculture during daytime (i.e. in light) may be more optimal to growth.

In conclusion, this study demonstrates that oxygen is a key factor controlling calcification of scleractinian corals. However, oxygen limitation is most likely not the causal factor underlying the inhibitory short-term effect of heterotrophy on dark calcification. Temporal energy reallocation induced by tissue acidosis may explain this phenomenon. These insights enhance our

understanding of how oxygen and heterotrophy affect coral calcification, both *in situ* as well as in aquaculture.

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Chapter 4

Water flow affects zooplankton feeding by the scleractinian coral *Galaxea fascicularis* on a polyp and colony level

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Abstract

Several factors may affect heterotrophic feeding of benthic marine invertebrates, including water flow rate and polyp context (i.e. the presence of neighbouring polyps). We tested the interactive effects of water flow rate and polyp context on zooplankton feeding by the scleractinian coral *Galaxea fascicularis*. Single polyps and colonies were incubated in a flow cell for 30 minutes with an ambient *Artemia* nauplii concentration of 10,000 L^{-1} and water flow rates ranging from 1.25 to 40 cm s^{-1}. Water flow rate and polyp context showed significant main and interactive effects on feeding rates of *G. fascicularis* polyps. More specifically, feeding rates were optimal at flow rates of 1.25 cm s^{-1} for single polyps and 5 to 10 cm s^{-1} for polyps inhabiting colonies. The presence of epizoic acoelomorph flatworms may have negatively affected the observed feeding rates, especially at high flow. Our results demonstrate that flow affects coral feeding and thus heterotrophic nutrient input at both a polyp and colony level. These findings are of relevance to our understanding of how biotic and abiotic factors interact on coral heterotrophy, and may serve to optimise coral aquaculture.

Introduction

Heterotrophy is vital to coral health, as it supplies the holobiont with essential nutrients including amino acids and fatty acids (reviewed by Houlbrèque and Ferrier-Pagès 2009). For scleractinian corals, profound effects of heterotrophy on the physiology of the coral host and its symbiotic dinoflagellates have been documented. Zooplankton feeding has been found to enhance coral calcification, organic matrix synthesis and photosynthetic rates (Ferrier-Pagès et al. 2003; Houlbrèque et al. 2004). Up to 100% of the daily metabolic carbon requirements can be supplied by zooplankton, both during bleaching episodes (Grottoli et al. 2006) or when high prey concentrations are used in aquaculture (Wijgerde et al. 2011a). These findings fit well with the long-term effects of zooplankton feeding on corals, which show that heterotrophy can be a limiting factor to growth (Houlbrèque and Ferrier-Pagès 2009; Osinga et al. 2011a).

Several factors may affect coral feeding rates, including bleaching status (Grottoli et al. 2006), prey density (Osinga et al. 2011b), symbiotic organisms such as epizoic flatworms (Wijgerde et al. 2011b), water flow rate (Hunter 1989; Dai and Lin 1993; Helmuth and Sebens 1993; Fabricius et al. 1995; Heidelberg et al. 1997; Sebens et al. 1997, 1998; Lin et al. 2002) and colony size (Hunter 1989; Helmuth and Sebens 1993; Sebens et al. 1997). Water flow is a key parameter in this respect, as sessile organisms including corals depend on water movement to provide them with prey items (Brusca and Brusca 2003). Increased flow rates will increase the encounter rate or flux of food particles (Best 1988; Hunter 1989; Fabricius et al. 1995; Sebens et al. 1998), but will also increase the kinetic energy of particles approaching coral polyps. A higher kinetic energy of food particles may constrain the capture abilities of coral polyps, as has been documented for octocorals (Wainwright and Koehl 1976; Patterson 1984; McFadden 1986). Moreover, drag forces caused by water flow can result in deformed feeding structures, decreasing capture efficiency (Wainwright and Koehl 1976; Leonard et al. 1988; Sebens and Johnson 1991; Dai and Lin 1993; Fabricius et al. 1995; Anthony 1997;

Sebens et al. 1997). Furthermore, corals may contract their tentacles if extension is no longer cost efficient (Dai and Lin 1993). These mechanisms explain why bell-shaped relationships between water flow rate and prey capture have been found for several coral species (Dai and Lin 1993; Helmuth and Sebens 1993; Sebens et al. 1997; Lin et al. 2002).

Colony size may also affect individual polyp feeding rates, both in negative and positive ways, due to polyp interactions within colonies. Negative effects may include polyp shading (i.e. polyps covering and obstructing one another) and local particle depletion, resulting in decreased prey capture by downstream polyps (Hunter 1989). Positive effects may include the generation of intracolonial turbulence and mucus secretion by upstream polyps, enhancing prey capture by downstream polyps (Helmuth and Sebens 1993; Sebens et al. 1997; Hii et al. 2009; Wijgerde et al. 2011a).

More insight into how different factors interact on zooplankton feeding by corals will contribute to our understanding of benthic-pelagic coupling on coral reefs. Furthermore, as heterotrophy is a limiting factor to growth [1,6], coral aquaculture may be optimised by taking factors that enhance coral feeding into consideration. Therefore, we determined how water flow rate affects zooplankton feeding by a scleractinian coral on both a polyp and colony level. To this end, we performed video analyses of the scleractinian coral *Galaxea fascicularis* (Linnaeus 1767) feeding on *Artemia* nauplii under different flow regimes. As this species experiences highly variable water flow in the field, ranging from approximately 5 to 50 cm s^{-1} at the depths at which this species is commonly found (9-12 m; Genin et al. 1994), we used a similar range of flow rates.

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 of 400 L,

with the following parameters: salinity 35±0.5 g L^{-1}, temperature 26±0.5 °C, pH 8.2±0.3, photon flux density 322 µmol m^{-2} s^{-1} (12h/12h light/dark regime), nitrate 0.25±0.08 mg L^{-1}, phosphate 0.02±0.01 mg L^{-1}, calcium 400±23 mg L^{-1}, magnesium 1300±40 mg L^{-1}. Water flow was provided by four Turbelle nanostream 6045 circulation pumps (Tunze Aquarientechnik GmbH, Penzberg, Germany) and an Eheim 1260 return pump (Eheim GmbH Co. KG, Deizisau, Germany), providing a total flow rate of 20,000 L h^{-1} or 5 to 10 cm s^{-1}. Both single polyps and colonies were used for video analysis.

Preparation of colonies and single polyps

Single polyps (approximate corallite length of 10 mm and diameter of 5 mm, respectively) were individually and randomly removed from a parent colony by using pincers, and subsequently glued onto 7x7 cm PVC plates with two-component epoxy resin (GroTech Aquarientechnik GmbH, Affalterbach, Germany). Small colonies of approximately 100 polyps (approximately 4 x 4 cm) were cut from a parent colony with an electrical hand saw (Dremel, Breda, The Netherlands). This size was chosen to ensure some distance (2.5-3 cm) between the corals and the walls of the flow cell, thereby reducing potential boundary layer effects. All single polyps and colonies were of the same genotype, since they all originated from a single parent colony.

Video analysis

For video analysis, *G. fascicularis* single polyps (*N*=4) and colonies (*N*=4) were incubated in a respirometric flow cell (Wageningen UR, Wageningen, The Netherlands) for 30 minutes (Figure 1). The outer dimensions of the flow cell were 51.8 x 29.1 x 14.3 cm (length x width x height), and its internal volume was 3.5 L. Water flow was created using a modified paddle wheel that was powered by a DC motor (Maxon motor Benelux B.V., Enschede, The Netherlands) with a three-channel incremental encoder and line driver that allows precise control of rotational speed. EPOS user interface software (version 2.3.1, Maxon motor Benelux B.V., Enschede, The Netherlands) was

used to create flow rates of 1.25, 5, 10, 20, 30 and 40 cm s^{-1}. Water flow rates were calibrated using particle tracking, according to Schutter et al. (2010). Water from the holding tank was used for the experiments to rule out artefacts resulting from changes in water chemistry. Temperature was kept at 26±0.5°C by means of a water jacket connected to a TC20 water cooler (Teco SRL, Ravenna, Italy). Photon flux density was set to holding tank intensity (322 µmol m^{-2} s^{-1}) with a T5 fluorescent lighting fixture containing four 24W fluorescent tubes with a colour temperature of 14,000 Kelvin (Elke Müller Aquarientechnik, Hamm, Germany). An HDR-CX505VE handy cam (Sony Corporation, Tokyo, Japan) was used for recording still and moving images in high resolution format (1440 x 1080 pixels, 25 fps). *Artemia* 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°C, and used immediately after hatching. Average nauplii size was 440 µm according to the manufacturer. A concentration of 10,000 *Artemia* nauplii L^{-1} was used for all experiments. This prey concentration was chosen as it reflects aquaculture conditions, and to ensure sufficient feeding events would occur during the short incubations. Polyps and colonies were acclimated in the flow cell for 15 minutes before the start of every incubation. Each polyp and colony was analysed individually, and once at each flow treatment. All treatments were randomised for each individual. Corals were allowed to rest in the holding aquarium for at least 48 hours between treatments, and they were never fed before any treatment. All experiments were carried out over a period of approximately four weeks. Capture, release and retention of *Artemia* nauplii by coral polyps were scored by analysing videos after experiments. For polyps within colonies, the most central polyp was consistently selected for all analyses. Nauplii capture by polyps was defined as prey that attached to the polyp surface for at least 10 seconds. Nauplii release was defined as prey that detached from the polyp surface and remained in suspension for at least 10 seconds. Nauplii retention 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 an aggregate. Retention of nauplii in aggregates was quantified as *G. fascicularis* has been found to mainly digest prey externally using mesenterial filaments (Wijgerde et al. 2011a).

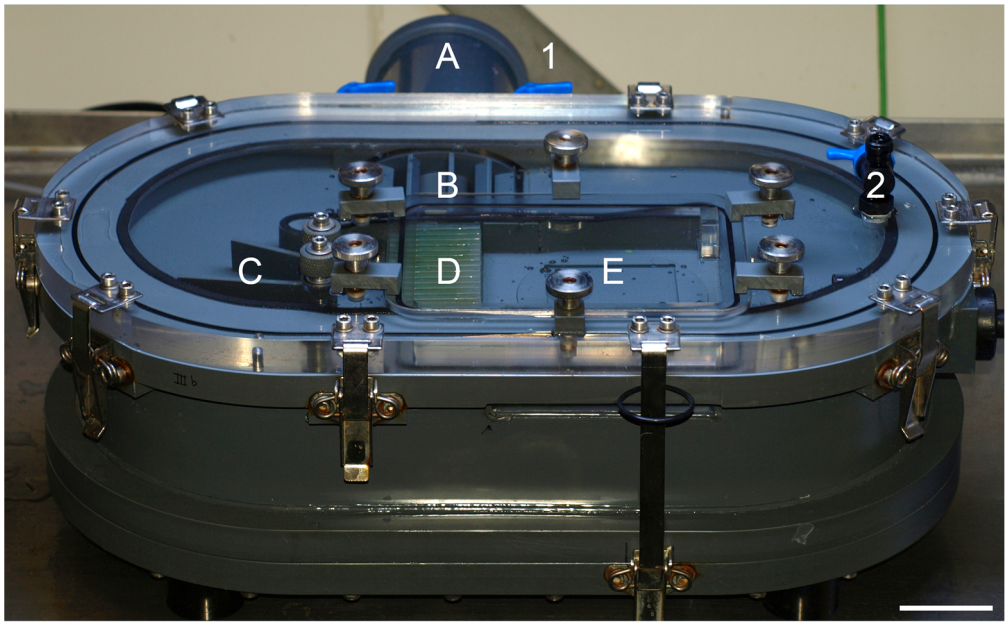

Figure 1: Overview of the respirometric flow cell used in this study. A: motor. B: paddle wheel. C: flow adjusters. D: flow laminator. E: coral plate holder. 1: water inlet. 2: water outlet. Scale bar: 5 cm.

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 was determined using Levene's test. Sphericity was determined with Mauchly's test. As capture and release data were not found to be normally distributed ($P<0.050$), a log10 transformation was used. After transformation, all feeding data were found to be normally distributed ($P>0.050$). Transformation also resulted in homogeneity of variance ($P>0.050$) and sphericity ($P>0.050$) of the data. We used a two-way mixed factorial ANOVA to test the (interactive) effects of water flow rate and polyp context on prey capture, release and retention by *G. fascicularis* polyps, where water flow was

considered a repeated measures factor (within-subjects factor). Bonferroni *post-hoc* tests were used to determine capture, release and retention differences between the various water flow rates, for both single polyps and polyps in colonies. Simple effects analysis was employed to infer capture, release and retention differences between single polyps and polyps in colonies at each water flow rate. A *P*-value <0.050 was considered statistically significant. Statistical analyses were performed with IBM SPSS Statistics 19.0 (IBM Corp., Armonk, USA). Graphs were plotted with SigmaPlot 11.0 (Systat Software, Inc., San Jose, USA). Data presented are expressed as means ± s.d unless stated otherwise.

Results

Video observations

During all treatments, *G. fascicularis* polyps were active and well expanded. All single polyps and polyps within colonies captured prey (Figure 2). Mucus excretion was apparent and resulted in clustering of captured nauplii in mucus aggregates (not shown). No ingestion of nauplii was observed during any of the treatments. Instead, mesenterial filaments were expelled through the actinopharynx and temporary openings in the ectoderm of the oral disc, which enveloped single nauplii and nauplii aggregates. Filament expulsion seemed to be random, however during several incubations this occurred in the vicinity of captured nauplii. On a few occasions, polyps that were part of colonies lost prey to neighbouring individuals, either passively by water current or actively by tentacle movement.

Deformation of polyps was observed at flow rates of 20 cm s^{-1} and higher, for both single polyps and those within colonies. No significant polyp contraction was observed for any of the flow rates.

The presence of epizoic acoelomorph flatworms (tentatively identified as *Waminoa* sp.) was also observed for all polyps. These epizoic worms,

approximately 1-2 mm in length, moved across coral polyps and actively preyed on *Artemia* nauplii.

Feeding rates

Prey capture, release and retention rates of *G. fascicularis* polyps were highly variable among the different flow treatments (Figure 2). Significant main effects of water flow rate and polyp context on prey capture rate were found (Table 1). A significant interactive effect was also found (Table 1), reflected by the fact that polyps in colonies captured significantly more prey compared to single polyps at water flow rates of 5, 10 and 30 cm s^{-1} (simple effects, P=0.001, P=0.007 and P=0.049, respectively, Figure 2).

Significant main effects of water flow rate and polyp context on prey release rate were found (Table 1). A significant interactive effect was also found (Table 1), reflected by the fact that polyps in colonies released significantly more prey compared to single polyps at water flow rates of 5, 10 and 30 cm s^{-1} (simple effects, P=0.011, P=0.008 and P=0.046, respectively, Figure 2).

Significant main effects of water flow rate and polyp context on prey retention rate were found (Table 1). A significant interactive effect was also found (Table 1), reflected by the fact that polyps in colonies retained significantly more prey compared to single polyps at water flow rates of 5, 10 and 20 cm s^{-1} (simple effects, P=0.000, P=0.016 and P=0.050, respectively, Figure 2).

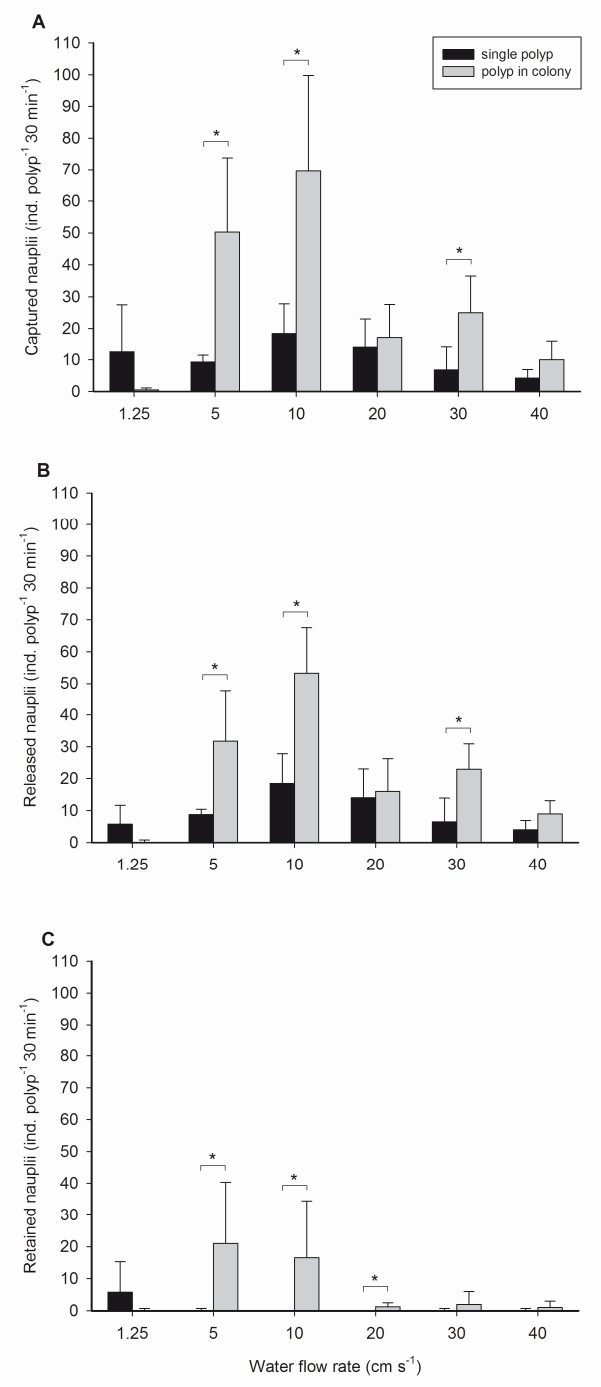

Figure 2: Prey capture (A), release (B) and retention (capture minus release, C) rates of *Galaxea fascicularis* single polyps (black bars) and polyps in colonies (grey bars) at water flow rates of 1.25 to 40 cm s^{-1}. Values are means + s.d. (*N*=4). *Indicates significant difference (*P*<0.050, simple effects analysis).

Table 1: Two-way mixed factorial ANOVA, showing main and interactive effects of water flow rate and polyp context on prey capture, release and retention by *G. fascicularis* polyps (*N*=4).

Factor	Variable	*F*	df	error	*P*
	prey capture				
Water flow rate		9.67	5	30	<0.001*
Polyp context		39.24	1	6	0.001*
Water flow rate * Polyp context		5.08	5	30	0.002*
	prey release				
Water flow rate		12.92	5	30	<0.001*
Polyp context		17.73	1	6	0.006*
Water flow rate * Polyp context		4.65	5	30	0.003*
	prey retention				
Water flow rate		3.21	5	30	0.019*
Polyp context		45.14	1	6	0.001*
Water flow rate * Polyp context		6.08	5	30	0.001*

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

Discussion

Effects of water flow and polyp context on coral feeding

This study revealed a significant main effect of water flow rate on capture rates of *G. fascicularis* in a relationship that approximated a bell curve, although the interaction with polyp context demonstrated that this curve was affected by the presence of neighbouring polyps. This finding is in accordance with previous studies on corals (Dai and Lin 1993; Helmuth and Sebens 1993; Sebens et al. 1997; Lin et al. 2002). More generally, a significant effect of flow rate on particle capture has been found for various benthic marine invertebrates, including alcyonaceans (Dai and Lin 1993; Fabricius et al. 1995; Lin et al. 2002), pennatulaceans (Best 1988), scleractinians (Helmuth and Sebens 1993; Heidelberg et al. 1997; Sebens et al. 1997, 1998; Purser et al. 2010), actiniarians (Anthony 1997), hydrozoans (Hunter 1989), bryozoans

(Pratt 2008), crinoids (Leonard et al. 1988) and barnacles (Larsson and Johnsson 2006). The ability of *G. fascicularis* to feed on zooplankton under a wide range of flow rates also correlates well with the different reef habitats in which this species is found, which are exposed to flow rates of 5 to 50 cm s^{-1} (Genin et al. 1994). Several authors have stated that the feeding capacity of suspension and filter feeding invertebrates can be affected by food particle encounter rate and deformation of feeding structures (Best 1988; Leonard et al. 1988; Hunter 1989; Sebens and Johnson 1991; Dai and Lin 1993; Fabricius et al. 1995; Anthony 1997; Sebens et al. 1997; Lin et al. 2002). In accordance with their claims, the initial positive effect of flow rate on prey capture rates we found for *G. fascicularis* is likely to have been caused by an increased encounter rate or particle flux of *Artemia* nauplii. At the same time, polyp deformation was absent under flow rates of 1.25 to 10 cm s^{-1}, favouring high capture rates. At flow rates of 20 cm s^{-1} and higher, polyp tentacles deformed significantly due to drag forces, resulting in reduced filter area facing the flow. This may have negatively affected prey encounter rate and capture at flow rates of 20 cm s^{-1} and above. Another limiting factor may have been the increased kinetic energy of nauplii at higher flow rates, which requires stronger adhesive and retention abilities of coral tentacles (Wainwright and Koehl 1976; Patterson 1984; McFadden 1986). This was illustrated by our observation that at higher flow rates, nauplii seemed effectively paralysed by cnidocytes, but were not as well retained by polyps. Hunter (1989) suggested that both the flux and kinetic energy of particles increase when flow rates increase, with positive and negative effects on feeding, respectively. As capture rates decreased with higher flow rates, the positive effect of higher prey flux did not compensate for the negative effects of increased kinetic energy of food particles and polyp deformation.

A significant main effect of water flow rate on overall release rates of *G. fascicularis* polyps was also found, in a pattern that matched capture rates. In other words, increased prey capture was followed by increased prey

release, which may not have been deliberate but a result of insufficient adhesive abilities of polyps (see below).

Water flow rate significantly affected overall prey retention rates, with much lower retention rates than previously found by Wijgerde et al. (2011a) (6±10 *versus* 32±33 *Artemia* nauplii polyp^{-1} 30 min^{-1}). This may have been due to the presence of epizoic acoelomorph flatworms, which were observed in high numbers during the incubations. Hii et al. (2009) and Wijgerde et al. (2011a) showed that *Galaxea fascicularis* secretes copious amounts of mucus for zooplankton entrapment, whereas Naumann et al. (2010) demonstrated that epizoic flatworms actively feed on this mucus. Therefore, mucus removal from the oral disc by epizoic flatworms could potentially affect the ability of the corals to capture and retain prey, especially at high flow rates. Indeed, Wijgerde et al. (2012c) recently demonstrated that epizoic flatworms reduce the capacity of *Galaxea* polyps to feed on zooplankton. Although Wijgerde et al. (2011a) also reported the presence of flatworms on polyps with high retention abilities, differences in flatworm hosting densities may explain the discrepancy. A reduced adhesive ability will especially affect single polyps, as no current shading effects of upstream polyps occur. Indeed, video analysis demonstrated that at flow speeds of 5 cm s^{-1} and higher, single polyps were unable to successfully retain prey. Moreover, Wijgerde et al. (2011b) demonstrated that epizoic flatworms actively compete with their coral host for zooplankton, which could further reduce prey capture by *G. fascicularis*. Future studies may reveal a negative impact of epizoic acoelomorph flatworms on other coral species, in terms of feeding impairment, as flatworms are common symbionts of many coral taxa, both *in situ* and in captivity (Barneah et al. 2007; Haapkylä 2009; Naumann et al. 2010).

Next to flow rate, turbulence, and thus flow direction, played a role in zooplankton capture by the corals. On the leeward side of both single polyps and colonies nauplii concentrated, which was clearly the result of eddy formation. From these eddies, zooplankton was regularly propelled in the direction of the coral after which capture sometimes followed. During several

measurements at 5 and 10 cm s^{-1}, the amount of nauplii captured directly from the water current was lower than the number captured from the eddy. Helmuth and Sebens (1993) and Sebens et al. (1997) described similar observations for the scleractinian corals *Agaricia agaricites* and *Madracis mirabilis*, respectively. They found that capture shifted from upstream to downstream regions with increasing flow rates. Based on their observations, they suggested that turbulent currents formed by polyps or branches aid in prey capture. This phenomenon contributed to the capture rates we observed (also see below on interactions).

Polyp context also had a significant main effect on prey capture, release and retention rates, as polyps inhabiting colonies generally captured, released and retained significantly more prey than single polyps. The apparent advantage of the presence of neighbouring polyps could be due to mucus secretion and paralysis of zooplankton prey by upstream polyps, allowing for more effective capture by downstream central polyps. This is in accordance with earlier findings by McFadden (1986) on octocorals, who found that colony aggregations displayed enhanced prey capture, and Wijgerde et al. (2011a), who showed that *G. fascicularis* polyps within a single colony can develop significant *Artemia* nauplii aggregates. However, the latter authors also found that polyp capture rates within a colony are patchy, as only 7.7% of polyps accumulate aggregates. This finding demonstrates that although certain individual polyps in a colony may capture prey more efficiently compared to solitary polyps, the colony as a whole may become less efficient in terms of average prey capture per polyp. Thus, if we had preselected different polyps inhabiting colonies for our observations, the results could have revealed less efficient feeding compared to solitary individuals. Polyps inhabiting colonies which do not capture prey may still benefit from the shared internal anatomy of scleractinians which enables nutrient redistribution (Gladfelter 1983; Domart-Coulon et al. 2006). The fact that polyps in the context of a colony capture less prey on average is in agreement with decreasing growth rates

with size observed for *G. fascicularis* (Schutter et al. 2010), possibly caused (in part) by decreased nutrient procurement per unit of biomass.

In this study, water flow rate and polyp context were found to have a significant interactive effect on prey capture, release and retention rates, demonstrating that the effect of water flow on feeding rates was modified by polyp context and vice versa. The interaction resulted from the different ways in which single polyps and central polyps in colonies responded to flow in terms of prey capture, release and retention. Polyps within colonies exhibited a distinct response to water flow, with virtually no prey capture and release at 1.25 cm s^{-1}, highest capture/release rates at 5 to 10 cm s^{-1}, and intermediate capture/release at even higher flow rates. Single polyps displayed a different response to flow, especially when regarding prey retention. This occurred only at the lowest flow rate, whereas polyps within colonies retained significantly more prey at intermediate flow rates. The interactive effect can also be illustrated with the fact that prey capture, release and retention rates were higher for polyps within colonies only at specific water flow rates. This interactive effect may be explained by intracolonial polyp interactions, including negative effects such as polyp shading and local particle depletion as described by Hunter (1989), and positive effects such as intracolonial turbulence and additional mucus production (Helmuth and Sebens 1993; Sebens et al. 1997; Wijgerde et al. 2011a). These negative and positive interactions are, turbulence excluded, absent for single polyps. At low flow and thus low particle flux, upstream polyps may reduce particle availability for their downstream clonemates, which as a result capture less prey. This could explain the low capture and release rates we found for central polyps at 1.25 cm s^{-1}. This, however, seems unlikely at the high prey concentrations that were used. Another explanation may be that at low flow, a thicker boundary layer results in advection of prey around the massive, hemispherical colonies, resulting in less prey availability for the densely packed central polyps. At high flow rates, on the other hand, upstream polyps may cover downstream polyps due to deformation caused by drag forces, thereby shading the feeding

structures of the latter. This could explain the distinctly lower capture and release rates we found for central polyps at high flow rates of 20 cm s^{-1} and beyond. Indeed, video analysis showed that at flow rates of 20 cm s^{-1} and higher, polyp deformation and thus shading, was significant. The reason why polyp interactions resulted in highest prey capture, release and retention at an intermediate flow of 5 to 10 cm s^{-1} may be that at these flow rates, an optimal trade-off exists between prey encounter rate on the one hand, and polyp shading effects and increased kinetic energy of prey on the other. As stated above, turbulence may further aid in prey capture, increasing contact time between prey and polyps. In a similar way, a favourable trade-off between prey encounter rate, drag force and kinetic energy may explain higher feeding rates by single polyps at intermediate and low flow rates.

Finally, as we used only one genotype, the results obtained here may not reflect the behaviour of this species in general. Future studies may reveal genotypic variability in terms of feeding ability under different flow regimes.

In conclusion, this study demonstrates that water flow and polyp context exert an interactive effect on zooplankton feeding by *G. fascicularis*, with optimal feeding rates at 1.25 and 5 to 10 cm s^{-1} for solitary and colonial polyps, respectively. These findings have implications for aquaculture of this species, as heterotrophic feeding can significantly enhance coral growth (Houlbrèque and Ferrier-Pagès 2009; Osinga et al. 2011a). Although the prey concentrations we used only exist in aquaculture, the relative differences reflect the important effects of water flow and polyp context on coral heterotrophy, which is relevant to the ecology of *G. fascicularis*. Exposure to high flow rates may significantly limit prey and nutrient acquisition by this species, and thus growth and survival, whereas low flow rates may enhance feeding rates of primary polyps. Future studies should address the potential interaction between water flow rate and prey concentration on the feeding rates of this species, similar to the study of Purser et al. (2010) for *Lophelia pertusa*. In addition, determining the effect of flow pattern, i.e. oscillating

versus unidirectional flow, would be relevant as Hunter (1989) demonstrated that this factor can affect feeding rates of benthic colonial invertebrates.

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Chapter 5

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

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Abstract

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* 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.

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 (Brown and Bythell 2005; Barneah et al. 2007b; 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 (Brown and Bythell 2005; Barneah et al. 2007b; 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.

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 (Aqua Holland 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°C, 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 7x7 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 fascicularis*, 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°C 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°C⁄5' – 2x (94°C⁄30" – 58°C⁄30" – 72°C⁄30") – 2x (94°C⁄30" – 56°C⁄30" – 72°C⁄30") – 34x (94°C⁄30" – 52°C⁄30" – 72°C⁄30") – 72°C⁄10'. 4FB⁄1806R: 95°C⁄5' – 2x (94°C⁄30" – 60°C⁄30" – 72°C⁄30") – 2x (94°C⁄30" – 58°C⁄30" – 72°C⁄30") ") – 2x (94°C⁄30" – 56°C⁄30" – 72°C⁄30") – 2x (94°C⁄30" – 54°C⁄30" – 72°C⁄30") – 2x (94°C⁄30" – 52°C⁄30" – 72°C⁄30") – 32x (94°C⁄30" – 50°C⁄30" – 72°C⁄30") – 72°C⁄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 analyze 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. (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°C 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 24W 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°C, 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 log10 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.

Results

Acoelomorph flatworms hosted by G. fascicularis

Galaxea fascicularis polyps hosted epizoic acoelomorph flatworms (Figure 1) at a density of 3.6±0.4 flatworms polyp^{-1}. The size of the flatworms varied, with the anterior-posterior axes 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 (Graff, 1905), phylum Acoelomorpha (Ehlers, 1985). The parenchyma of the flatworms contained high densities of symbiotic algae, possibly *Symbiodinium* or *Amphidinium* sp.

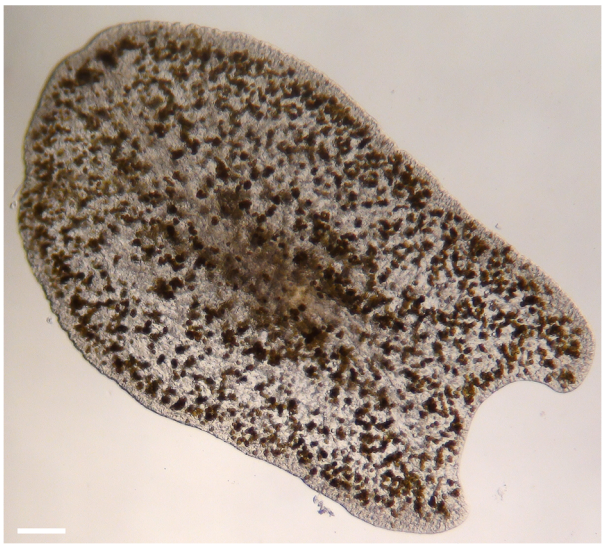

Figure 1: Photomicrograph of an epizoic acoelomorph flatworm (*Waminoa* sp.) isolated from *Galaxea fascicularis*. Note the abundant symbiotic dinoflagellates in the worm's parenchyma. Scale bar: 100 µm.

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 (Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 2C). Polyps 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 (Figure 2C). These retention rates were 57.1±1.2, 57.6±1.8 and 19.8±1.9% relative to dewormed polyps, respectively.

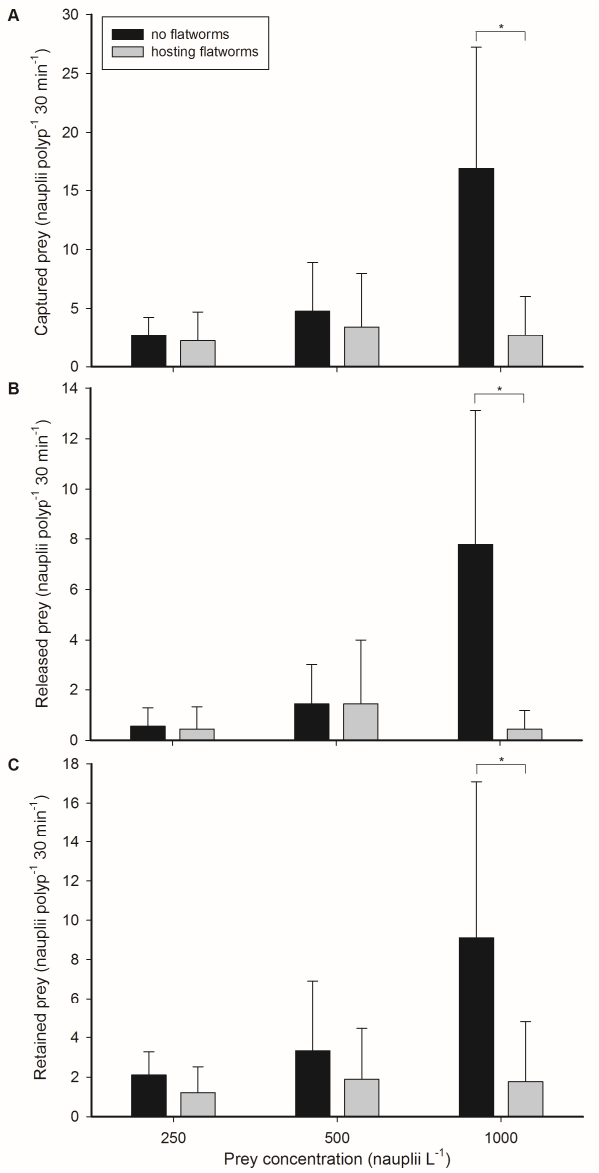

Figure 2: *Galaxea fascicularis* feeding rates with and without flatworms at different prey concentrations. (A) Captured, (B) released and (C) retained prey by *G. fascicularis* single polyps, expressed as nauplii polyp^{-1} 30 min^{-1}, at three different prey concentrations; 250, 500 and 1,000 nauplii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

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).

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 ($P<0.050$).

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 (Figure 3). Subsequent paralysis of prey was observed, which was possibly followed by ingestion and digestion in the worm's syncytial digestive tract. Some flatworms captured additional prey whilst digesting previously captured prey, with a maximum of two prey items per worm (Figure 3), although this behaviour was rare.

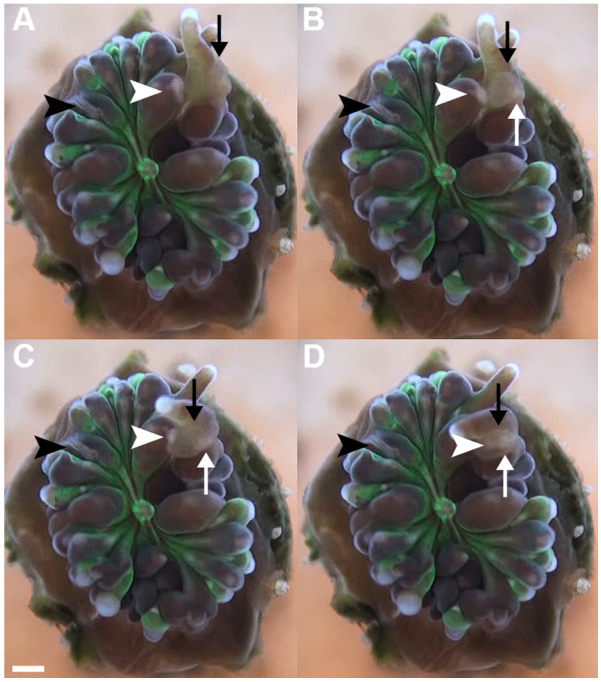

Figure 3: Overview of an epizoic flatworm capturing a single *Artemia* nauplius. (A) Flatworm (*Waminoa* sp.) on the oral disc of its coral host (*G. fascicularis*), (B) raising its anterior edge from the polyp surface, (C) curling down over its prey (*Artemia* sp.) and (D) pressing its prey onto the 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.

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 (Figure 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 (Figure 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).

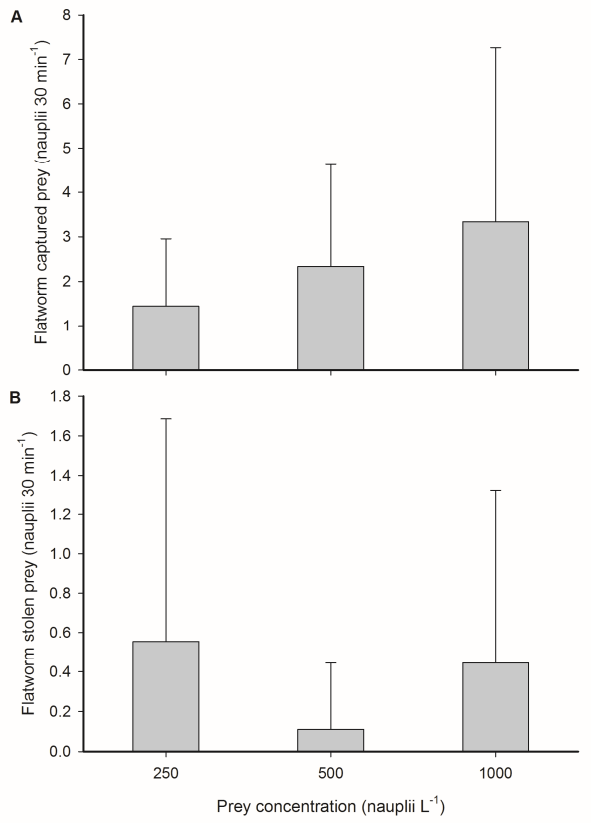

Figure 4: Prey capture and kleptoparasitism by epizoic flatworms. (A) Total captured prey from the water column and (B) stolen prey from the host coral by epizoic flatworms inhabiting a single

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).

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 (Figure 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 (Figure 5B).

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, Figure 6).

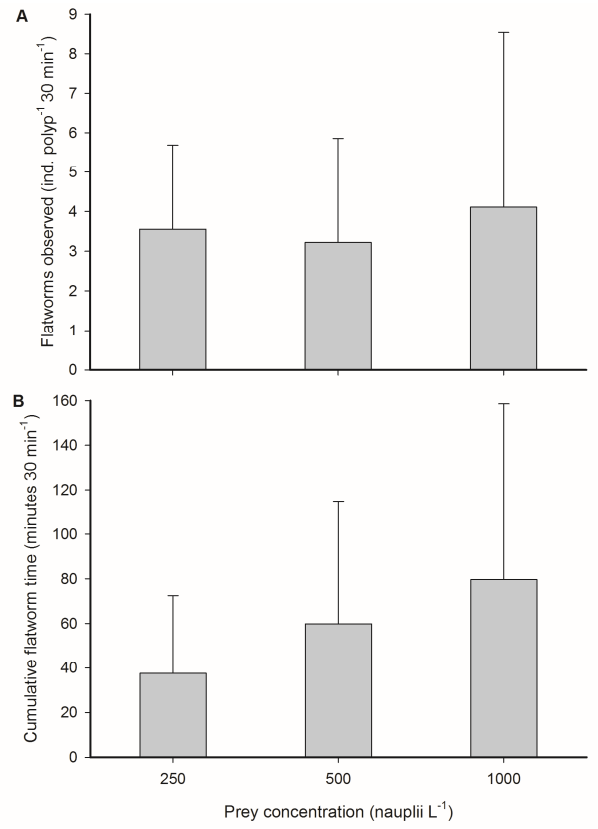

Figure 5: Flatworm density and activity on coral polyps. (A) Flatworms observed on the oral disc of their host coral, expressed as individuals polyp^{-1} 30 min^{-1}, and (B) cumulative flatworm time spent on the oral disc, expressed in minutes 30 min^{-1}, at three different prey concentrations; 250,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

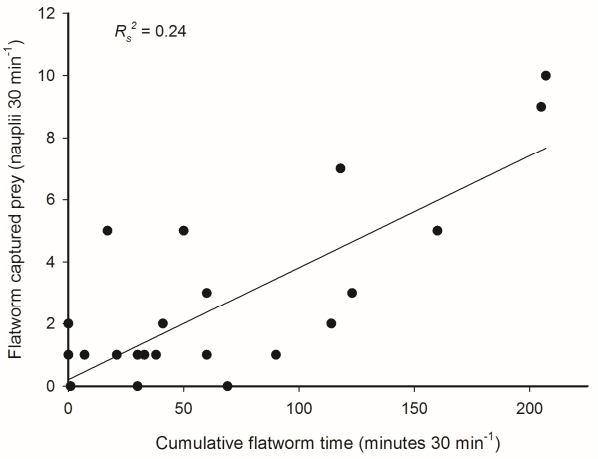

Figure 6: Correlation between activity and prey capture of epizoic flatworms. Correlation between cumulative flatworm time, expressed as minutes 30 min^{-1}, and total captured prey, expressed as nauplii 30 min^{-1}, by epizoic flatworms (Spearman's rho, r_{est}=0.49, R_s^2=0.24, P=0.010, two-tailed).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

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,b; 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.

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. (2009) and Wijgerde et al. (2011a) 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. Finally, kleptoparasitism clearly contributed to a reduction of coral feeding by decreasing prey retention rates of the coral host (also see next section).

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; Lasker 1982; Lewis 1992; Ferrier-Pagès et al. 1998a, 2003; Houlbrèque et al. 2004a). 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. (2011a) on the feeding dynamics of *G. fascicularis*, who showed that prey capture and release are coupled, and decrease over time concomitantly.

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±2.1 to 4.1±4.4 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±3.3 to 50.0±2.1%) 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 (Sebens et al. 1996, 1998; Palardy et al. 2006). 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).

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Chapter 6

General discussion

Introduction

The aim of this thesis was to increase our understanding of the role of heterotrophic feeding in the nutrient budget and growth of the scleractinian coral *Galaxea fascicularis*, and how (a)biotic factors affect this role.

The research questions for this thesis were:

1. What is the potential role of heterotrophic feeding in the nutrient acquisition and budget for the scleractinian coral *Galaxea fascicularis*? (**Chapters 2 and 6**)
2. What mechanism explains the inhibitory short-term effect of zooplankton feeding on skeletal growth of *G. fascicularis*? (**Chapter 3**)
3. How does water flow rate affect zooplankton feeding by solitary polyps and colonies of *G. fascicularis*? (**Chapter 4**)
4. What is the effect of epizoic acoelomorph flatworms on zooplankton feeding by *G. fascicularis*, and is this effect dependent on prey availability? (**Chapter 5**)

In this chapter, I will discuss and integrate the obtained results in the context of existing literature, and provide recommendations for future research.

Heterotrophy: an underestimated source of nutrient acquisition for corals

Over the last decade, the important role of heterotrophic feeding in organic nutrient acquisition has been well documented (reviewed by Houlbrèque and Ferrier-Pagès 2009). However, as discussed in the general introduction, the feeding rates measured to date may have been underestimations. To obtain more realistic estimates of coral feeding rates, we quantified prey capture, external and internal digestion, and release by the scleractinian coral *Galaxea*

fascicularis using video analysis (**Chapter 2**). Indeed, our video data revealed significantly higher prey capture rates compared to clearance rate data from *G. fascicularis* (Hii et al. 2009; Osinga et al. 2011b) under similar conditions (93±0 *versus* 50±30 and 40 nauplii polyp^{-1} hour^{-1}, respectively). Interestingly, Hii et al. (2009) also documented the extracoelenteric feeding behaviour of *G. fascicularis* with photographs, although they did not recognise the process as such. The mesenterial filaments in their images are simply described as coral mucus. Based on our data, corals may digest 98.6% of their captured prey externally, suggesting that intracoelenteric particle analysis alone may grossly underestimate coral feeding rates and benthic-pelagic coupling (i.e. nutrient exchange between the pelagic and benthic zones). This theory is supported by the fact that many corals have been reported to possess the ability of extracoelenteric feeding (Duerden 1902; Carpenter 1910; Matthai 1918; Yonge 1930,1973; Abe 1938; Goreau et al. 1971; Lang 1973; Logan 1984; Lang and Chornesky 1990; Goldberg 2002). At present, it is still unclear what factors determine the feeding behaviour of corals, in terms of intra- or extracoelenteric feeding. It is likely that the latter feeding mechanism allows corals to process much more prey compared to internal feeding. This is plausible as corals have to ingest prey before internal digestion, which requires energy expenditure, i.e. muscle contraction and ciliary movement. In addition, the coelenteric volume may have a limited available volume for prey. Future studies should therefore focus on determining thresholds for extracoelenteric zooplankton feeding in terms of prey size and concentration, both in captivity and *in situ*.

Heterotrophic feeding can be a crucial survival strategy for corals, when they temporarily lose their autotrophic symbionts during mass bleaching events (Grottoli et al. 2006). Therefore, accurate measurements of zooplankton feeding rates during periods of bleaching are of high importance, to determine whether corals are able to maintain a positive energy balance in a time of global climate change. Future studies should therefore use video

analysis to compare feeding rates of healthy corals with those of bleached individuals.

Another key issue which needs to be addressed is the current methodology for estimating nutrient gain from individual (zoo)plankton particles. Many studies have assumed a 100% assimilation of available carbon from zooplankton during intracoelenteric digestion (Fabricius et al. 1995; Sebens et al. 1996, 1998; Houlbrèque et al. 2004; Grottoli et al. 2006; Purser et al. 2010), which may not be accurate. During our study, we measured depletion values of total organic carbon, nitrogen and phosphorous at only 43.1%, 51.3% and 50.9% respectively (**Chapter 2**), suggesting that a significant amount of refractory organic material may not be digested and assimilated. In addition, studies have only focused on organic carbon and nitrogen depletion, and have not provided data on organic phosphorous uptake, an important element for tissue growth (Sorokin 1973; D'Elia 1977). When video analyses are combined with comprehensive plankton analyses, before and after prey digestion, realistic nutrient uptake and budgets may be calculated under a variety of environmental conditions. Measuring and comparing the isotopic signatures of prey and coral tissue (^{13}C and ^{15}N) can further substantiate assimilation of prey-derived compounds into coral biomass (Ferrier-Pagès et al. 2011).

Temporal metabolic acidosis: an explanation for the contrasting effects of heterotrophy on coral growth

It is now widely accepted that coral calcification, the precipitation of aragonite from calcium and carbonate ions, is enhanced by heterotrophic feeding (Houlbrèque and Ferrier-Pagès 2009). As outlined in the general introduction, three mechanisms have been proposed to explain this phenomenon; supply of the organic matrix precursor aspartic acid (Allemand et al. 1998, 2004; Houlbrèque et al. 2004a), increased photosynthesis rates (Dubinsky et al. 1990; Houlbrèque et al. 2003, 2004a; Titlyanov et al. 2000a,b), and enhanced

DIC production through higher metabolism (Szmant-Froelich et al. 1984; Furla et al. 2000; Ferrier-Pagès et al. 2003). The substantial uptake of inorganic phosphorus from zooplankton by *G. fascicularis* described in **Chapter 2** supports the second mechanism, as it may be partially responsible for the increased zooxanthellae growth and higher areal rates of photosythesis observed after prolonged feeding. In addition, zooxanthellar uptake of inorganic nitrogen and carbon from zooplankton and metabolic waste products (e.g. NH_4^+ and CO_2) produced by the coral (Piniak et al. 2003) may also enhance zooxanthellae density and thus photosynthetic capacity. Unfortunately, we were unable to measure uptake of inorganic nitrogen from zooplankton.

Although the enhancement of coral calcification by heterotrophy has been demonstrated on a time scale of weeks (reviewed by Houlbrèque and Ferrier-Pagès 2009), conflicting results have been obtained with short-term experiments. Zooplankton, glucose or glycerol supplementation actually decrease dark calcification rates of the scleractinian corals *Galaxea fascicularis* (Al-Horani et al. 2007) and *Montastrea faveolata* (Colombo-Pallotta et al. 2010), which is not well understood. A possible explanation is that in darkness, the ATP-driven process of calcification (Ip et al. 1991; Babcock and Wikström 1992) is temporarily oxygen-limited during prey capture and nutrient uptake, without photosynthetic oxygen production to compensate for this. To provide more insight into the effects of heterotrophic feeding on coral calcification, we measured light and dark calcification rates of the scleractinian coral *Galaxea fascicularis* with and without zooplankton supplementation under a range of ambient oxygen saturations (**Chapter 3**). When corals were fed with zooplankton in light conditions, we detected no adverse effects on calcification rates at normoxia (110% oxygen saturation). At 50 and 150% saturation, a slight positive effect of feeding was found. We only found a negative effect of feeding in light at 280% saturation. Under dark conditions, however, feeding resulted in a complete shutdown of calcification between 50 and 150% saturation. This clearly showed that a discrepancy

between the short- and long-term effects of feeding on calcification only exists in darkness. As oxygen supplementation did not alleviate the inhibitory effect of feeding on dark calcification, oxygen limitation of calcifying calicoblastic cells does not seem to be a valid hypothesis. An alternative mechanism underlying inhibition of dark calcification by feeding may be tissue acidosis, caused by increased respiration rates (**Chapter 3**). This, in turn, could result in a temporal decrease of tissue pH through the production of protons from carbon dioxide and water by the enzyme carbonic anhydrase, resulting in reduced calcification rates. Tissue acidosis may induce a transient energy reallocation to processes other than calcification, including soft tissue growth and organic matrix synthesis, as this may be energetically favourable.

Future studies may determine the threshold zooplankton concentration or zooplankton to coral biomass ratio below which no short-term reduction of dark calcification can be detected. It is likely that a typical dose-response relationship exists between zooplankton supplementation and inhibition of dark calcification (Figure 1).

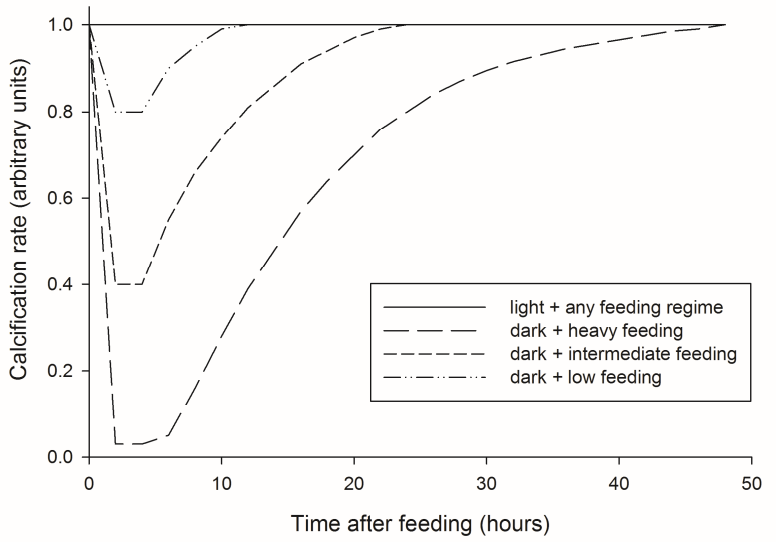

Figure 1: Hypothesised temporal effect of feeding on calcification rates. In light, no impairment of calcification occurs as photosynthesis titrates protons produced by respiration. In darkness, heavy feeding (150 nauplii polyp^{-1}) induces high respiration rates, disrupting calcification for several hours, after which respiration and calcification rates normalise around 48 hours after

feeding. Intermediate and low feeding (75 and 37.5 nauplii polyp^{-1}) regimes will result in lower respiration increases, less disruption of calcification, and a faster return to baseline calcification rates. Model based on Szmant-Froelich and Pilson (1984), Hii et al. (2009) and Wijgerde et al. (2011a). See text for further details.

Heavy feeding (e.g. 150 *Artemia* nauplii polyp^{-1}) may induce high respiration rates, significantly disrupting dark calcification through acidosis, after which both respiration and calcification will return to baseline levels. Intermediate and low feeding (e.g. 75 and 37.5 nauplii polyp^{-1}) will likely result in lower respiration increases and acidosis, and thus a less pronounced impairment of dark calcification. This theory is supported by the findings of Szmant-Froelich and Pilson (1984), who measured for the scleractinian coral *Astrangia danae* that higher prey quantities resulted in higher respiration increases after feeding, and vice versa. They also found that respiration rates take longer to normalise to baseline levels when feeding higher amounts of prey. At the highest feeding dosage (1186 µg C per colony), respiration stabilised to normal levels after approximately 48 hours. Based on a nauplii digestion time of 3 to 6 hours for *Galaxea fascicularis* (Hii et al. 2009; Wijgerde et al. 2011a), a high feeding dosage is expected to inhibit dark calcification for several hours, after which it may return to a baseline level within 48 hours (Figure 1). Intermediate and low feeding may result in less dramatic calcification impairment, in terms of intensity and duration (Figure 1). To confirm whether respiration-induced acidosis is the underlying mechanism for calcification impairment, tissue measurements with pH microsensors (Al-Horani et al. 2003) are required before and during feeding experiments.

A temporal negative effect of heterotrophic feeding on dark calcification rates would explain the discrepancies between the short- and long-term effects of feeding. Although dark calcification is temporarily inhibited during zooplankton feeding, in between feeding events, corals can benefit from enhanced organic matrix synthesis, photosynthesis rates and metabolic DIC supply which promote calcification. Although it is still unclear whether feeding rates on reefs are high enough to induce negative effects, the

nocturnal feeding behaviour of corals may impose a significant physiological cost in terms of impaired dark calcification rates. Future research should determine to what extent other scleractinian corals, next to *G. fascicularis* and *M. faveolata*, are sensitive to dark calcification impairment during feeding.

Modulation of heterotrophy by (a)biotic factors: effects on nutrient budget

It is clear that heterotrophic feeding plays an important role in the nutrient budget and growth of scleractinian corals. As several biotic and abiotic factors are known to affect coral feeding rates, it logically follows that the role of heterotrophic feeding in nutrient procurement and growth varies. In **Chapter 4**, the effects of water flow rate and colony size on heterotrophic feeding of *G. fascicularis* were presented. In **Chapter 5**, the effects of epizoic flatworms and prey concentration on feeding rates were studied. These results demonstrate that the effects of biotic (colony size, flatworms and prey concentration) and abiotic (water flow rate) factors on heterotrophic feeding are profound. Based on nutrient depletion data resulting from zooplankton digestion (**Chapter 2**), carbon input from zooplankton feeding can be calculated under a range of conditions. When combining these data with photosynthesis, respiration (Schutter et al. 2010) and excretion (Ferrier-Pagès et al. 1998b) rates of *G. fascicularis*, carbon budgets for different scenarios can be calculated (Tables 1 and 2). By combining daily organic carbon input from autotrophy and heterotrophy, and subtracting losses through respiration and excretion, the amount of carbon left for growth is obtained, called scope for growth (Warren and Davis 1967). This value is often used as a proxy for the overall health or stress status of an organism (Maltby 1999). The term scope for growth is used as not all carbon may be directed towards skeletal or tissue growth, or organic matrix synthesis, as it can also be stored in coral tissue, mainly in the form of lipids (Anthony et al. 2002). Negative scope for growth values suggest that coral growth is impaired, whereas positive values suggest that sufficient

carbon is left for growth. However, even in the latter case, a carbon limitation may still exist, i.e. higher scope for growth values may result in higher coral growth. A limitation of scope for growth is that it will, to some degree, underestimate the true growth potential of corals, as respiration can also drive inorganic growth (i.e. calcification).

Table 1: Estimated nutrient budget for *Galaxea fascicularis* at various water flow rates, for single polyps and polyps in colonies. Data are based on an ambient prey concentration of 10,000 *Artemia* nauplii L^{-1}, a feeding time of 6 hours, 6.2 polyps cm^{-2} and a prey aggregation density of 0.08 aggregates $polyp^{-1}$. Photosynthesis and respiration based on Schutter et al. (2010), excretion based on Ferrier-Pagès et al. (1998b). Values are means (*N*=3-9).

	Input (µg C cm^{-2} day^{-1})		Output (µg C cm^{-2} day^{-1})		
single polyps					
water flow rate ($cm\ s^{-1}$)	photosynthesis	feeding	respiration	excretion	scope for growth
1.25	101.09	22.10	77.76	25.27	20.16
5	101.09	16.40	77.76	25.27	14.46
10	101.09	32.80	77.76	25.27	30.86
20	89.86	24.80	86.40	22.46	5.79
30	70.85	12.00	89.86	17.71	-24.72
40	70.85	7.50	89.86	17.71	-29.22
polyps in colonies					
water flow rate ($cm\ s^{-1}$)	photosynthesis	feeding	respiration	excretion	scope for growth
1.25	101.09	0.90	77.76	25.27	-1.04
5	101.09	89.00	77.76	25.27	87.06
10	101.09	123.50	77.76	25.27	121.56
20	89.86	30.60	86.40	22.46	11.59
30	70.85	44.30	89.86	17.71	7.58
40	70.85	17.70	89.86	17.71	-19.02

Table 1 reveals that under most water flow conditions, *G. fascicularis* has a positive scope for growth. However, at high flow rates of 30 to 40 cm s^{-1}, single

polyps have a negative scope for growth. This is due to the fact that both heterotrophic feeding (**Chapter 4**) and photosynthesis are reduced. Polyps in colonies show a different trend; at both low and high flow rates, scope for growth is negative. This is due to the fact that at a low flow rate of 1.25 cm s^{-1}, heterotrophic feeding is reduced, whilst at a high flow rate of 40 cm s^{-1}, both heterotrophy (**Chapter 4**) and photosynthesis are reduced. Interestingly, during a study on the effects of flow regime on *G. fascicularis* growth rates, Schutter et al. (2010) found that the lowest flow rate applied (0 cm s^{-1}) resulted in lowest growth. This may have been, in part, due to the fact that heterotrophic feeding, in the form of *Artemia* capture and DOM (dissolved organic matter) uptake, was lower in the absence of flow. At 30 and 40 cm s^{-1}, although feeding is less impaired, photosynthesis is also reduced. Schutter et al. (2010) also found a lower scope for growth at higher flow rates, together with decreased tissue growth, although skeletal growth was higher. At 10 cm s^{-1}, *G. fascicularis* appears to attain the highest scope for growth, due to the fact that heterotrophic feeding is highest at this flow rate. Interestingly, Schutter et al. (2010) also found a highest scope for growth at 10 cm s^{-1}, which translated to higher tissue growth. This suggests that intermediate flow rates favour tissue growth by optimising heterotrophy. This theory is in good agreement with the current heterotrophy paradigm, which states that heterotrophy provides essential nutrients such as amino and fatty acids for (tissue) growth (reviewed by Houlbrèque and Ferrier-Pagès 2009).

Although scope for growth is likely to be much lower in the field due to limited prey availability, the capacity of *G. fascicularis* to feed on zooplankton under a wide range of flow rates correlates well with the different reef habitats in which this species is found. These habitats are exposed to highly variable flow rates, ranging from approximately 5 to 50 cm s^{-1} (Genin et al. 1994). Interestingly, *in situ* observations showed that *G. fascicularis* is more abundant at protected sites compared to areas exposed to higher flow rates. According to the authors, the dominant mechanism behind this finding is interspecific competition between *G. fascicularis* and other coral species by

means of sweeper tentacles. However, they acknowledge the possible role of other factors, which may also include higher feeding (and thus growth and survival) rates at lower flow speeds of 5 to 10 cm s^{-1}. Either way, the budget analysis above underscores that water flow rate and colony size have profound effects on coral energy balance. Future studies should address the potential interaction between water flow rate and prey concentration on the feeding rates of this species, similar to the study of Purser et al. (2010) for *Lophelia pertusa*. In addition, determining the effect of flow pattern, i.e. oscillating versus unidirectional flow, would be relevant as Hunter (1989) demonstrated that this factor can affect feeding rates of benthic colonial invertebrates.

Table 2: Estimated nutrient budget for *Galaxea fascicularis* at various prey densities, without and with epizoic flatworms. Data are based on a water flow rate of 5 cm s^{-1}, a feeding time of 6 hours, 6.2 polyps cm^{-2} and a prey aggregation density of 0.08 aggregates $polyp^{-1}$. Photosynthesis and respiration based on Schutter et al. (2010), excretion based on Ferrier-Pagès et al. (1998b). Values are means (*N*=3-9).

	Input (µg C cm^{-2} day^{-1})		Output (µg C cm^{-2} day^{-1})		
without flatworms					
prey concentration (nauplii L^{-1})	photosynthesis	feeding	respiration	excretion	scope for growth
250	101.09	4.73	77.76	25.27	2.79
500	101.09	8.47	77.76	25.27	6.53
1000	101.09	29.96	77.76	25.27	28.01
with flatworms					
prey concentration (nauplii L^{-1})	photosynthesis	feeding	respiration	excretion	scope for growth
250	101.09	2.95	77.76	25.27	1.00
500	101.09	5.91	77.76	25.27	3.97
1000	101.09	3.95	77.76	25.27	2.01

Next to water flow and colony size, episymbiont presence and ambient prey concentration affect coral feeding rates in an interactive way (**Chapter 5**). This is reflected in Table 2, which clearly shows that a positive relationship between prey concentration and feeding rates is only found for polyps without epizoic flatworms. When flatworms are present, no positive effect of increased prey concentration is detected. The inhibitory effect of flatworms on coral feeding rates translates to a lower scope for growth under all conditions. At the lowest prey concentration applied (250 nauplii L^{-1}), this value is close to zero, suggesting a carbon limitation for flatworm-hosting corals when prey concentrations fall below this threshold. On coral reefs, carbon limitation is likely to occur, as prey densities range between 0.4-6.3 individuals L^{-1}; Heidelberg et al. 2004, 2007; Holzman et al. 2005; Yahel et al. 2005a,b; Palardy et al. 2006). It must be noted that excretion rates could be reduced, in favour of maintaining respiration and growth, when corals cannot feed sufficiently. This would, however, increase their sensitivity to environmental stressors including sedimentation, as mucus forms an important protective layer around coral tissue (Brown and Bythell 2005). Future studies should determine to what extent flatworms compromise the growth and health of *G. fascicularis* and other coral species *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).

Implications for aquaculture

Coral aquaculture, as an alternative to mariculture or wild collection, is an emerging activity (Wijgerde et al. 2012a). To make this practice economically viable, however, culture protocols require optimisation. The results presented in **Chapter 2 to 5**, together with the budget analysis above, have clear implications for culture protocols.

First of all, when considering the importance of nutrition for coral aquaculture, the timing of feeding seems essential. In **Chapter 3**, it was demonstrated that dark calcification of *G. fascicularis* was disrupted by zooplankton supplementation, while light calcification was unaffected under normoxic conditions. This suggests that feeding scleractinian corals during daytime promotes efficient aquaculture. The effect of daytime feeding is further enhanced by hyperoxia; at 150% oxygen saturation, light calcification rates of fed corals were significantly higher compared to unfed corals (**Chapter 3**). Although it is difficult to maintain such high oxygen concentrations in aquaculture, it seems vital to maintain normoxia, as hypoxia resulted in reduced light and dark calcification rates of *G. fascicularis*.

Related to feeding, water flow rate is key to successful coral aquaculture. Previous studies have shown that water flow promotes coral growth (Schutter et al. 2010) and photosynthesis (Mass et al. 2010; Schutter et al. 2010), and that water flow acts synergistically with irradiance on the growth of *Galaxea fascicularis* (Schutter et al. 2011). The findings presented in **Chapter 4** reveal that water flow also affects heterotrophic feeding of *G. fascicularis*, with an optimum at 10 cm s^{-1} for polyps in colonies. This finding corresponds well with previous research by Schutter et al. (2010), who found highest scope for growth and tissue growth at the same flow rate (Schutter et al. 2010). Although single polyps retain the highest amount of prey at 1.25 cm s^{-1} (**Chapter 4**), scope for growth is higher at 10 cm s^{-1} when prey capture rather than retention data are used for budget analysis. Using prey capture data may be favourable as polyps may partially digest prey before release, after which predigested prey may be recaptured and further digested, at least in an aquaculture setting.

Next to water flow rate, colony size modulates the effect of water flow on heterotrophic feeding (**Chapter 4**). Although polyps in colonies may exhibit higher feeding rates and scope for growth compared to their solitary conspecifics, the colony as a whole is less efficient as only 7.7% of all polyps generate prey aggregates (**Chapter 2**). This suggests that nutrient

procurement per unit of biomass decreases with size for *G. fascicularis*, resulting from intracolonial polyp competition. This fits well with the findings of Schutter et al. (2010), who measured decreased specific growth rates with size for this species; after 245 days of incubation, a decrease of 76% (from 0.025 to 0.006 day^{-1}) was found. Although decreased feeding efficiency is but one possible factor that contributes to decreased specific growth rates of *G. fascicularis*, it is clear that maintaining small colonies favours growth in aquaculture.

Epizoic flatworms are another important biotic factor to consider in the context of aquaculture. In **Chapter 5**, it was demonstrated that acoelomorph flatworms negatively affect coral feeding rates, which is reflected by the budget analysis above. The lower scope for growth values suggest a possible growth-limitation for flatworm-hosting corals. In aquaculture, it may be prudent to control captive flatworm populations by introducing natural predators to the system. There is evidence that certain wrasses (e.g. *Halichoerus* spp.), dragonets (e.g. *Synchiropus splendidus*) and nudibranchs (*Chelidonura varians*) actively prey on flatworms (Carl 2008; Nosratpour 2008). Chemical treatment of corals with anthelmintics such as levamisole works well (**Chapter 5**), but this is laborious and could negatively affect long-term coral health.

The effect of prey density is the last factor that was addressed during this study, and its effect on coral feeding was found to be dependent on flatworm presence (**Chapter 5**). For worm-free corals, a positive effect of prey concentration on prey capture rate was found. Coral polyps which hosted flatworms, however, did not exhibit higher feeding rates at higher prey availability. This insight is highly relevant to coral aquaculture, as it appears that providing corals with more prey does not necessarily result in higher feeding and growth rates when flatworms are present. Indeed, flatworms are commonly found in aquaculture (Carl 2008; Leewis et al. 2009). As nutrition is a costly aspect of coral aquaculture (Osinga et al. 2011a), it is important to consider that flatworms may prevent or reduce the beneficial effect of feeding on coral growth, thereby reducing the cost-effectiveness of aquaculture. When

flatworms are removed, a positive effect of prey concentration on growth is expected, due to higher feeding rates and nutrient procurement (Houlbrèque and Ferrier-Pagès 2009).

Ecological implications

Although several ecological implications of this thesis have been discussed above, the role of oxygen deserves special attention. In **Chapter 3**, a strong control of oxygen on light and dark calcification rates was presented. The pronounced inhibition of dark calcification by hypoxia, and the impairment of light calcification by hyperoxia have implications for our understanding of *in situ* calcification rates. Corals inhabiting lagoons and reef flats regularly experience hypoxia and hyperoxia due to reduced water flow rate and exchange during low tide, resulting in oxygen saturations ranging from approximately 30 to 194% (Kinsey and Kinsey 1967; Kraines et al. 1996). In addition, coral tissue and the coral-water interface become anoxic (1% saturation) during night time and hyperoxic (up to 373% saturation) during the day (Shashar et al. 1993; Kühl et al. 1995; Wangpraseurt et al. 2012). This suggests that corals may exhibit highly variable calcification rates throughout the day and night, especially on reefs that experience low tides with reduced water flow.

Although the oxygen saturation of seawater by itself exerts a strong control over calcification, its effect may be modulated by other factors. At present, many studies on coral ecology focus on climate change and anthropogenic disturbances, investigating the (interactive) effects of seawater pH, temperature and eutrophication on coral growth and survival (e.g. Langdon and Atkinson 2005; Fabricius 2011). The role of oxygen saturation in these processes, however, is not well understood. In fact, hypoxia and hyperoxia may exacerbate the negative effects exerted by these factors. First of all, low oxygen saturation, a common phenomenon in reef waters during night-time (Kinsey and Kinsey 1967; Kraines et al. 1996), may aggravate the

negative effect of ocean acidification on reef accretion. The current theory which explains the reduction of coral growth in response to ocean acidification is that the aragonite saturation state (Ω) decreases at lower pH (Kleypas et al. 1999). This occurs because when pH decreases, the carbonate (CO_3^{2-}) concentration is reduced in favour of the bicarbonate (HCO_3^-) concentration. Thus, the solubility of calcium carbonate, the major constituent of coral skeleton, is higher when seawater pH decreases, possibly resulting in higher dissolution of skeleton directly exposed to seawater. It is also possible that calcification requires more energy in the form of ATP, as more protons have to be removed from the calcification site (calicoblastic medium) when coral tissue is exposed to more acidic seawater, in a similar way as described in **Chapter 3**. When oxygen levels in reef waters are low, ATP production through aerobic respiration by calicoblastic cells may be impaired, possibly rendering corals even more sensitive to ocean acidification as they may be unable to allocate sufficient metabolic energy to pH maintenance of the calicoblastic medium.

In a similar way, oxygen saturation may interact with temperature on coral growth. High sea surface temperatures are known to negatively affect calcification when coral bleaching occurs, during which corals lose their symbiotic dinoflagellates (Hoegh-Guldberg 1999; Hughes et al. 2003). As translocation of photosynthates (e.g. glycerol) by the zooxanthellae provides corals with the required energy source for calcification (Muscatine et al. 1981; Muscatine 1990), coral growth is greatly reduced when these dinoflagellates are absent. Although seawater hypoxia may not further aggravate this negative effect, hyperoxia may increase energy stress by forcing corals to allocate energy to antioxidant enzymes such as superoxide dismutase and catalase.

Finally, the combined effects of oxygen saturation and eutrophication may indirectly lead to reduced coral growth. Eutrophication is known to increase (macro)algal cover (Fabricius 2005), which may lead to higher oxygen fluctuations during the day and night due to increased algal

photosynthesis and respiration, respectively. As corals seem highly sensitive to high and low oxygen saturations (**Chapter 3**), the currently observed phase shifts from coral-dominated to algae-dominated reefs (Hughes et al. 2003; Hoegh-Guldberg et al. 2007) may be accelerated by extreme hypoxia and hyperoxia. This theory is further supported by the findings of Wangpraseurt et al. (2012), who showed that the coral-turf algae interface becomes hypoxic (30% saturation) at night. In addition, eutrophication can induce harmful algal blooms (Bauman et al. 2010), which may deplete reef waters of dissolved oxygen (Guzmán et al. 1990) and thus reduce coral calcification.

It is likely that hypoxia and hyperoxia augment the negative effects of ocean acidification, global warming and pollution, which should be addressed in future studies. Multi-factorial experiments which address the short- and long-term interactive effects of these stressors on coral growth will shed more light on this matter.

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Summary

Coral reefs are of tremendous ecological and economic importance, and are currently in global decline due to climate change and anthropogenic disturbances. Overfishing is a threat to reefs in Asia, where corals are collected for the aquarium trade. This trade appears unsustainable, as wild collection of reef organisms has led to elimination of local populations and significant changes in age structure. Therefore, a major incentive exists to grow corals sustainably, so that local degradation of coral reefs can be reduced. To optimise coral aquaculture, detailed knowledge of factors controlling growth is required. Zooplankton feeding is considered important to coral growth, as it supplies the coral with nutrients such as fatty acids and amino acids. However, a realistic quantification of the carbon, nitrogen and phosphorous acquisition from heterotrophic feeding is currently lacking, obscuring nutrient budgets for corals. In addition, contrasting short- and long-term effects of heterotrophy on coral growth have been found. To further complicate matters, (a)biotic factors, including water flow rate, coral size, the presence of episymbionts and prey density affect zooplankton feeding, and thus nutrient input and growth, and knowledge of these factors is still limited. The relevance of addressing the knowledge gaps above is twofold. First, it contributes to our fundamental understanding of the role of heterotrophy in the coral nutrient budget and growth, and how (a)biotic factors affect this role. Second, these findings allow coral aquaculture protocols to be improved, which benefits the sustainable trade in these endangered species. The research questions for this thesis were: what is the potential role of heterotrophic feeding in the nutrient acquisition and budget for the scleractinian coral *Galaxea fascicularis* (**Chapters 2 and 6**)?; what mechanism explains the inhibitory short-term effect of zooplankton feeding on skeletal growth of *G. fascicularis* (**Chapter 3**)?; how does water flow rate affect zooplankton feeding by solitary polyps and colonies of *G. fascicularis* (**Chapter 4**)?; and finally, what is the effect of epizoic acoelomorph flatworms on zooplankton feeding by *G. fascicularis*, and is this effect dependent on prey availability (**Chapter 5**)?

In **Chapter 2**, the acquisition of organic compounds through heterotrophic feeding by the scleractinian coral *G. fascicularis* is presented. First, the feeding behaviour of single polyps was investigated using video analysis. A highly dynamic feeding process was observed. A single *G. fascicularis* polyp captured 558±67 *Artemia* nauplii, and released 383±75 nauplii (*N*=3) over a 6 hour interval. On average, 98.6% of prey captured was not ingested. Instead, prey items were clustered in aggregates that were digested externally by mesenterial filaments. In addition, carbon, nitrogen and phosphorus analysis of zooplankton was conducted before and after digestion by *G. fascicularis* colonies (*N*=6). For total organic carbon (TOC), 43.1% (0.298±0.148 µg *Artemia*$^{-1}$) was lost after 6 hours of digestion. For total organic nitrogen (TON), total organic phosphorus (TOP) and orthophosphate (PO_4^{3-}), these values were 51.3% (0.059±0.028 µg *Artemia*$^{-1}$), 50.9% (0.009±0.004 µg *Artemia*$^{-1}$) and 84.6% (0.0019±0.0008 µg *Artemia*$^{-1}$), respectively. For extracoelenteric zooplankton feeding alone, total estimated nutrient inputs for *G. fascicularis* colonies were 76.5±0.0 µg organic carbon, 15.2±0.0 µg organic nitrogen, 2.3±0.2 µg organic phosphorus and 0.5±0.8 µg inorganic phosphorus per cm^2 coral tissue per day. These values exceed calculations based on intracoelenteric feeding by up to two orders of magnitude, and demonstrate that extracoelenteric zooplankton feeding is a key mechanism of nutrient acquisition for a scleractinian coral.

In **Chapter 3**, the short-term effects of zooplankton feeding on light and dark calcification rates of *G. fascicularis* colonies (*N*=4) at various oxygen saturation levels are discussed. Significant main and interactive effects of oxygen, heterotrophy and light on calcification rates were found. Light and dark calcification rates of unfed corals were severely affected by hypoxia and hyperoxia, with optimal rates at 110% saturation. Light calcification rates of fed corals exhibited a similar trend, with highest rates at 150% saturation. In contrast, dark calcification rates of fed corals were close to zero under all oxygen saturations. It is concluded that oxygen exerts a strong control over light and dark calcification rates of corals. Nevertheless, the inhibitory effect

of heterotrophy on dark calcification appears to be oxygen-independent. A new hypothesis is that dark calcification is impaired during zooplankton feeding by a temporal decrease of the pH and aragonite saturation state of the calcifying medium, caused by increased respiration rates. This may invoke a transient reallocation of metabolic energy to soft tissue growth and organic matrix synthesis.

In **Chapter 4**, the effects of water flow rate and polyp context (the presence of neighbouring polyps) on zooplankton feeding by *G. fascicularis* are described. Single polyps (*N*=4) and colonies (*N*=4) were incubated in a flow cell for 30 minutes with an ambient *Artemia* nauplii concentration of 10,000 L^{-1} and water flow rates ranging from 1.25 to 40 cm s^{-1}. Water flow rate and polyp context showed significant main and interactive effects on feeding rates of *G. fascicularis* polyps. More specifically, feeding rates were optimal at flow rates of 1.25 and 5 to 10 cm s^{-1} for single polyps and those inhabiting colonies, respectively. These results demonstrate that flow affects coral feeding and thus heterotrophic nutrient input at both a polyp and colony level.

In **Chapter 5**, the effect of epizoic flatworms on zooplankton feeding by *G. fascicularis* is reported. The feeding behaviour of single polyps (*N*=9) was studied using video analysis, 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. It is suggested to classify the coral-associated *Waminoa* sp. as an epizoic parasite, as its presence may negatively affect growth and health of the host.

In **Chapter 6**, the role of heterotrophic feeding in the nutrient budget and growth of *G. fascicularis* is discussed, including how (a)biotic factors affect this role. It is clear that the importance of heterotrophy in the coral nutrient budget has been underestimated, and that its relative contribution to the budget depends on water flow rate, coral size, flatworm presence and prey density. In addition, the short-term effects of heterotrophic feeding on coral growth are variable, and depend on ambient light and oxygen conditions. These insights are of relevance to coral ecology and optimisation of sustainable coral aquaculture. Future work will have to address important knowledge gaps, including the mechanism underlying impairment of dark calcification during feeding, the effect of epizoic flatworms on coral growth (both in *in situ* and in aquaculture), and the interactive effects of oxygen and seawater pH on coral growth.

Samenvatting

Koraalriffen zijn van groot ecologisch en economisch belang, en zijn in achteruitgang vanwege klimaatverandering en antropogene verstoringen. Overbevissing vormt een bedreiging voor koraalriffen in Azië, waar koralen worden verzameld voor de aquariumhandel. Deze handel lijkt niet duurzaam, aangezien de verzameling van riforganismen heeft geleid tot lokale uitsterving van populaties en veranderingen in leeftijdsstructuur. Om deze redenen bestaat de motivatie om koralen duurzaam te kweken. Om koraalkweek te optimaliseren is kennis nodig van factoren die koraalgroei beïnvloeden. Het vangen van zoöplankton is van belang voor de groei van koralen, omdat het voedingsstoffen zoals vetzuren en aminozuren aanlevert. Echter, een realistische kwantificering van de koolstof-, stikstof- en fosforopname via heterotrofie ontbreekt, waardoor een nutriëntbalans niet kan worden berekend. Ook zijn de gevonden korte- en lange-termijn effecten van heterotrofie op koraalgroei tegenstrijdig. Verder beïnvloeden (a)biotische factoren zoals waterbeweging, koraalgrootte, de aanwezigheid van episymbionten en prooidichtheid de prooivangst en dus de heterotrofie en groei van koralen, en kennis van deze factoren is nog beperkt. Het belang van het invullen van deze kennisgaten is tweeledig. Ten eerste draagt het bij aan ons fundamenteel inzicht in de rol van heterotrofie in het nutriëntbudget en de groei van koralen, en hoe (a)biotische factoren deze rol beïnvloeden. Ten tweede kunnen deze inzichten leiden tot een verbetering van de aquacultuur van koralen, wat de duurzame handel in deze bedreigde dieren ten goede komt. De onderzoeksvragen voor dit proefschrift waren: wat is de potentiële rol van heterotrofie in nutriëntacquisitie en –budget voor het steenkoraal *Galaxea fascicularis* (**Hoofstuk 2 en 6**)?; welk mechanisme verklaart het korte-termijn remmende effect van zoöplanktonvoeding op de skeletgroei van *G. fascicularis* (**Hoofdstuk 3**)?; hoe beïnvloedt waterbeweging de zoöplanktonvangst door enkelvoudige poliepen en kolonies bij *G. fascicularis* (**Hoofdstuk 4**)?; en ten slotte, wat is het effect van epizoïsche acoelomorfe platwormen op de zoöplanktonvangst door *G. fascicularis*, en is dit effect afhankelijk van prooibeschikbaarheid (**Hoofdstuk 5**)?

In **Hoofdstuk 2** wordt de opname van organische stoffen door het steenkoraal *G. fascicularis* via heterotrofie besproken. Om de prooivangst te meten werd een gedetailleerde videoanalyse uitgevoerd van enkelvoudige poliepen tijdens toediening van *Artemia*-naupliën. Een dynamisch voedingsproces werd geobserveerd. Een enkele koraalpoliep ving 558±67 naupliën, en liet 383±75 naupliën (*N*=3) los in een periode van 6 uur. Gemiddeld werd 98,6% van de prooien niet ingeslikt. In plaats daarvan werden prooien in aggregaten extern verteerd door middel van mesenteriële filamenten. Verder werd de hoeveelheid koolstof, stikstof en fosfor in de prooien voor en na vertering door *G. fascicularis* geanalyseerd (*N*=6). 43,1% (0,298±0,148 µg *Artemia*$^{-1}$) van het totaal organisch koolstof (TOC) verdween na 6 uur vertering. Voor totaal organisch stikstof (TON), totaal organisch fosfor (TOP) en anorganisch fosfor (PO_4^{3-}) werden waarden van respectievelijk 51,3% (0,059±0,028 µg *Artemia*$^{-1}$), 50,9% (0,009±0,004 µg *Artemia*$^{-1}$) en 84,6% (0,0019±0,0008 µg *Artemia*$^{-1}$) gemeten. De totaal geschatte nutriëntopname vanuit extern verteerd plankton voor *G. fascicularis* was 76,5±0,0 µg organisch koolstof, 15,2±0,0 µg organisch stikstof, 2,3±0,2 µg organisch fosfor en 0,5±0,8 µg anorganisch fosfor per cm^2 koraalweefsel per dag. Deze waarden zijn tot twee orden van grootte hoger vergeleken met cijfers op basis van interne vertering, en tonen aan dat externe vertering van zoöplankton een belangrijk mechanisme van nutriëntacquisitie is voor een steenkoraal.

In **Hoofdstuk 3** worden de korte-termijn effecten van zoöplanktonadditie op de licht- en donkercalcificatie van *G. fascicularis* kolonies (*N*=4) onder diverse zuurstofverzadigingen belicht. Significante interactieve effecten van zuurstof, heterotrofie en licht op calcificatiesnelheden werden gevonden. De licht- en donkercalcificatie van niet-gevoede koralen werden sterk beïnvloed door hypoxie en hyperoxie, met optimale waarden bij 110% zuurstofverzadiging. De lichtcalcificatie van gevoede koralen vertoonde een vergelijkbare trend, met de hoogste waarde bij 150% zuurstofverzadiging. De donkercalcificatie van gevoede koralen lag

dicht bij nul onder alle zuurstofverzadigingen. Geconcludeerd wordt dat zuurstof een sterke invloed heeft op licht- en donkercalcificatie van koralen, en dat calcificatiesnelheden in de natuur een hoge dynamiek hebben. Desondanks lijkt het remmende effect van heterotrofie op donkercalcificatie zuurstofonafhankelijk te zijn. De nieuwe hypothese is dat donkercalcificatie wordt geremd tijdens de planktonvangst door een tijdelijke daling van de pH en aragoniet-verzadiging van het calcificerende medium, veroorzaakt door verhoogde respiratiesnelheden. Dit kan een tijdelijke reallocatie van metabole energie naar de aanmaak van weefsel en synthese van de organische matrix stimuleren.

Hoofdstuk 4 behandelt de interactieve effecten van waterbeweging en poliepcontext (de aanwezigheid van naburige poliepen) op de zoöplanktonvangst door *G. fascicularis*. Enkelvoudige poliepen (*N*=4) en kolonies (*N*=4) werden 30 minuten geïncubeerd in een stroomcel met een prooiconcentratie van 10.000 L^{-1} en een waterbeweging van 1,25 tot 40 cm s^{-1}. Waterbeweging en poliepcontext vertoonden een significant interactief effect op de planktonvangst van *G. fascicularis*. De planktonvangst was optimaal bij stroomsnelheden van 1,25 en 5 tot 10 cm s^{-1} voor respectievelijk enkelvoudige poliepen en poliepen in kolonies. Deze resultaten tonen aan dat waterbeweging de planktonvangst en heterotrofe nutriëntopname beïnvloedt op een poliep- en kolonieniveau.

In **Hoofdstuk 5** wordt het effect van epizoïsche platwormen op de planktonvangst door *G. fascicularis* besproken. De prooivangst van enkelvoudige poliepen (*N*=9) werd bepaald middels videoanalyse, met en zonder symbiose-platwormen. 18S DNA analyse toonde aan dat de platwormen gehuisvest door *G. fascicularis*, met een dichtheid van 3,6±0,4 individuen $poliep^{-1}$, behoorden tot het genus *Waminoa* (Convolutidae). Poliepen met platwormen vertoonden een prooivangst van 2,2±2,5, 3,4±4,5 en 2,7±3,4 naupliën $poliep^{-1}$ 30 min^{-1} bij prooiconcentraties van 250, 500 en 1.000 naupliën L^{-1}, respectievelijk. Ontwormde poliepen vingen 2,7±1,6, 4,8±4,1 en 16,9±10,3 naupliën $poliep^{-1}$ 30 min^{-1}. Significante interactieve

effecten van platwormaanwezigheid en prooidichtheid werden gevonden, weerspiegeld door het feit dat platwormen de prooivangst van het koraal alleen remden bij een prooidichtheid van 1.000 naupliën L^{-1}. Ook vertoonden platwormen kleptoparasitisme, en verwijderden 0,1±0,3 tot 0,6±1,1 naupliën 30 min^{-1} van de gastheerpoliep, of 5,3±3,3 tot 50,0±2,1% van de door het koraal gevangen prooien. Aanbevolen wordt de koraal-geassocieerde *Waminoa* sp. te classificeren als een epizoïsche parasiet, aangezien de aanwezigheid van de worm de groei en gezondheid van het gastheerkoraal negatief kan beïnvloeden.

In **Hoofdstuk 6** wordt de rol van heterotrofie in het nutriëntbudget en de groei van *G. fascicularis* bediscussieerd, en hoe (a)biotische factoren deze rol kunnen beïnvloeden. Het is duidelijk dat het belang van heterotrofie voor het nutriëntbudget in het verleden is onderschat, en dat de relatieve bijdrage aan het budget afhankelijk is van waterbeweging, koloniegrootte, aanwezigheid van platwormen en prooidichtheid. Verder zijn de korte-termijn effecten planktonvoeding op koraalgroei variabel en afhankelijk van licht- en zuurstofcondities. Deze inzichten zijn relevant voor de ecologie van koralen en de optimalisatie van duurzame koraalkweek. Toekomstig werk zal belangrijke kennisgaten moeten invullen, waaronder het achterliggende mechanisme van calcificatieremming in het donker tijdens planktonvangst, het effect van epizoïsche platwormen op koraalgroei (zowel *in situ* evenals in cultuur), en ten slotte de interactieve effecten van zuurstof en zeewater-pH op koraalgroei.

Acknowledgements

John Lennon is claimed to have once said: “Life is what happens to you, while you're busy making other plans.” When I look back at my Ph.D. years in Wageningen, I have to say that he was right. Even though the process of science involves careful planning of experiments, you are bound to stray off course as you encounter technical problems and gain new insights. Eventually, you end up with a dissertation that is different from what you had in mind in the beginning. This is not a bad thing at all, as the end result can be quite satisfactory. However, obtaining a good end result requires the aid of individuals around you, who support you both professionally and personally.

In my particular case, there have been many of these individuals.

This is my attempt to thank those around me who have been pivotal in my success. First of all, I have to extend a big thank you to all the Master and Bachelor students who have assisted me during my experiments. I could never have finished my Ph.D. in three years without the support of these fine young people: Rara Diantari, who analysed coral feeding rates and nutrient uptake from zooplankton prey using our respirometric flow cell. Pascal Spijkers, who used the same setup to determine the effects of water flow rate on coral feeding rates. Marleen Hoofd and Saskia Jurriaans, who measured coral calcification rates under a wide range of conditions. Eline van Onselen and Pauke Schots, who used video analysis to determine how flatworms affect coral feeding rates. The great work of these students was rewarded by co-authorships on my research papers. I wish you all the best for your future careers.

I would like to thank Professor Johan Verreth, chair at Aquaculture and Fisheries, for allowing me to obtain my Ph.D. degree as an "unofficial" student. Thank you for your support, especially during the last months of writing. I hope we can continue our successful collaboration in the future.

I had a lot of colleagues from WUR who were interested in my research, and some of them provided helpful comments on the experimental design and statistics of my experiments. Marc Verdegem and Paul van

Zwieten helped me along with my first statistical analyses, and Johan Schrama kept me and my students thinking critically about the limitations of our designs. Gab van Winkel, my carpool colleague, and I spent many hours talking about coral research during our trips to and from Wageningen. His ideas gave me a lot of inspiration during the conceiving of new experiments. A big thank you is reserved for the women at the AFI secretary, who have helped me solve many practical issues. Helene Willems, Linda Kaster, Vera Kindermans-Elbertsen, Laura Graus, Eugenia Halman and of course Gera den Dikken, thank you for your kind assistance. Ronald Booms has been of great help over the years, teaching me how to do lab work and ordering aquarium pumps and reagents for me well after closing hours. Menno ter Veld, who succeeded Sietze Leenstra as head of the experimental facility, has been very important during my research. He helped me solve many practical issues in the coral lab, providing me with a lot of things essential for experimentation, ranging from laboratory tables to technical material such as ion exchangers. These services were also kindly provided by our biotechnicians: Wian Nusselder, Sander Visser, Truus Wiegers-van der Wal and Aart Hutten. I have to include Sietze Leenstra, who retired during my second Ph.D. year. Sietze, thanks for all your help over the years and for sharing your wonderful stories with me. I would also like to thank Tino Leffering, Roel Bosma, Leon Heinsbroek, Ep Eding, Leo Nagelkerke, Karen van de Wolfshaar and Geertje Schlaman for the nice conversations we had during the coffee breaks. Miriam Schutter, although no longer a WUR colleague, has to be included here. Her work on coral physiology at AFI paved the way for my research. Together with our technical department, she developed the tools I used for my experiments, including the respiration and respirometric flow cells. This gave me a very smooth start.

Although I can hold a pipette and prepare buffers in the lab, I am not exactly a craftsman. A robust technical setup, however, is essential when conducting reliable, reproducible experiments. Fortunately, I had access to a great technical department known as the "Ontwikkelwerkplaats", with skilled

people who could build anything that we required. The services provided by Eric Karruppannan were essential to my studies. Together with his colleagues Evert Janssen, Hans Meier and Reinoud Hummelen, he designed and built the respiration and flow cells that were used for our work. In addition, Eric and Evert assisted me during other experiments, and supplied and built outboard motors, aluminium frames and power supplies. Without their aid, I would have produced few results.

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Finally, there is one special individual to thank. A person without whom I never would have made it this far. Ronald Osinga. We first met in 2006, when I was doing a Master's at Utrecht University. At that time, I was a passionate aquarium hobbyist, maintaining corals and reef fishes in my student dormitory. I had decided to pursue a doctorate in some field of coral research, but was struggling to find a suitable place to start. I stumbled across AFI's website, which contained references to Ronald's project at the time, CORALZOO. It was clear to me he was doing exciting things with corals, and our initial email contact revealed we had some common interests. As we had no funding for a Ph.D. project, I started working with Ronald in May 2007 as a technical research assistant, tasked with maintaining the experimental setups. I left AFI in January 2008 to pursue a Ph.D. at a different institution, but I soon realised

that I really wanted to work with corals. We applied for funding at different agencies, but were unsuccessful. Thanks to Ronald, I returned to AFI temporarily in 2008 to work on a protocol book for coral aquaculture. In 2010, new funding allowed Ronald to hire someone to assist him, and again he gave me the chance to work with corals professionally. Although I was again appointed as a research assistant, we were able to conduct a lot of experiments with the help of students, which finally resulted in an "unofficial" Ph.D. project and this dissertation. Ronald, your support, expertise and commitment to me were pivotal over the years. I am convinced I would not have pursued a scientific career without you. I will always consider you a dear colleague and friend.

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About the author

I was born on January 27th 1982 in Oosterhout (NB), The Netherlands. After attending high school (VWO, Monseigneur Frencken College, Oosterhout), I enrolled in the study Biology at Utrecht University in 2001, still a doctorate study at the time. In 2003, the international Bachelor/Master system was adopted by the university. After obtaining my Bachelor's degree I continued at the same university with the Master's programme entitled Cancer Genomics and Developmental Biology. I did my first thesis with the VHL research group of professor Emile Voest at the University Medical Centre Utrecht in 2005, where I worked on molecular pathways in renal cancer cells. Following that, I did my major thesis with the chair group Endocrinology of professor Dick van der Horst in 2006-2007, where I worked with Fernanda Almeida and Rüdiger Schultz on the sexual differentiation of cod larvae.

Although I was fascinated by the inner workings of organisms on a cellular and molecular level, I struggled to find a future career path. In 2005, I started keeping tropical marine invertebrates and fish in my student dorm, a hobby which absorbed me completely. In fact, I found it even more interesting than what I did or learnt at the university. In 2010, I finally got the opportunity to work on a Ph.D. project, with corals at the center stage.

At the same time, together with the company EcoDeco BV, I applied for funding at SenterNovem (now Agentschap NL) to establish a nursery for the sustainable aquaculture of corals. This attempt was successful, and at this moment, the first experimental coral nursery in The Netherlands is in operation.

Next to working with corals, I enjoy reading Stephen King novels and cycling. I am also an enthusiastic amateur photographer.

The author at an experimental coral nursery in The Netherlands.

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