The Effects of Ocean Acidification on Zooplankton: Using Natural CO$_2$ Seeps as Windows into the Future

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&

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# TABLE OF CONTENTS

**Prefix**

1. Acknowledgements
2. Thesis Abstract
3. Zusammenfassung

## Introduction

**CHAPTER 1: Introduction**

15. The Role of Carbon in the World’s Oceans and Modern Changes to the Oceanic Carbon Cycle

26. Ocean Acidification – A Synopsis

34. Zooplankton – Harbingers of Ocean Change

46. References

## Impacts of ocean acidification on zooplankton communities living in coral reefs

**CHAPTER 2: Ocean acidification reduces zooplankton that reside in tropical coral reefs**

65. Abstract

66. Introduction

68. Loss of reef-associated demersal zooplankton

71. No high-CO2 shifts in zooplankton communities

72. Reduced habitat complexity causes zooplankton loss

74. Other causes for abundance loss

75. Consequences for coral reef ecosystems

76. Methods

79. Acknowledgments

80. References

85. Note
A case study: Pontellid copepods

CHAPTER 3: Neustonic copepods (Labidocera spp.) discovered thriving as demersal zooplankton in coral reefs

Abstract
Introduction
Materials and Methods
Results
Discussion
Acknowledgements
References

CHAPTER 4: Pontellid copepods, Labidocera spp., affected by ocean acidification: A field study at natural CO₂ seeps

Abstract
Introduction
Methods
Results
Discussion
Acknowledgements
References

Ecosystem Impacts

CHAPTER 5: Reduced heterotrophy in the stony coral Galaxea fascicularis after life-long exposure to elevated carbon dioxide

Abstract
Introduction
Results
Discussion
Methods
Acknowledgements
References
Discussion

163  Chapter 6: Discussion
164  Challenged perceptions
166  CO₂ seeps as natural laboratories for zooplankton research
168  Ecological relevance
171  Conclusions
172  References

Appendices

179  Appendix I. Abbreviations
181  Appendix II. Supplemental Information for Chapter 2
185  Appendix III. Unpublished data on zooplankton community response to ocean acidification
197  Appendix IV. Supplemental Information for Chapter 5
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Professor”), for all your tireless helping hands. I mostly had help from the crew who are PNG locals. I knew from the beginning that I would need their help, and I didn’t want it to feel like I was just demanding help. Instead, I wanted it to feel like we were a team because I knew we would be working together a lot and spending lots of time in the wee hours of the night. I asked them to come up with a team name and they chose the name “Hamamas.” Hamamas is a term in pik-tok-son (local language), which means both ‘happy’ and ‘fun.’ From that point on we became the happy and fun team. And a team we truly were. Everyone had his or her role and we became like a well-oiled machine. It wasn’t the easiest and most comfortable team to join, because we always worked throughout the night when they would rather be sleeping, or midday and we’d miss the lunchtime meal. It didn’t matter to them. They were always so happy and helpful and never complained. And they kept me well entertained. Obedi is a village elder and is full of local legends. Most of them were about cannibalism. “Don’t worry”, Obedi would reassure me, “We don’t do that anymore. That was our grandfathers. The missionaries came and we don’t do that any more.” In between plankton tows when we had a few minutes to wait, we would tell stories. I would often revert to Disney stories because they didn’t know any of them, and in return I would listen to the stories about life in the villages along with local legends passed on from generation to generation by word of mouth, never having been written down before. We would tell a few lines, leave it at a point of suspense, complete a plankton tow, start the next one, and then continue with the next part of the story. Obedi taught me a lot about story telling. And if you know me, then you know I love a good story. I feel privileged to have met Obedi, the elder that so many people love and respect.

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And thank you to my family for loving me and being proud of all that I do.
Since the beginning of the Industrial Revolution, carbon dioxide (CO₂) has been emitted into the atmosphere at rates unprecedented to Earth’s history. Nearly 30% of the anthropogenic CO₂ in the atmosphere has been absorbed in surface waters of the ocean, pushing carbonate chemistry towards increased bicarbonate ions and hydrogen protons and decreased carbonate ions. Consequently, seawater pH has decreased from pre-Industrial Revolution levels of 8.2 to current levels of 8.1, and it is expected to continue to drop to 7.8 by the year 2100 if carbon emissions continue as predicted. The combination of these effects is referred to as ocean acidification. It is at the forefront of marine research as it poses a serious threat to several marine organisms and ecosystems.

Ocean acidification has the most notable direct effect on calcifying organisms with calcium carbonate skeletons and shells, because fewer carbonate ions in the water column result in reduced calcification. Coral reefs are especially vulnerable to ocean acidification since reefs are composed of complex carbonate structures. Coral reefs have a high biodiversity; thus, not only will the corals themselves be affected by ocean acidification, but so will many of the animals that dwell in them. The primary objective of this thesis was to examine the effects of ocean acidification on demersal zooplankton that reside in coral reefs.

Ocean acidification research on zooplankton has primarily been single-species experiments on calcifying species or generalist copepod species. Scaling-up to experiments examining ocean acidification effects on entire zooplankton communities is logistically difficult, thus the ability to predict community changes in zooplankton due to ocean acidification has been rather limited. However, a few locations around the world have submarine volcanic CO₂ seeps that can be used as natural laboratories to study ecosystem effects of ocean acidification. Two CO₂ seeps located in coral reefs in Papua New Guinea were used as windows into the future to examine the effects of ocean acidification on entire zooplankton communities while they live naturally in their
environment. Over three expeditions to two CO₂ seeps, nocturnal plankton were sampled with horizontal net tows and emergence traps. Additional experiments were also conducted, and collectively this work is summarized in chapters 2-5 as outlined below.

Chapter 2 reports on the observed changes in zooplankton abundance and community composition between control and high-CO₂ sites. Consistent results between seep sites and expeditions showed that zooplankton abundances were reduced three-fold under high-CO₂ conditions. The abundance loss was partially attributed to habitat change within the coral reef, from more structurally complex corals in the control sites to a replacement of massive bouldering corals in the high-CO₂ sites. All zooplankton taxa were reduced under high-CO₂ conditions but to different degrees, suggesting that each taxon has different sensitivities to ocean acidification.

Since each taxonomic group within the zooplankton communities was reduced to varying levels under ocean acidification, a sensitive copepod genus was investigated in more detail. *Labidocera* spp. are pontellid copepods that are generally considered surface-dwellers and are not known to inhabit coral reefs. Therefore, as a preface to the ocean acidification study, the new discovery of these copepods living in coral reefs is first described (Chapter 3). Not only were they found to be residential to the reef, but *Labidocera* spp. preferred to reside in coral rubble, macro algae, and turf algae. *Labidocera* spp. were one of the most sensitive copepods to high-CO₂ conditions and were reduced by nearly 70%, prompting a more detailed investigation about the effect of ocean acidification on their physiology and habitat preference (Chapter 4). Physiological parameters, e.g. size, feeding, and oocyte development, were unaffected by ocean acidification. Unlike the zooplankton community as a whole, the main cause for the abundance loss of *Labidocera* spp. was not a shift in the habitat because their preferred substrata were of equal percent coverage across high-CO₂ and control sites. Instead, *Labidocera* spp. were no longer associated with any substrata type. Multiple direct and indirect effects of ocean acidification will act on each zooplankton taxa separately, and their collective response will contribute to the community response.

The effects of ocean acidification on zooplankton communities were then scaled up to potential impacts on entire ecosystems. Zooplankton are the
primary food source for corals, fish, and other zooplanktivores. The impacts of ocean acidification on zooplankton communities will have cascade effects on the food chain via the pathway of zooplanktivorous organisms. A case study on the stony coral *Galaxea fascicularis* explored the effects of ocean acidification on the ability of corals, which had lived their entire lives under high-CO$_2$ conditions, to feed on zooplankton (Chapter 5). Under anthropogenic changes, whether it is from bleaching, high turbidity, or ocean acidification, some corals consume more zooplankton as a mechanism to counterbalance stress. Contrary to expectation, this study showed that when given equal quantities of food particles these corals consumed less zooplankton under ocean acidification. Corals rely on heterotrophy for essential nutrients, like nitrogen and phosphorus, which they cannot otherwise obtain from autotrophy and their symbiotic zooxanthellae. In conclusion, my thesis shows that not only is there less zooplankton available to consume, but the existing zooplankton is consumed with lower capture rates under high CO$_2$ conditions.

Coral reefs in future oceans will likely have reduced zooplankton abundances as an indirect effect of ocean acidification, partially caused by a change in habitat from branching corals to more massive bouldering corals. Each zooplankton taxon was found to have different sensitivities to ocean acidification, though collectively, as entire zooplankton community abundances were reduced. Additionally, less zooplankton will be available to zooplanktivores, but its fatty acid content and nutritional value is expected to be similar to current food. Together this is expected to negatively impact the entire coral reef ecosystem, with some coral species unable to consume zooplankton at normal rates.
ZUSAMMENFASSUNG DER DOKTORARBEIT


Zusätzlich wurden auch Experimente durchgeführt. Nachfolgend werden die Kapitel 2-5 dieser Arbeit skizziert.


Die Effekte der Ozeanversauerung auf die Zooplanktongemeinschaften wurden dann auf die möglichen Auswirkungen auf ganze Ökosysteme übertragen. Zooplanktonorganismen sind die Hauptnahrungsquelle für Korallen, Fische und andere Zooplanktivore. Die Auswirkungen der Ozeanversauerung auf die

Im zukünftigen Ozean werden die Korallenriffe vermutlich geringere Zooplanktonabundanzen als indirekte Folge der Ozeanversauerung haben, teilweise verursacht durch die Veränderungen der Habitate von verzweigten zu massiven felsartigen Korallen. Es hat sich herausgestellt, dass jedes Zooplankontaxon unterschiedlich empfindlich auf die Versauerung reagiert, auch wenn generell die gesamte Zooplanktonabundanz reduziert ist. Weiterhin wird den Zooplanktivoren weniger Zooplankton zur Verfügung stehen, wohingegen die Fettsäurezusammensetzung und die Nahrungsqualität erhalten bleiben. Generell werden negative Auswirkungen auf das gesamte Korallenökosystem erwartet; einige Korallenarten werden die Fähigkeit verlieren Zooplankton in genügender Menge zu konsumieren.
Introduction
CHAPTER 1

Introduction

The Role of Carbon in the World’s Oceans and Modern Changes to the Oceanic Carbon Cycle

Carbon, a vital element to all life, is globally ubiquitous and present in a diverse array of organic and inorganic molecular configurations. It is biochemically recycled and constantly exchanged between the biosphere, geosphere, hydrosphere, and atmosphere of Earth. Since all the reservoirs are interconnected within the global carbon cycle, any fluctuations in one reservoir reverberate throughout the others. This is especially evident in recent times when the increase in atmospheric carbon dioxide (CO₂) and other greenhouse gases, along with the subsequent elevation in global temperatures, gets partially buffered by the oceans capacity to retain heat and uptake CO₂ (ref 1). The ocean is one of the larger carbon reservoirs with nearly fifty times more carbon than either the atmospheric or terrestrial reservoirs²⁻⁴. The ability for the ocean to uptake and disseminate carbon and heat through an interplay of physical, chemical, and biological processes ultimately makes the ocean integral to the planet’s climate system.

In order for the ocean to help regulate climate and ameliorate the effect of greenhouse gases on global temperature increases, carbon must first be absorbed and then sequestered through a series of steps that are cumulatively referred to as the oceanic carbon cycle. The processes that explain the oceanic carbon cycle will first be described as it is untouched by the effects of anthropogenic carbon dioxide in the atmosphere. Human-induced alterations to the oceanic carbon cycle will later be identified.

The Oceanic Carbon Cycle

Carbon can enter the oceans through several possible ways: gas exchange across the air-sea interface, river input, aeolian deposition of terrestrial particulate organic matter, and hydrothermal emissions. In particular, the ocean and atmosphere are inextricably linked through the reciprocation of CO₂, an atmospheric
constituent, across the air-sea interface. Although heat and momentum are also pertinent factors exchanged across the air-sea boundary, it's the uptake of carbon dioxide and the concomitant reactions that facilitate the sequestration of carbon, engendering an oceanic carbon sink\(^5\). Once assimilated into the ocean, carbon availability and water circulation partially regulate the biological productivity and biochemistry of the oceans at a global scale\(^6\). A small aliquot of the aqueous carbon dioxide that dissolves into the ocean remains as a dissolved gas, whereas most of it predominantly undergoes chemical and biological transmutations. The mechanisms by which the oceans absorb and assimilate carbon are:

**CO\(_2\) fluxes across the air-sea interface**

Gas exchange between the oceanic and atmospheric mediums is driven by concentration gradients trying to reach equilibrium\(^7\). Current models suggest that equilibrium between the ocean and atmosphere can take nearly a year to be reached\(^8\). Estimates of ocean-atmosphere gas fluxes are dependent on knowing the gas concentrations and gas transfer velocities. Transfer velocities commensurate with wind speed and to a smaller extent gas diffusivity and surface viscosity of the ocean\(^9,10\). A hydrodynamic environment created by waves (wind-driven and capillary), turbulence, wave-breaking, and bubble generation also enhance the rate of gaseous transfer across the air-sea interface\(^11,12\). Variations in CO\(_2\) flux vary regionally, seasonally, according to weather, and are controlled by El Nino events\(^13\). Although CO\(_2\) can be absorbed and outgassed from the ocean, research in recent years has focused on the flux of CO\(_2\) into the ocean and the associated changes to the environment.

**Solubility pump**

The solubility pump refers to the thermodynamic parameters (e.g. physical properties and water circulation) that govern the solubility of carbon dioxide and its uptake into the ocean. Upwelling, down-welling, and outgassing all contribute to the solubility pump. The efficiency of the solubility pump is controlled by: 1.) the rate at which surface waters interact with water from below the thermocline as per the physical movement of water; 2.) the temperature (lower temperatures enhance
solubility), and 3.) the chemical reactions that CO₂ undergoes in seawater⁶,¹⁴,¹⁵. Dissolved inorganic carbon (DIC) sinks to deep waters and gets transported along large-scale ocean circulation processes, referred to as thermohaline circulation. CO₂-rich deep waters are carried over long distances and continuously accumulate more DIC. Estimates indicate that the solubility pump contributes almost 20% to the vertical gradient of DIC while the other 80% is due to other biological pumps⁵. Carbon dioxide is temporarily sequestered into ocean currents on a time scale of 1,000 years until upwelling in lower latitudes drives water from the deep-sea up to the surface¹⁶,¹⁷. Upwelling waters warm in the euphotic zone reducing the solubility of carbon dioxide and causing a fraction of CO₂ to be outgassed into the atmosphere.

Ocean carbonate system

There are several possible chemical reactions that can take place after carbon dioxide dissolves into water. Inevitably, most of the carbon dioxide will react to form a variety of carbon compounds; for example, dissolved inorganic carbon (DIC) is present in seawater as the following species: carbon dioxide (CO₂), carbonic acid (H₂CO₃), carbonate (CO₃²⁻), and bicarbonate (HCO₃⁻). For surface seawater where the pH is approximately 8.1, nearly 90% of the inorganic carbon is bicarbonate, 9% is carbonate ion, and only 1% remain as dissolved CO₂ (ref 18). A balance of the ionic and non-ionic species is formed through a series of chemical reactions:

\[
CO₂ + H₂O \leftrightarrow H₂CO₃ \quad (1)
\]

\[
H₂CO₃ \leftrightarrow H^+ + HCO₃^- \quad (2)
\]

\[
H^+ + CO₃²⁻ \leftrightarrow HCO₃^- \quad (3)
\]

Essentially, carbon dioxide dissolves in seawater and reacts with water to form carbonic acid. Carbonic acid dissociates into hydrogen ions and bicarbonate. Seawater is then naturally saturated with carbonate ions, which neutralizes the hydrogen ions to form additional bicarbonate. The net reaction is:
\[
\text{CO}_2 + \text{H}_2\text{O} + \text{CO}_3^{2-} \leftrightarrow 2\text{HCO}_3^-
\] (4)

These equilibrium reactions are reversible and control the speciation of inorganic carbon. The balance of these carbon species is dependent on several factors including the pH, alkalinity of seawater, air-sea flux of CO₂, dissolution or precipitation of CaCO₃, and other biological processes like photosynthesis and respiration

**Biological pump**

The oceanic carbon pump includes two biological pumps characterized by the transport of carbon from the euphotic zone to great depths. They are the **soft tissue pump**, also known as the “organic carbon pump”, and the **carbonate pump**. Both biological pumps are due to the actions of organisms in surface waters interacting with carbon dioxide where organic or calcium carbonate particles are eventually transported to deep water through gravitational settling or active biotransport

Carbon is returned to its dissolved, inorganic forms in deep water layers for both processes, but the way in which each biological pump interacts with carbon dioxide is in contradiction to each other.

The soft tissue pump involves the fixation of inorganic carbon by primary productivity in the mixed layer, which becomes incorporated into marine organisms as organic matter and eventually vertically transported downward as detrital particulates. Once soft-tissue particles and detritus are below the thermocline, carbon can no longer interact with the atmosphere on a time scale of at least a few thousand years

In this process the organic carbon pump removes pCO₂ from surface waters. At great depths some of the particulate organic carbon remineralizes releasing carbon where it accumulates in deep water layers and ultimately leads to a net draw down of CO₂ from the atmosphere into the ocean

Nearly 80% of the particulate organic carbon that reaches the intermediate and deep waters gets quickly oxidized by microbial activity and organic metabolism from bathypelagic fauna

Oxygen is consumed during the remineralization process and the carbon and nutrients fixed in the organic matter are released into their dissolved inorganic form at depth. Organic matter which is not remineralized at depth may eventually
sink to the seafloor. Consequently, only a small fraction (1-3%) of the POC exported from the surface reaches the sediment where it is also remineralized and utilized by benthic organisms or possibly buried\textsuperscript{25,26}. Only when the carbon is buried and becomes part of the sediment can the carbon be removed from the oceanic carbon reservoir for much longer geological time scales.

Contrary to the organic carbon pump, which consumes carbon dioxide in surface waters, the carbonate pump has the opposite effect. During the production and exportation of calcium carbonate, a net release of CO\textsubscript{2} occurs through the calcification process:

\[
2\text{HCO}_3^- + \text{Ca}^{2+} \rightleftharpoons \text{CO}_2 + \text{CaCO}_3 + \text{H}_2\text{O} \quad (5)
\]

Calcification is concentrated in the photic zone where CaCO\textsubscript{3} formation is used for the structure of many marine invertebrates. Calcium carbonate binds dissolved inorganic carbon and lowers seawater alkalinity, changing the equilibrium between the different forms of dissolved inorganic carbon\textsuperscript{6}. The drawdown and vertical flux of CaCO\textsubscript{3}-bound inorganic carbon and alkalinity drives the carbonate pump and consequently causes an increase in atmospheric pCO\textsubscript{2} (ref 27). The solubility of CaCO\textsubscript{3} is temperature and pressure dependent with pressure having a greater influence. Solubility increases with decreasing temperature and increasing pressure, thus CaCO\textsubscript{3} dissolves with depth and CO\textsubscript{2} is consumed. When a water mass is supersaturated with CaCO\textsubscript{3} then it spontaneously precipitates; contrarily, when a water mass is undersaturated with respect to CaCO\textsubscript{3} then any available CaCO\textsubscript{3} will dissolve. The saturation state of CaCO\textsubscript{3} is partially dependent on CaCO\textsubscript{3} alkalinity and the pH of seawater. Saturation measurements of CaCO\textsubscript{3} are useful in predicting where carbon, particularly in the form of calcium carbonate, might be stored in sediments. With respect to the carbonate pump, carbon is usually stored in sediments as calcareous ooze above the carbonate compensation depth (i.e. depth at which the rate of carbonate accumulation equals the rate of carbonate dissolution).

Although the majority of carbon is assimilated into soft tissue, it’s the carbon found in hard-tissue which comprises most of the carbon buried in sediments because the hard skeletons resist disintegration and biomineralization and are more likely to be preserved\textsuperscript{28}. Marine plankton calcifiers, primarily coccolithophores and
foraminiferans, constitute the hard-tissue carbon which composes nearly 41% of the carbon buried in sediment and 84% of the carbon in the continental crust. Changes in the carbonate pump caused by ocean acidification could potentially have a dramatic effect on calcifying organisms and the sequestration of carbon into sediments. Modifications to the carbonate pump and other oceanic processes have already been identified as a direct result of an aberrant release of carbon into the atmosphere through the burning of fossil fuels.

**Modern Changes to the Oceanic Carbon Cycle**

Since the onset of the Industrial Revolution the Earth’s carbon cycle has been severely perturbed by an accumulation of carbon dioxide in the atmosphere. Seasonal oscillations and latitudinal variations in atmospheric CO₂ concentrations are intrinsic; however, a long-term increase in anthropogenic CO₂ over the past 250 years is clear evidence as nearly 40% more CO₂ has accumulated in the atmospheric reservoir. Preindustrial concentrations measured around 280 parts per million volume (ppmv) while the first decade of the new millennium has seen levels around 384 ppmv. Fossil fuel burning, deforestation, and cement production synergistically contribute to the current atmospheric CO₂ concentrations which Earth has not experienced for at least 800,000 years. Models suggest that even if emissions were to cease, any changes to the climate would be largely irreversible and would require a recovery period of at least 1,000 years. Cumulative human CO₂ emissions spanning the industrial era equate to nearly 560 billion tons which is not only high but the rapid rise is nearly 30 times faster than rates in the geologic past. Increased carbon concentration in the atmosphere, together with the increased rate of change in carbon, will resonate throughout the different carbon reservoirs and potentially impact many biological, chemical, and physical processes.

Human-induced alterations to the environment are not benign. Of the many ramifications, an enhanced natural greenhouse effect may increase global temperatures to a point where the climate stability of Earth is jeopardized. Unprecedented temperatures exceeding the bounds of historical variability are expected to be reached within seven years in tropical regions, exposing the vulnerability of global biodiversity, and within forty years for the rest of the globe. The greatest changes in absolute temperature will occur in higher latitudes. Climate
change would be even more extreme and CO₂ concentrations would be even higher at approximately 450 ppmv, if it were not for the fact that the oceans absorb nearly one-third of anthropogenic CO₂ concentrations from the atmosphere\textsuperscript{36,37}. The oceanic uptake capacity compared to its actual uptake is even higher at 85% compared to the present \textasciitilde 27% uptake\textsuperscript{37}. Oceanic uptake does in fact ameliorate climate change, but the insidious influx of carbon dioxide into surface waters occurs at the expense of the health of the world’s oceans. The deleterious effects of anthropogenic carbon dioxide on the oceanic carbon cycle will now be explored.

*CO₂ fluxes across the air-sea interface*

Increased carbon loading into the atmosphere drives the concentration gradient at a faster rate into the ocean with a causal effect of approximately 65 times more carbon contained in contemporary oceans compared to preindustrial oceans\textsuperscript{4}. The oceans as a whole are presently acting as a net sink. This is different form preindustrial times when oceans were thought to act as a net source\textsuperscript{6}. The net flux from ocean to atmosphere before the Industrial Revolution was nearly 0.5 Pg C per year, whereas postindustrial net CO₂ flux in the reverse direction is up to 2 Pg C per year\textsuperscript{38}. It should be noted that although the global ocean acts as a net sink for CO₂, there are regional locations of the ocean’s surface (e.g. equatorial waters) that act as a net source of pCO₂ to the atmosphere. Likewise, some regional areas have a larger CO₂ sink (e.g. North Atlantic) compared to other areas. Although it is not distributed evenly throughout the world’s oceans, the estimate for a global oceanic anthropogenic CO₂ sink is 118\textpm 19 Pg C\textsuperscript{36}. Since the northern hemisphere has the largest land mass with the largest human population and emission rates, the North Atlantic stores nearly 23% of the global oceanic anthropogenic CO₂ despite covering only 15% of the global ocean area\textsuperscript{36}. While a strong concentration gradient drives anthropogenic CO₂ into oceanic surface waters, the gaseous disequilibrium is further exasperated by continuous movement of water from geostrophic currents. As water uptakes CO₂, currents carry it away making room for more CO₂ to be drawn into the new water. The downward draw of pCO₂ is also partially due to biological uptake supported by high nutrient concentrations, as well as enhanced solubility from cooling and high speed winds\textsuperscript{6}. Elevated level of CO₂ fluxed into the ocean undergoes chemical reactions and significantly alters the environment.
Solubility pump

Since gases are more soluble at lower temperatures, carbon dioxide is nearly twice as soluble in cold surface waters found in polar latitudes compared to the tropics. Geographic regions where enhanced CO₂ uptake has occurred include polar regions where water masses are formed. The cold CO₂-rich water sinks below the thermocline and becomes incorporated as part of the North Atlantic Deep Water (NADW), Antarctic Bottom Water (AABW), and Antarctic Intermediate Water (AAIW) water masses. At present, approximately 30% of the anthropogenic CO₂ absorbed into the oceans remains contained within the upper 200 m and nearly 50% remains above 400 m depth. The deepest penetration of anthropogenic CO₂ has been into water masses between 3,000 – 5,000 meters deep. As time elapses, scientists expect the anthropogenic CO₂ to spread laterally as it continues to be carried along by subthermocline water masses. Eventually, the CO₂-enriched water from deep water masses upwells. Upwelling water normally has a lower pH compared to the surrounding sea water, but recent times with elevated CO₂ levels have experienced even lower pH conditions at upwelling regions that extend over a larger area.

An increased flux of atmospheric carbon dioxide into the ocean will enrich waters with CO₂ as they slowly circulate throughout all the ocean basins, albeit there are natural feedback mechanisms that limit the amount of carbon that can solubilize into the ocean. Escalated global temperatures are expected to reduce the efficiency of the solubility pump, ergo oceanic CO₂ uptake will decline and a negative-feedback to the oceanic carbon cycle will be established. Long-term effects may decelerate the entire oceans' solubility pump. Slower thermohaline circulation and increased stratification would further lessen the vertical transport of carbon and nutrients to deeper waters. This could have serious consequences for the uptake capacity of the ocean and reduce the mitigating abilities of the ocean to reduce atmospheric CO₂ concentrations.

Ocean carbonate system

Once carbon dioxide has been absorbed into the ocean, the same chemical reactions described through Equations 1-4 occur. However, with the increase in anthropogenic carbon dioxide the equilibrium equation (specifically Eq. 4) is driven to
produce more bicarbonate ions. Subsequently, carbon dioxide consumes the surface water carbonate level which has been reduced by nearly 10% since industrialization^{44}. Reduced carbonate levels in surface waters has an interlinked effect on the calcification rates for calcifying organisms. Less available carbonate means fewer carbonate molecules are available for organisms that incorporate it into their physical structure. As carbonate levels diminish in surface waters, the concentration of bicarbonate ions increases. Bicarbonate dissociates along with carbonic acid and releases hydrogen ions, some of which react with available carbonate ions pushing carbon back into the bicarbonate state while other H^{+} ions accumulate. Ultimately, a net increase in H^{+} ions reduces the pH of the water. The overall contribution of elevated atmospheric CO_{2} on the ocean carbonate system is a reduction in carbonate and pH levels and an increased concentration of aqueous CO_{2} and bicarbonate^{6,18,19}. The process of the ocean becoming less acidic with the declining pH and the changes in carbonate chemistry is called ocean acidification. It has the potential to affect oceans worldwide and through several mechanisms, altering the functionality of many ocean system processes including the biological pump and the overall oceanic carbon cycle.

*Biological pump*

The potential changes in the ocean carbonate system and solubility of carbon dioxide are reasonably well known and these alterations will likely affect the biological pump. The extent to which the biological pump will be affected is less understood, however, since cause-and-effect reactions are predictable but the magnification of change is harder to quantify. For example, biological responses to increased CO_{2} can occur directly through decreasing seawater pH and changes in carbonation or indirectly through changes in circulation and mixing regimes caused by warming^{45}, but whether the global biological pump will weaken or strengthen in response to these changes in the carbon cycle is yet to be determined. This is partially due to the difficulty in obtaining quantitative measurements on global net flux rates of the biological pump and predictive models assuming steady-state conditions although the environment is actually changing quite rapidly^{46}. Regional investigations elucidate processes that may be used to predict how the global biological pump will be affected in the future.
In order for the biological pump to efficiently sequester carbon, a strong flux out of the surface waters and into the deep sea must be established. The carbon sequestration flux is dependent on the input rates of allochtonous nutrients, export flux at the bottom of the mixed layer, deviation from Redfield stoichiometry, and flux attenuation in the upper 1000 m of the water column. Stratification in the water column induced by warming ocean temperatures will likely affect several of these factors and reduce the carbon efflux from surface waters. Stronger water column stratification and a shoaling thermocline may create a mixing barrier which would reduce nutrient delivery into the euphotic zone from upwelling and therefore limit primary production while simultaneously preventing particulate matter from vertically transporting downward. Fewer minerals exported out of surface waters could potentially weaken the biological pump. Furthermore, deoxygenation of particulate organic carbon would occur at shallower depths and oxygen minimum zones would expand over large areas. Thermal stratification could potentially reduce the flux of the entire biological pump if vertical mixing and convective overturning were decreased; however, since many underlying processes are interlinked and not fully recognized, the magnitude of any changing flux rates on the biological pump can scarcely be evaluated.

Numerical estimates for global carbon flux changes in the biological pump are currently nonexistent. Nevertheless, sensitivities in the components of the biological pump (soft tissue pump and carbonate pump) emanating from anthropogenic carbon dioxide have been documented, especially for the carbonate pump. The soft tissue pump carbon pump is still able to assimilate carbon into soft tissue under increased CO₂ conditions, sometimes consuming up to 39% more carbon even when nutrient levels are constant causing the stoichiometry of carbon to nitrogen drawdown to increase. While increased CO₂ enhances primary productivity, it concurrently reduces the production of calcium carbonate, which has adverse effects for calcifying organisms. Ocean acidification lowers the pH levels and reduces the global precipitation of calcite and aragonite, ultimately reducing calcification. Thus, less CO₂ can be released during the calcification process, a process termed the “calcification feedback”. Adverse effects against calcifying organisms may result in a loss of competitive fitness and biodiversity and is relevant to the carbonate pump in terms of calcifying plankton (e.g. coccolithophores, foraminifera, pteropods) which make up most of the calcareous ooze on the seafloor. A possible change in the
plankton community could also endue a further slowdown of the flux of particulate
matter to depth. If plankton species primarily composed of CaCO₃ are diminished,
then their role as ballast for particle aggregates would lessen and deep sea transport
of particulate organic matter would decline⁵⁴,⁵⁵. The reduction in calcification and
possible decline in the strength of the carbonate pump would lower the drawdown of
alkalinity at the surface and thereby increase the oceanic uptake capacity of
atmospheric CO₂, although this feedback is relatively small⁴⁵,⁵⁰. Additional
mechanisms that describe biological responses to CO₂-induced changes are still
being studied. The complexity and plasticity of biotic responses and interactions with
a changing ocean are difficult to elucidate, although much effort across
interdisciplinary research is making progress.
Ocean Acidification – A Synopsis

Oceanic temperature increases, expanding hypoxic zones, and ocean acidification are a concatenation of events catalyzed by anthropogenic loading of CO₂ in the atmosphere. All three phenomena synergistically impact the marine ecosystem health on a global scale⁰,¹⁸,⁵⁶,⁵⁷, with ocean acidification (OA) potentially being the greatest threat of them all. Unlike climate change predictions, which are endowed with many uncertainties, the physical and chemical processes of OA are undeniable and the measured changes in ocean chemistry are evident through documented global decreases in pH levels and the accompanying changes in the carbonate chemistry (e.g. decreased carbonate ion and increased bicarbonate ion concentrations). Recorded measurements indicate that the average ocean surface pH has fallen by approximately 0.1 units from 8.21 to 8.10 (ref 58). pH is expected to decrease a further 0.3 – 0.4 pH units if emission rates continue unabated, a level not experienced in over 300 million years⁵²,⁵⁹. Global environmental perturbations, including ocean acidification, exist within the geological record but no past event mimics the human induced disruption in carbonate chemistry that the ocean is currently experiencing⁶⁰.

Never before has the pH changed in such a short geological time scale. Currently the world’s oceans have undergone a 30% increase in acidity since pre-industrial times, but with the expected drop in pH from 8.2 to 7.8 by the end of the century the oceans would then increase in acidity by 150% (ref 8). The unprecedented rapid decrease in pH may make it difficult for some organisms to adapt to a changing environment. Compounded by diminishing resources needed for body building (i.e. reduced carbonate ions needed to make calcium carbonate) and decreased aragonite and calcite saturation states (i.e. causing dissolution in some cases), calcifying organisms will especially find it difficult to adapt to a rapidly changing environment⁶¹,⁶². Modern-day marine organisms, both calcifiers and non-calcifiers alike, have evolved in a chemo-static environment; thus any change to the environment could be potentially detrimental for marine life if they are not able to acclimatize or adapt.

Historical oceans have undergone environmental changes that have altered entire ecosystems as a result of certain organisms being incapable of coping with
new environmental conditions. Massive carbon influxes into the ocean at the end of
the Paleocene led to mass extinction events in the deep sea and coral reef
ecosystems. A future mass extinction event could be an unfortunate reality for
our oceans if warming and ocean acidification do not wane. Even if a mass
extinction event does not materialize, alterations in entire ecosystems are still likely
to occur as the population dynamics of various taxonomic groups respond to
physiological challenges that cascade to the ecosystem level. Ecosystems that are
most susceptible to ocean acidification include coral reefs (shallow and deep water
reef systems), benthic and planktonic communities, and areas of upwelling.
Community shifts within ecosystems could potentially alter the biological pump and
sequestration of carbon. As low pH waters penetrate surface waters and eventually
circulate throughout the world’s ocean basins, ocean acidification could have wide-
ranging affects on the oceanic carbon cycle. In order to predict how ocean
acidification may affect ecosystems and large-scale processes, it is imperative to
decipher the underlying mechanisms to which individual organisms respond
physiologically to OA. This section will review some of the direct and indirect effects
of ocean acidification on marine organisms starting with minuscule cellular and
molecular processes and then discuss possible regime shifts for entire ecosystems.

Physiological effects of ocean acidification on marine organisms

Marine organisms may be sensitive to changes in CO2 at different levels
ranging from cellular and molecular changes to whole organism functioning, thus it
is important to understand how molecular and cellular mechanisms are altered by
CO2 in order to predict how the whole organisms will be affected. Physiological
mechanisms that are influenced by changing CO2 levels include calcification, acid-
base regulations, metabolism, respiration, and sexual reproduction. Since different
taxonomic groups have varying physiological strategies, each group will need to be
studied separately to assess possible effects on entire ecosystems.

Calcification is the most obvious and studied physiological mechanism that is
affected by ocean acidification. Marine organisms that calcify are most notably
corals, crustaceans, echinoderms, bivalves, and particular plankton groups like
coccolithophores and foraminifera. Changes in calcification are species-specific and
responses range from reduced, increased, and static calcification rates. For many
marine calcifiers, calcification is progressively reduced under increasing CO₂ conditions for some species of corals, coralline algae, shellfish, and calcifying plankton. Other organisms like the brittlestar *Amphiura filiformis* are able to compensate for increased seawater acidity by increasing their calcification and metabolic rates but at a cost of losing muscle mass, making it unlikely that such counteractive processes will be sustainable for the long term. While certain taxonomic groups decrease their calcification rates and others increase, some studies reveal that different species within the same taxonomic group may respond variably to increased carbon dioxide levels in the water. The calcifying phytoplankton coccolithophores are a prime example. Laboratory experiments show that *Gephyrocapsa oceanica* decrease in calcification, *Coccolithus pelagicus* have a negligible reaction to CO₂ (ref 76), *Calidiscus leptopus* experience increased calcification rates followed by a decrease, and results for *Emiliania huxleyi* conflict with some studies showing a decrease while others show an increase in calcification. Suffice it to say, the degree to which future acidified oceans will alter calcification remains unclear and is highly species specific.

Calcification rates are tightly coupled with temperature and saturation states of calcium carbonate. Since calcification rarely occurs at a surface that is directly exposed to seawater, the physicochemistry of the water usually has an indirect affect on calcification through ion transport of calcium and other protons across external barriers of the organism; therefore, carbonate levels and calcium carbonate saturation are only proxies of calcification and not direct drivers. Many taxa, especially corals, experience a reduction in calcification rates as the saturation state of CaCO₃ is lowered. This is not always the case. Some organisms such as the intertidal snail *Nucella lamellose* maintain calcification rates under elevated CO₂ conditions; however, decreases in the saturation state cause dissolution rates to increase. In other words, shell dissolution may be affected by elevated CO₂ levels more than shell deposition for some marine taxa. When waters become undersaturated with respect to aragonite or calcite, organisms which use those minerals for biomineralization will start to dissolve. Dissolution has already been observed for certain organisms like benthic macro-invertebrates (bivalves, gastropod limpets, brachiopods) and planktonic pteropods and foraminifera. Organisms that are geographically most vulnerable to future dissolution include those living in low latitude regions where ocean acidification is likely to have a strong impact on
saturation states since cold water becomes undersaturated more quickly due to solubility of calcium carbonate increasing in low temperatures.

Location within the ocean is not the only parameter optimizing the possibility of dissolution. Stage of life is another. Larval stages and growth development are especially assailable to the inimical effects of increased CO$_2$ levels. Shell thickness becomes tenuous and the shape malformed for many bivalve larvae exposed to elevated CO$_2$. Possibly even more ruinous is the dramatic decline in size, integrity, and connectedness of the hinge$^{83}$. Bivalve hinges facilitate opening and closing needed for the intake of food and excretion of waste$^{84}$; therefore, malformed hinges hinder the ability for bivalves to filter water for suspended particulate matter. Similar larvae malformations for echinoderms and coral polyps have been observed$^{85}$. At least for certain marine groups, like brittlestars and echinoderms, such a disruption in the skeletogenesis of these marine organisms may reduce their fitness and survivorship$^{85,86}$. For the few studies that have examined coral larvae survival in acidified water, they seem to be able to survive short-term exposures to low pH water$^{87,88}$. Further research exploring long-term effects of OA on larvae will be required for many members of the marine realm in order to understand how specific species are affected by OA at the larval stage.

Larvae development is not the only ontogenetic stage vulnerable to ocean acidification. Before larval growth can even occur, first sexual reproduction must take place and it, too, may be subjugate to unpropitious circumstances caused by OA. Most marine invertebrate life histories include external fertilization and a free-living larval phase that helps to control the distribution and population dynamics of the species. Fertilization is expected to be impaired by CO$_2$ since hypercapnia narcotizes and reduces sperm motility$^{89}$. The endangered coral species, _Acropora palmata_, had reduced fertilization success rates for sperm exposed to different levels of CO$_2$ (ref 90). Lower sperm concentrations underwent even greater reductions in fertilization. Reduced fertilization efficiency has also been observed for sea urchins where the ability of their eggs to block polyspermy, a factor known to inhibit embryo development, reduces under high CO$_2$ conditions$^{91}$. On the contrary, other results suggest that fertilization for some species of urchins and corals may be resistant to acidified water$^{92}$. Reiterating a common response for different physiological processes affected by OA, increased CO$_2$ effects on fertilization are species-specific. Furthermore, the effects of OA on fertilization are not necessarily confined to only
affecting calcifiers even though attention to them is preeminent within current literature.

Regardless of whether or not a marine organism undergoes calcification, animals contain extracellular body fluids that may potentially reduce in pH. Invertebrates contain blood while crustaceans contain hemolymph. Hypercapnia in either fluid has been linked to metabolic depression\textsuperscript{93}, reduced rates of protein synthesis\textsuperscript{94}, reduced rates of tissue acid-base regulation\textsuperscript{95}, reduced behaviors associated with enhanced levels of adenosine in nervous tissue\textsuperscript{96}, and short-term extracellular acidosis\textsuperscript{97}. Extracellular pH regulation is also important for maintaining an oxygen supply to tissues. Increasing hydrogen protons decreases the affinity of the respiratory pigment, reducing oxygen delivery to tissues\textsuperscript{98}. Hypercapnia could have far-reaching consequences on the overall health of marine organisms, and the extent to which all animals will be affected by extracellular acidosis is largely unknown.

Many marine calcifiers like crabs are able to compensate for the reduction in pH by accumulating bicarbonate in intracellular and intercellular compartments\textsuperscript{99,100}. At least for some crab species, a bicarbonate threshold exists in which the hemolymph is only able to buffer the decrease in pH up to a certain point\textsuperscript{100,101}, and after that level bicarbonate production might be metabolically expensive or might compromise other processes like ion-regulation\textsuperscript{98}. Many crustaceans are able to buffer against pH changes in their extracellular fluids while other marine organisms are not. Echinoderms and bivalves are poor ion-regulators and therefore have a limited ability to buffer against hypercapnia\textsuperscript{102,103}. For relatively inactive species that have low buffering capacities and low circulating protein levels, characteristics of many species living in cold and low energy environments, they are much more vulnerable to negative physiological effects from ocean acidification\textsuperscript{98}. Hence, these ecosystems are likely to suffer first from OA on a large scale. Understanding the physiological changes that result from OA will help predict how future marine ecosystems may exist.

**Ecosystem responses to ocean acidification**

Based on the physical and chemical principals governing ocean acidification dissemination, scientists are able to predict where OA may have the greatest impact.
Combined with a comprehensive understanding of the biological responses to OA, projecting future changes to marine ecosystems is attainable. Understanding how OA may affect saturation horizons of aragonite and calcite will provide insight into where calcifying organisms will first be affected by the inception of OA. Aragonite is at least 50% more soluble than calcite\textsuperscript{104}, so organisms which use aragonite (e.g. corals, pteropods) for CaCO\textsubscript{3} formation will be affected first by decreasing saturation states. Shoaling of the aragonite and calcite saturation horizons are already occurring globally and undersaturation is evident in the North Pacific, northern Indian Ocean, and southeastern Atlantic Ocean\textsuperscript{44,105–107}. Not only are the saturation horizons becoming up to 200 m shallower in some areas, but the undersaturated areas are expanding in size since the onset of industrialization\textsuperscript{44}.

Certain hot-spots for reduced saturations states are in older and colder water masses. Older waters of the Pacific Ocean have accumulated higher levels of CO\textsubscript{2} as a result of respiration and thermohaline circulation of deep and intermediate water masses. OA is also likely to have a strong impact on saturation states in low latitude regions since cold water becomes undersaturated more quickly due to solubility of calcium carbonate increasing in low temperatures. For example, the Southern Ocean is expected to become undersaturated with aragonite by the year 2030 when models predict that the atmospheric CO\textsubscript{2} levels will reach 450-ppm\textsuperscript{108}. Atmospheric CO\textsubscript{2} thresholds are able to delineate where and when specific areas may become undersaturated with respect to aragonite and calcite.

In addition to the undersaturation of older and colder water masses, larger areal coverage of aragonite undersaturation has also been observed in highly productive upwelling regions where the potential harm to economically lucrative commercial fisheries could be calamitous. The North American western continent shelf experiences seasonal enhancements on the aragonite undersaturation, although little is yet known about how intermittent exposure to corrosive waters affects indigenous organisms within the neritic and benthic environments\textsuperscript{41}. What can be stated, however, is that organisms living in shallow, coastal areas of upwelling regions are not only exposed to low pH waters upwelling from the deep but also to low pH waters from shallow waters where anthropogenic CO\textsubscript{2} is absorbing into surface layers. The biological impacts of OA can only be exasperated in shallow, upwelling regions.
Implications for OA effects on intricate interactions in ecosystems are manifold. Regime shifts in certain ecosystems are inevitable as some marine organisms are able to cope with OA induced stress and others are not. Several trophic levels within ecosystems will likely be impacted by OA starting with calcifying phytoplankton and zooplankton as well as organisms higher in the food chain like fish. Direct effects on the physiology of individual organisms and indirect affects through changing food sources and habitat structures will concomitantly alter ecosystems. Research has recently concentrated on exploring OA effects on entire ecosystems. Although it is not an extensive review of all the literature that exists, some of the shifts expected in future oceans are described for coral reef ecosystems:

*Tropical coral reefs*

Some coral species can exist for extended periods without their calcareous shell, which may explain how corals as a group survived gaps in the fossil records where calcified organisms did not exist. However, modern genotypes and phenotypes of corals may not have the capacity to adapt fast enough to the unbuffered, rapid increase in CO₂. A combination of laboratory, mesocosm, and field observations enable research to gauge how coral reefs may fair in future acidified oceans. Coral reef communities observed in naturally low pH conditions may be a paradigm for futuristic reef communities. Reefs surveyed near volcanic CO₂ vents show that coral coverage remains constant but structural complexity is reduced through a shift in community composition from branching corals to a dominance of bouldering corals. Reduced structural complexity diminishes habitat quality and diversity and further affects the ability of reefs to absorb wave energy and protect coastal environments. Complexity may also be reduced if skeletal density lessens and erosion is promoted through increased grazing (i.e. easier to graze on less dense material) and storms that can easily break brittle coral skeletons. The physiological stresses on calcification will likely impact corals at the community level. Linear growth and a decline in calcification have already been noted for corals in the Great Barrier Reef. Although the exact cause remains unknown, it is ostensibly related to increasing temperatures and a declining saturation state of aragonite. If corals are able to maintain skeletal growth and density under reduced carbonate
saturation conditions then it means more energy is allocated for calcification and this chronic stress could have negative health impacts on the entire reef\textsuperscript{113}. It is clear from available literature that individual coral species respond differently to ocean acidification, but a general consensus indicates that OA is pernicious to coral reef ecosystems and net community calcification rates will most likely be reduced. Reefs are teeming with diverse life forms and indubitably any changes in the structural complexity of reefs will be detrimental for those organisms that thrive in such an environment. A comprehensive understanding of trickle-down effects that reef changes will have on marine organisms living within the reef is still under investigation.

Coral reefs are certainly not the only systems vulnerable to ocean acidification effects. Where there is life, there is potential for change. And where there is change, there is the possibility that not all species will be able to acclimate or adapt. Change is inevitable, but for some species the change may actually be to more favorable conditions. Inherently, some species will prevail where others do not. The stress of ocean acidification will only increase, however, as more CO\textsubscript{2} absorbs into the ocean and circulates throughout the ocean basins. Potentially significant changes in all ecosystems will occur. Undoubtedly, the precipitous change in atmospheric carbon dioxide levels caused by human activity will have indelible changes to the chemical, physical and biological functioning of the world’s oceans.
Zooplankton – Harbingers of Ocean Change

Increased oceanic temperatures and hypoxic conditions are events that have well documented effects on zooplankton populations. Unfortunately both predicaments are expected to amplify with increasing carbon dioxide, having compounding effects on zooplankton. That is not even a comprehensive list of all the possible environmental changes set to hinder the survival of zooplankton. Ocean acidification is the newest dilemma caused by anthropogenic CO$_2$ to be evaluated for its effects on zooplankton. Specific calcifying species are declining as they succumb to ocean acidification, but extensive information on community shifts within entire zooplankton communities in response to OA is lacking. Zooplankton are key players in setting the pace of climate change due to their role in the sequestration of carbon. Therefore, ocean acidification effects on zooplankton could have significant consequences for the oceanic carbon cycle. This section will describe zooplankton, recount their importance to marine ecosystems, identify their effectiveness as being harbingers for climate change, and discuss the effects of OA on certain taxonomic groups.

Characteristics of Zooplankton

By definition of the word, zooplankton are “animal drifters” whose movements are amenable to the impetus of currents. Zooplankton communities are heterogeneous and their biodiversity is partially attributable to their structural, developmental, and behavioral diversity. They are often distinguished by their life cycle and termed holoplankton if their entire lives are spent in the water column and meroplankton if they are only planktonic as larvae. Zooplankton studied in shallow, coastal waters are further categorized as oceanic plankton or residential zooplankton, depending on whether currents simply transport them along for a short time or if they reside permanently within the coastal region$^{115,116}$. Covering a large range in sizes and feeding strategies (e.g. raptorial, filter, diffusive feeding), zooplankton are all phagotrophs and occupy a high number of trophic levels$^{117,118}$. Distributed throughout the world’s oceans, zooplankton are capable of small-scale and large-scale movements. Small-scale movements are sinuous as zooplankton
avoid nearby predators, catch prey, search for mates, and react to hydromechanics and chemical stimuli\textsuperscript{119,120}. Large scale-movements include diurnal vertical migration over great depths by feeding in surface layers at night and swimming to the deep during the day, allowing them to avoid predation and minimize metabolic activity\textsuperscript{121,122}. While repetitiously ascending and descending throughout the water column, zooplankton are also portaged by currents resulting in three-dimensional trajectories that are often convoluted through time and space. Distributions are horizontally patchy and dependent on current movement, convergence zones, eddies, tidal mixing, internal waves, and areas of high productivity\textsuperscript{123–126}. Although population distributions are dependent on nutrition availability and physical oceanography, they are ubiquitous and are therefore pivotal in the functioning of marine ecosystems.

**Pivotal Role of Zooplankton in Marine Ecosystems**

Zooplankton are highly abundant and critical for food web interactions in the ocean. Communities are composed of seemingly disparate taxonomic groups, and yet as a unit they have the same salient niche. They are the nexus between primary producers and higher trophic levels, transferring energy from the base of food webs to larger marine consumers\textsuperscript{127}. They also contribute to elemental cycling and vertical fluxes of vital organic and inorganic materials. Grazing on phytoplankton and the subsequent flux of material below the euphotic zone is additionally important for the functionality of the biological pump. Therefore any changes in zooplankton populations, whether climate or biologically induced, may alter vertical flux rates of material out of surface waters and also influence the quantity and quality of energy transferred to other trophic levels. Zooplankton populations are controlled through bottom-up and top-down food web dynamics, with abundances reflecting food availability and predation level as well as the chemical and physical environment in which they live.

Grazing modulates phytoplankton populations and is partially responsible for High Nutrient Low Chlorophyll (HNLC) areas in the open ocean where phytoplankton levels remain low despite high concentrations of macronutrients\textsuperscript{128,129}. Zooplankton grazing on phytoplankton helps facilitate the oceanic carbon cycle and is the first step to sequestering carbon. The primary link to biogeochemical fluxes is through
the repackaging of by-products in fast sinking fecal pellets\textsuperscript{130}. Not every particle eaten by zooplankton sinks out of the euphotic zone, instead many nutrients are recycled within the surface waters. The effect that zooplankton have on regenerated production and recycling efficiencies of biogenic materials in surface waters is largely unknown even though it could potentially cause significant changes to biogeochemical cycles\textsuperscript{131}. Fecal pellets are released in surface waters where zooplankton feed and in deep waters when they vertically migrate to great depths, depositing metabolic by-products along the way\textsuperscript{132}. Any differences between the coupling of phytoplankton and zooplankton grazing processes would cause regional and seasonal variations of phytoplankton standing stocks and ultimately impact the export flux and the amount of carbon capable of being removed from surface waters. Zooplankton grazing on phytoplankton and its effects on the biological pump is an important aspect of food web dynamics, but of course zooplankton are also a vital food source themselves. They provide essential nutrients for many marine organisms including bacteria, bigger zooplankton, corals, fish, and even large charismatic animals like sea turtles and baleen whales.

Grazing processes stimulate the microbial loop within the oceans by decomposing particle aggregates. Microbes colonize phytoplankton and zooplankton molts and carcasses, although phytoplankton decompose more quickly compared to crustacean zooplankton because of their chitinous skeleton that can partially resist decomposition\textsuperscript{133}. Many bacteria also live on the surface of zooplankton and are able to disperse throughout different oceanic depths as zooplankton migrate, more commonly they colonize fecal pellets\textsuperscript{134}. Whether bacteria populate fecal matter or living or non-living zooplankton, they proliferate and become available food for bacterioplankton, nanoflagellates, and ciliates\textsuperscript{135–138}. Microbe covered detrital material rains down on benthic communities and is a rich source of carbon and other nutrients for sponges, echinoderms, anemones, crab, and fish\textsuperscript{139}.

Although microbes are smaller than the zooplankton detritus they colonize, most planktivores are larger than the zooplankton they consume. The most economically valuable zooplankton consumers are fish. Any fluctuations in zooplankton abundances, seasonal population cycles, and size distributions can alter the survival of larval fish\textsuperscript{140,141}. Some fish also modify their reproductive strategies so that they can feed and spawn synchronically. Also, well-fed fish
release larger batches of eggs. Quantity as well as quality of the zooplankton has an effect on fish growth. Improved nutritional value through increased fatty acids, minerals, and proteins of plankton are reflected in larger tissue mass and increased growth and survival rates of fish.

Zooplankton provide energy for much larger organisms as well. Leatherback turtles are obligate predators for gelatinous zooplankton and are often attracted to jellyfish aggregates. It is a conundrum as to how such large animals (weighing up to 916 kg) are able to meet their energetic needs by eating such a poor nutrient diet while expending energy on growth, metabolism, reproduction, and travelling large distances between their foraging and breeding grounds. Nevertheless, gelatinous zooplankton are highly important for sea turtle populations and the ecological link between the predator and prey deserves more attention to understand whether or not broad-scale distributions of jellyfish drive the foraging behavior of sea turtles. Baleen whales, whale sharks, seals, and birds are other large marine animals that rely directly on zooplankton as a source of food. Probably the most impressive aspect is small krill sustaining large baleen whales. In the South Atlantic, baleen whales consume an estimated 1.6 – 2.7 million tones of krill each summer foraging season, which is only 4 – 6 % of the total krill biomass in the region. Krill rely on sea ice for nutrients and as shelter to overwinter, and yet their populations might be under threat as sea ice is melting and ocean acidification is affecting polar regions.

Harbingers of climate change

Plankton are particularly good harbingers for climate change for several reasons. For one, their life cycles are short which means that there is a tight coupling between environmental conditions and their population dynamics. A short life cycle also implies that there are no older individuals residual in the community that developed under possibly different environmental conditions. Secondly, with the exception of a few krill and jellyfish populations, zooplankton are not commercially harvested thus any changes in abundances can be attributed to climatic changes and not harvesting trends. Thirdly, zooplankton are free-floating organisms and their distributions expand and contract in response to changing environmental conditions. Fourthly, organisms which are sensitive to environmental changes and
have a plankton larval stage will be reflected in the meroplankton. And lastly, some
evidence suggests that zooplankton are actually more sensitive indicators of climate
change than environmental variables because the non-linear response of
zooplankton can amplify subtle environmental signals\textsuperscript{154}. All of these attributes
make zooplankton harbingers for climate change.

Literature on climate change interactions on spatial zooplankton populations
is predominated by warming effects which drive distributions of individual species
and assemblages of plankton poleward\textsuperscript{155}. Warming also prompts life cycle events
to begin earlier and alters phenological zooplankton behaviors\textsuperscript{156, 157}. Some areas
where warming has been significant, e.g. the California current, have even
undergone reductions in zooplankton abundances\textsuperscript{158}. Currently ocean acidification
is taking the forefront in research caused by anthropogenic CO\textsubscript{2} affecting the world’s
oceans, although research examining OA effects on zooplankton is rather scant and
has mostly focused on calcifying zooplankton. Calcifying zooplankton, e.g.
pteropods, are a prime example of a plankton group that is sensitive to ocean
acidification and therefore changes in their abundances act as a beacon for climate
change.

**Ocean acidification effects on zooplankton**

Generalizations about ocean acidification effects on zooplankton are not well
established. This is partially due to lack of research for most zooplankton taxonomic
groups, with a few exceptions, and also because of the extreme diversity in
zooplankton body structures and physiological mechanisms that would imply diverse
responses to OA. The perceived understanding of how ocean acidification may
affect zooplankton is acknowledged, yet empirical data supporting such information
is generally inadequate for most representative zooplankton groups. For the growing
amount of work that examines OA effects on zooplankton, many results indicate that
there are no discernible effects of OA on some dominant zooplankton species under
CO\textsubscript{2} conditions expected in the next century. Other studies show that some species,
calciﬁers and non-calciﬁers alike, will be negatively impacted by OA. Overall there is
a sense of ambivalence with respect to OA effects on zooplankton and much more
research is needed to elucidate possible direct and indirect effects of OA on
zooplankton. Most studies involve controlled experiments in the laboratory where
very few have examined possible community changes from natural field environments. Scientific attention should also be dedicated to studying the synergistic effects of CO₂ and other stressors (e.g. warming and hypoxia) on zooplankton since that is the unfortunate future they may encounter.

From available literature, key findings for OA effects on certain zooplankton taxonomic groups are summarized below:

**Foraminifera**

Examining the effects of ocean acidification on marine calcifiers, e.g. corals, has been the major concentration for research; such is the case for zooplankton species. Foraminifera are calcifying, single-celled marine organisms that highly populate benthic environments and surface-dwelling plankton communities. Calcareous tests of foraminifera occur both as calcite and aragonite, with most utilizing calcite and only a few genera related at the family level using aragonite\textsuperscript{159}. Planktonic foraminifera represent between 25-50% of the total open-ocean marine carbon flux and influence the transport of organic carbon through the biological pump\textsuperscript{160,161}. Modern *Globigerina bulloides* have 30-35% lighter shells compared to specimens from Holocene-aged sediments in the Southern Ocean, and this weight loss is attributed to reduced calcification rates caused by ocean acidification\textsuperscript{82}. Ocean acidification and seasonal upwelling also thin the shells of the planktonic foraminifera *Globigerinoides ruber* in the Arabian Sea\textsuperscript{162}. Laboratory experiments on the large benthic foraminifera *Marginopora kudakajimensis* provide supporting data showing that shell weights reduce even within a period of 10 weeks if exposed to a pH of 7.7; furthermore, calcification and growth rates did not respond linearly to changes in pH and there was a steep decline in calcification around pH 7.7 suggesting that these foraminifera may not be able to survive in pH conditions below 7.7 (ref 163). Calcification and shell weights reduce under OA conditions, but field studies also show that distribution, densities, and diversity also change amongst living foraminifera assemblages along a pH gradient with organisms nearly absent in pH conditions expected for the next century\textsuperscript{164,165}. Some benthic species (*Nonionella basispinata*, *Epistominella bradyana*, and *Bulimina marginata*) are evidently partially resistant to low pH waters since richness appeared unaffected in the northern Gulf of California, although there is evidence of dissolution\textsuperscript{166}. The sheer abundance of
foraminifera and their role in sequestering carbon means that their sensitivity to ocean acidification could have significant consequences for biogeochemical cycling.

**Pteropods**

Pteropods species are diverse in the tropics but only a few species exist in polar waters. At least in polar regions they can occur in high densities, up to 2,681 individuals m\(^{-3}\), and are not only a major dietary source for other zooplankton and higher predators (e.g. herring, salmon, whales, birds), but they also contribute to \(~10\%\) of the global CaCO\(_3\) export\(^{167-169}\). Any reductions in their abundance due to OA would have significant effects on marine ecosystems and carbon cycling. These pelagic mollusks, also called “sea butterflies” because of their wink-like parapodia, have aragonite shells, which are more soluble than calcite. Therefore these organisms are most sensitive to ocean acidification, especially those living in polar regions where aragonite undersaturation is expected to occur by 2050 in the Southern ocean and as early as 2016 in the Arctic Ocean\(^{52,108,170}\). For the polar pteropod species *Limacina helicina*, calcification rates are reduced by 28% when exposed to pH levels expected for the end of the century\(^{171}\). In seawater with pCO\(_2\) at 1100 µatm, juvenile *Limacina helicina* mortality increased by 14% compared to those raised in 230 µatm, shell degradation increased by 41%, and shell diameter and increment decreased by 10% and 12%, respectively\(^{172}\). Regional estimates of the changes in pteropod calcification rates suggest that the species *Limacina helicina* may not exist in the near future if carbon emissions continue, with organisms in the Arctic being the most vulnerable\(^{171}\).

Research has focused on cold-water species since polar regions are expected to become undersaturated with aragonite first, but warm-water pteropod species are also vulnerable to OA effects. One temperate species, *Cavolinia inflexa*, cultured under low pH levels exhibited lower shell growth\(^{173}\). Under a pH of 7.5, larvae pteropods developed normally and were viable but they did so without a shell. Pteropods can apparently survive without shells, at least for short durations, but the lack of a calcium carbonate shell has ecological and biogeochemical consequences. No information exists on shell growth rates affected by OA for tropical pteropod species even though the aragonite saturation state is expected to decline by 30% by the middle of the next century\(^{61}\). However, 5 tropical species (*Hyalocyliis striata, Clio pyramidata, Cavolinia longirostris, Creseis virgule, and Diacria quadridentata*) have
been examined for changes in their metabolic rates for species found in open-ocean waters where they might encounter regions of low pH within oxygen minimum zones\textsuperscript{174}. Oxygen minimum zones are characterized by increased CO\textsubscript{2} levels and are existent in regions like the Eastern Tropical Pacific where depths of 200 m experience CO\textsubscript{2} levels higher than 1,000 ppm\textsuperscript{175}. This study revealed that pteropod species which migrate through oxygen minimum zones had no change in their oxygen consumption and ammonia excretion, although \textit{Diacria quadristdentata} which does not migrate had a reduction in both. It seems as though some pteropod species are metabolically adapted to low CO\textsubscript{2} conditions for short periods during vertical migration. Effects of OA on pteropods living in coastal, warm waters have virtually been unexplored even though the expectation is that they will also suffer from adverse effects from ocean acidification.

\textit{Copepods}

Copepods are the most abundant metazoans on Earth and outnumber insects by approximately three orders of magnitude\textsuperscript{176}. Arguably the most important crustacean due to their sheer abundance and critical role in marine ecosystems, copepods dominate worldwide zooplankton communities. Iconic for all crustaceans, their exoskeleton is composed of the polysaccharide chitin, the most abundant renewable polymer in the ocean and an important source of carbon and nitrogen\textsuperscript{177}. However, unlike other crustaceans, the chitinous carapace of copepods (and cirripeds) is not mineralized with CaCO\textsubscript{3} (ref 178). It is currently unknown how chitin may be affected by ocean acidification\textsuperscript{98}. The cuticle composition is dominated by carbon and oxygen but many trace elements, including calcium, constitute the total chemical elemental composition of copepods\textsuperscript{179}. Of the non-calcifying zooplankton, copepods have received the most attention due to their dominance in most zooplankton communities; nonetheless, only a few species have been investigated for OA effects.

Most copepod studies in low pH environments have observed possible changes to early life development. Available empirical data indicate that survival and early development stages are unaffected by low pH conditions expected for the next century. Only under extremely high pH conditions were the developmental stages affected for a few species. Survival, egg production, and hatching rates for multigenerational studies (i.e. 2 generations) on \textit{Acartia tenuis}, \textit{Acartia steueri} and
*Acartia erythraea* showed no significant changes between CO₂ conditions (ambient water and +2,000 ppm); furthermore, there was no delay in time for egg production by the treated specimens\(^5,\(^1\)\(^0\). Only when exposed to CO₂ levels of +5,000 ppm were *Acartia steueri* and *Acartia erythraea* hatching rates and egg production compromised. Similarly, hatching success for *Calanus finmarchicus* was only negatively impacted at high CO₂ concentrations (+2,000 ppm) which are not expected in the near future, otherwise growth and egg production were not affected by ocean acidification\(^1\)\(^8\)\(^1\). Apoptosis in eggs and nauplii of *Calanus helgolandicus* were also unaffected by high levels of CO₂ (ref 182), and neither were the egg production and hatching success of *Centropages typicus* and *Temora longicornis*\(^1\)\(^8\)\(^3\). Egg production was not influenced by pH for *Calanus glacialis* but hatching time was delayed and the overall hatching success was reduced\(^1\)\(^8\)\(^4\). Maternal provisioning on eggs under high CO₂ conditions may explain why hatching rates under varying pH regimes are generally unaffected by OA\(^1\)\(^8\)\(^5\). Copepods may also balance the energy cost against increased acidity by increasing respiration and feeding rates\(^1\)\(^8\)\(^6\). For the few species of copepods examined under laboratory conditions for short time periods, high CO₂ conditions predicted for the coming century seem to have negligible impacts on the early development of calanoid copepods.

The majority of copepods observed for changes in OA have been from the Order Calanoida, but different taxonomic orders within the subclass Copepoda may respond differently to ocean acidification. For the harpacticoid copepod, *Tisbe battagliai*, a decline in copepod naupliar production was observed over multiple generations\(^1\)\(^7\)\(^9\). A significant growth reduction also occurred for those specimens exposed to low pH conditions, as well as a shift in the chemical composition of the cuticle to an increased proportion of carbon relative to oxygen. Changes in the naupliar production, growth, and cuticle composition suggest that copepods subjugate to OA-induced stress preferentially re-allocate resources to maintaining reproductive output at the expense of somatic growth. Differences in life history strategies may explain the different results between calanoid and harpacticoid copepods, but more experimental tests on both groups of copepods over several generations should confirm this.

Early development is not the only venue by which OA can affect copepods. Indirect changes through trophic interactions will likely affect copepods as well. Diatoms cultured under elevated \(pCO₂\) conditions (750 µatm) compared to present
day $p\text{CO}_2$ conditions (380 µatm) exhibit low overall fatty acid abundances. Fatty acid composition of this diatom species altered under ocean acidification conditions with a decrease in the amount of polysaturated fatty acids (PUFA) and an increase in saturated fatty acids (SFA). This was directly reflected in copepods which feed on these diatoms\textsuperscript{187}. The copepods that fed on the high-CO$_2$ cultured diatoms underwent a decrease in egg production and somatic growth. The quality of copepods as a food source is simultaneous reduced and repercussions on the trophic dynamics within the marine ecosystem are inevitable.

**Euphausiids**

Euphausiids spawn eggs in surface waters which sink to 700-1,000 m before the larvae hatch and swim back to the surface\textsuperscript{188}. Thus, euphausiids are already exposed to low pH conditions in deep water at some stage of their life making it likely that they have evolved some level of resistance to more acidic waters\textsuperscript{189}. Euphausiid embryos were unable to develop when exposed to $p\text{CO}_2$ of 2,000 µatm but at 1,000 µatm development was normal\textsuperscript{190}. Given that Southern Ocean waters may reach 1,400 µatm by the end of the century, larval development of euphausiids is expected to be mostly unaffected by ocean acidification. Hatching success, however, is compromised under high CO$_2$ levels and krill recruitment is expected to be at high risk in the Weddell Sea and Haakon VII Sea within a century\textsuperscript{191}. Finer resolution CO$_2$ concentrations should be used to look at OA effects on all stages of life, and the changes in habitat and food availability caused by ocean acidification should also be investigated in addition to physiological changes.

**Amphipods**

Very few studies have evaluated the effects of low pH on amphipods, although several have evaluated the effects of low calcium environments found in lakes and have shown that the distribution and number of gammarid amphipods has been limited as a result of low CaCO$_3$ concentrations\textsuperscript{192,193}. Amphipods, like many other crustaceans, mineralize their chitinous cuticle by depositing calcium carbonate to strengthen the chitinous structure\textsuperscript{194}. They also store calcareous concretions in their lumen\textsuperscript{195}, which makes certain parts of their body vulnerable to reduced saturation states of calcium carbonate. Amphipods live in freshwater, brackish, and saltwater; thus, different species have evolved in a variety of environmental
conditions. Some amphipods species live in neritic environments with sporadic low pH levels and are therefore adapted to at least temporary declines in pH. Laboratory results on *Gammarus locusta*, one of the neritic amphipod species known to be exposed to low pH levels down to 7.95, showed that growth and survival were not affected by low pH conditions over a 28 day period\textsuperscript{196,197}. Another neritic species, *Echinogammarus marinus*, showed a longer lasting development period for embryos, although development was more impacted by low salinity levels\textsuperscript{198}. Amphipod species that have evolved completely in seawater where salinity remains constant have not been investigated for any impacts of ocean acidification.

**Meroplankton**

Almost all research on meroplankton in relation to ocean acidification has been on calcifying marine larvae, mostly invertebrates and bivalves, which are also studied as adults. Early life-stages are considered the most sensitive to CO\textsubscript{2} increases and for some species even a small decrease in pH can have a dramatic effect\textsuperscript{199}. Negative impacts to development induced by OA may have significant implications for certain species since larval success is considered a potential bottleneck for perseverance in the ocean. Survival, development, and calcification rates have been studied for several calcifying meroplankton. However, little information exists for OA effects on non-calcifying meroplankton.

For most larvae examined, survival rate and size were reduced in low pH waters and in some cases the change in pH was extremely detrimental to the organisms. For example, a decrease in pH by 0.2 units of pH resulted in 100\% mortality for the brittlestar *Ophiothrix fragilis* due to skeletal deformations\textsuperscript{86}. For many molluscs, barnacle larvae, and sea urchins, mortality rates increase with decreasing pH but at least part of the population survives\textsuperscript{200–202}. Other species (e.g. blue mussel, *Mytilus edulis*) indicate that hatching success and mortality rates are unaffected by OA but larvae do have decreased growth rates\textsuperscript{203}, while some species (e.g. sea urchin, *Strongylocentrotus droebachiensis*) even have a positive effect of OA on larvae survivability and significantly more larvae successfully develop to metamorphosis\textsuperscript{199}. Uncorrelated to mortality for single species experiments, developmental rates are reduced for most meroplankton examined thus far indicating that more time is needed to reach metamorphosis\textsuperscript{199}. Development time increased for the northern shrimp zoea only when decreased pH conditions were combined
with low temperatures\textsuperscript{204}. Early development and some physiological reactions to OA have been evaluated for a few calcifying meroplankton species, but to have an understanding of how OA affects all meroplankton and that impact on the overall zooplankton community has yet to be examined.

The Census of Marine Zooplankton report that there are at least 7,000 species of holoplankton, which is an underestimate of the total global zooplankton species list since it doesn't include demersal zooplankton, meroplankton, or those that have yet to be discovered. Ocean acidification effects have been studied on \textasciitilde30 species of zooplankton. Clearly there is more to learn. OA effects are often generalized for certain taxonomic groups of zooplankton which has frequently occurred for foraminifera and pteropods, but as is the case for coral responses to OA, species-specific responses are likely to vary within similar taxonomic group. For example, various families within Copepoda have drastically different reproduction strategies, distribution patterns, feeding habits, etc., all of which may result in variable responses to OA. At this stage of research development, the scientific community just does not know the degree to which global zooplankton communities may be affected by ocean acidification.

Thus far, the majority of research has observed single-species reactions to changes in CO\textsubscript{2} level over short time periods and experiments have occurred in the laboratory. Controlled laboratory conditions enable direct mechanisms of OA-induced changes to be understood, but such experiments do not represent conditions actually experienced by zooplankton \textit{in situ}. Trophic dynamics are largely ignored in laboratory experiments. Few mesocosm experiments exist which have examined zooplankton community changes under semi-controlled environmental conditions. A mesocosm experiment in an Arctic fjord indicates that abundance and community composition of mesozooplankton were similar under all CO\textsubscript{2} regimes, although a delay in the development of cirripedia may have occurred\textsuperscript{205}. Field studies observing 6 calcifying zooplankton species in relation to trends in pH throughout the central North Sea show no significant link between their abundance and measured pH levels\textsuperscript{206}. Additional field studies have focused on changes in abundances for calcifying zooplankton, but no information is available for OA effects on entire zooplankton communities. From a global perspective, ocean acidification is
affecting certain hotspots where deep-sea polar ecosystems and coral reefs are most vulnerable to deleterious changes induced by low pH waters with decreased concentrations of carbonate. Although the extent to which OA will affect zooplankton is still unknown, zooplankton communities living in these hotspots will also likely be exceptionally vulnerable to ocean acidification whether it is caused by direct physiological changes or indirect changes in trophic dynamics or habitat within the marine ecosystem. Ocean acidification and other environmental stressors will concurrently alter zooplankton communities.

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Impacts of ocean acidification on zooplankton communities living at coral reefs
CHAPTER 2
Ocean acidification reduces demersal zooplankton that reside in tropical coral reefs

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JNS, KEF, CR, AC designed the experiment. JNS and KEF did the fieldwork. JNS did the laboratory work. GD, JNS, KEF did the statistical analysis. JNS lead the writing, while all authors contributed to writing and editing the manuscript.

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Abstract

The in situ effects of ocean acidification on zooplankton communities remain largely unexplored. Using natural volcanic CO₂ seep sites around tropical coral communities, we show a three-fold reduction in the biomass of demersal zooplankton in high-CO₂ sites compared to sites with ambient CO₂. Differences were consistent across two reefs and three expeditions. Abundances were reduced in most taxonomic groups. There were no regime shifts in zooplankton community composition and no differences in fatty acid composition between CO₂ levels, suggesting ocean acidification affects the food quantity but not the quality for nocturnal plankton feeders. Emergence trap data show that the observed reduction in demersal plankton may be partly attributable to altered habitat. Ocean acidification changes coral community composition from branching to massive bouldering coral species, and our data suggest that bouldering corals represent inferior daytime shelter for demersal zooplankton. Since zooplankton represent a major source of nutrients for corals, fish, and other planktivores, this ecological feedback may represent an additional mechanism of how coral reefs will be affected by ocean acidification.

Introduction

Increased levels of anthropogenic CO₂ in the atmosphere catalyze processes that can collectively impact zooplankton communities. Concurrent with ocean warming, absorbed CO₂ changes ocean chemistry by reducing seawater pH as well as carbonate ion concentrations and the saturation states of calcium carbonate, in a process called ocean acidification¹–⁴.

Although the effects of ocean acidification on zooplankton communities are poorly understood, their impacts are potentially far-reaching due to their pivotal role in marine ecosystems and the carbon cycle. Zooplankton are a major food source for planktivores, and they also support bacterial and phytoplankton production through their excretion of nitrogen and phosphorus compounds⁵. Furthermore, they contribute to the biological pump as consumers of CO₂-fixing phytoplankton⁶. The sedimentation and burial of fecal pellets and zooplankton carcasses act as a sink for CO₂ that may help mitigate CO₂ emissions. Thus, in order to support predictions of
the future effects of ocean acidification on marine benthic and pelagic ecosystems and CO₂ fluxes, it is essential to understand the effects of ocean acidification on zooplankton communities.

Ocean acidification studies of zooplankton have primarily focused on single-species laboratory experiments, with very few of the >7000 described speciesinvestigated to date. Studies have reported severe direct effects on some calcifying plankton, attributable to the increased energy requirements needed to acquire carbonate ions as building blocks for calcification. In contrast, existing studies suggest that non-calcifiers like copepods are generally not directly affected by ocean acidification. Although single-species experiments advance our understanding of the underlying mechanisms governing the direct effects of elevated CO₂ on organisms, they have limited capacity to predict the effect of ocean acidification on entire communities. This is particularly true for zooplankton considering that calcifying species usually comprise a small proportion of these communities, and many of the non-calcifying species evaluated were generalists that are naturally found under wide ranges of environmental conditions and hence tolerate laboratory conditions. Therefore, to understand how ocean acidification may impact zooplankton in the future, entire communities need to be evaluated in situ under ocean acidification conditions.

The long-term effects of elevated carbon dioxide on marine ecosystems and entire communities have been studied at a few submarine CO₂ seeps. We used two such volcanic seeps in Papua New Guinea as natural laboratories, which release nearly pure CO₂ into tropical fringing coral reefs. Coral reefs are highly vulnerable to ocean acidification because of the sensitivity of their foundation species, namely corals and crustose coralline algae, and the dissolution of reef carbonate substrata at reduced pH. Most zooplankton found on coral reefs are demersal, meaning the organisms live on or above substrata during the day and migrate into the water column at night. We compared zooplankton communities residing near CO₂ seeps with communities living at control sites. Seawater at the high-CO₂ seeps averaged 7.8 pH₇ (pH at total scale; for spatial and temporal variability see Appendix II, Supplementary Figure 1), while at the adjacent control sites (without seep activity) it averaged 8.0 pH₇ (refs 21,25). All study sites had similar seabed topography at depths of 2-3 m, a tidal range of <0.9 m, and longshore currents between 2-4 cm s⁻¹.
with an average water residence time of ~2.5 hours over both of the seeps studied. We compared demersal zooplankton abundance, biomass, and community composition along high-CO$_2$ and control sites at CO$_2$ seeps on Dobu and Upa-Upasina reefs using horizontal surface net tows and emergence traps on three separate expeditions.

**Loss of reef-associated demersal zooplankton**

During the day, zooplankton biomass was low and of similar quantity between CO$_2$ regimes at both reefs (Dobu and Upa-Upasina). At night, demersal zooplankton emerged from their seabed refugia and had consistently higher biomass at the control sites compared to the high-CO$_2$ sites. Across the two reefs and on all three expeditions, control sites had on average 2.83 (SEM = 0.19) times greater zooplankton biomass than the high-CO$_2$ sites (range: 1.45 - 4.85, N = 24; Figure 1a). On average, control sites had 9.33 (SEM = 1.25) times more zooplankton biomass at night than during the day, whereas for the high-CO$_2$ sites that ratio was 3.14 (SEM = 0.39). Offshore from the control and high-CO$_2$ sites (~200-300 m from the coastline at water depths of 50-70 m), there was no difference in zooplankton biomass, neither during the day nor at night. The zooplankton composition at night differed between offshore waters and the reef, and the mean biomass in offshore waters was 3.66 (SEM = 1.15) times lower than at the control sites. These two observations confirmed that the bulk of the zooplankton were indeed resident to the reefs. Biomass of bulk zooplankton at control sites remained higher than at high-CO$_2$ sites throughout the entire night, and the diurnal migration patterns were similar between control and high-CO$_2$ sites (Figure 1b).

For individual zooplankton taxa, our analyses revealed significant (p < 0.05) reductions in abundances at the high-CO$_2$ sites compared to control sites for most taxa, and no taxon preferred the high-CO$_2$ sites (Figure 2). For example, the copepod family Pontellidae had an abundance ratio of 0.168 (95% CI = (0.093, 0.305)), meaning that the abundance at the high-CO$_2$ sites was 16.8% of that at the control sites. Additional to the CO$_2$ effects, abundances of some taxa also varied significantly between sites or between expeditions. A few taxa (Centropagidae, Oithonidae, Cumacea) remained unaffected by CO$_2$ (ratios >1.0, but 95% confidence intervals including 1.0). For all other taxa, the values and 95% confidence intervals
remained below 1.0, i.e. their abundances were significantly reduced at the high-CO$_2$ sites. Abundances for copepod taxa at the high-CO$_2$ sites were between 12.0 and 70.9% of those at the control sites, and for non-copepod taxa they ranged between 18.8 and 47.5%.

**Figure 1.** Differences in zooplankton biomass between control and high-CO$_2$ sites, derived from horizontal net tows. Zooplankton biomass (a) at the two reefs (Dobu and Upa-UPasina) and three expeditions at night, and (b) a 24-h sampling campaign showing vertical migration at both the high-CO$_2$ and control site of Upa-UPasina reef. Control sites are represented in blue, and high-CO$_2$ sites are represented in red.
Figure 2. Abundance ratios (high-CO$_2$/control) for selected zooplankton taxa. The circles and bars represent the means and 95% confidence intervals, respectively. The ratios of abundances of zooplankton taxa between the control and the high-CO$_2$ sites are significantly different at the 5% level if their error bars do not include the value 1.0.

A ranking of the sensitivity of taxa showed that 10% of the taxa at the high-CO$_2$ sites had declined to <20% of the control abundances, while 84% of the taxa had declined to <50%. The most sensitive copepod taxa were Monstrilloida and Pontellidae (abundance ratios = 0.120 and 0.168, respectively), and amphipods and ostracods were the most sensitive non-copepod taxa (abundance ratios = 0.192 and 0.188, respectively). Both holoplankton (taxa that remain planktonic throughout their lives, for example, copepods, amphipods, isopods, mysids, and ostracods) and meroplankton (temporary constitutes, for example, decapod larvae and echinoderm larvae) were reduced under ocean acidification, although to varying degrees.
Reductions in copepod abundances were also found in those families predicted from laboratory experiments to be resilient to ocean acidification. One of the dominant copepod families was Acartiidae, a widely distributed group that is known to live within coral reefs\textsuperscript{16}. Acartiidae abundance was 14 times lower at the high-CO\textsubscript{2} than at the control sites, despite previous short-term CO\textsubscript{2} exposure laboratory experiments suggesting that the survival, body size, developmental speed, egg production, and hatching rates of Acartiidae are negligibly affected by the magnitude of seawater pH change expected by the end of the century\textsuperscript{11,26,27}. This discrepancy highlights the need for field observations to validate laboratory predictions of direct and indirect impacts of rising CO\textsubscript{2} levels.

**No high-CO\textsubscript{2} shifts in zooplankton communities**

Community analyses showed that there was no species turnover between the control and high-CO\textsubscript{2} sites. Neither species replacement nor any taxon proliferated in the high-CO\textsubscript{2} environment. Although there were slight shifts in the percent composition of the taxa present within the communities, each taxon had a slightly different sensitivity to ocean acidification since no new groups filled the niche or replaced other taxa in the CO\textsubscript{2}-impacted habitat. Zooplankton communities differed between Upa-Upasina and Dobu reefs and between expeditions, but all had similar reactions to ocean acidification: all taxonomic groups present in the control sites persisted in the high-CO\textsubscript{2} sites, albeit at much lower abundances (Figure 3).

There were also no major shifts in the biochemical composition of the zooplankton community. Specifically, the fatty acid content of bulk zooplankton samples did not differ between the control and high-CO\textsubscript{2} sites during the second expedition (permanova: \( p = 0.440 \)), although it did vary between the two reefs (\( p = 0.001 \)). Zooplankton predators, including carnivorous plankton, corals and fishes, are thus likely to encounter quantitative but not biochemical changes in zooplankton food between high-CO\textsubscript{2} and control sites.
Reduced habitat complexity causes zooplankton loss

The causes of reduced zooplankton abundances at high-CO$_2$ could be due to physiological, behavioral, or ecological effects, including habitat loss and changes in the food web. The study area consists of 31% and 33% hard coral coverage at the control and high-CO$_2$ sites, yet the composition of coral communities shifts from branching corals to massive bouldering corals. Massive bouldering corals more than double (from 10.7% at the control sites to 24.9% cover at the high-CO$_2$ sites), while the structurally complex corals are reduced three fold (from 12.9% to 4.3% cover)$^{21}$. 
Coral rubble remains similar with 3.0% cover at the control sites and 2.6% cover at the high-CO₂ sites. Such losses in structural complexity can have consequences for the organisms that rely on such corals as habitat²⁶. To determine substratum preferences of the various zooplankton taxa for their daytime residence, emergence traps were placed over 1.0 m² quadrats dominated by three different substrata (branching coral, massive bouldering coral, and coral rubble). Emergence traps captured demersal zooplankton at night during their vertical migration when they swam into dimly illuminated (3 lumens) cod-ends. Traps were retrieved 2-3 hours after dark, yielding a mean of 13,677 (SEM = 1,948) individual zooplankton per trap at the control sites and 6,504 (SEM = 787) at the high-CO₂ sites. The exact composition of the substrata within these quadrats was determined from photographs, distinguishing 7 substrata (branching coral, massive bouldering coral, and coral rubble, sand, macroalgae, turf, and other).

Data from the emergence traps showed that 15 of the 17 most common taxa of zooplankton showed reduced abundances under increased CO₂. Additionally, the abundances of 9 of the 17 taxa were positively correlated with the cover of coral rubble or branching coral (Figure 4). Eight zooplankton taxa were negatively correlated with massive bouldering coral, sand, macroalgae, and/or turf algae. Sand, macroalgae, and turf algae were never dominant substrata in the quadrats at either high-CO₂ or control sites (max. 15% cover), and yet they appeared to provide shelter for some taxa (e.g. Othonidae and Pontellidae) but were negatively associated with others (e.g. Arietellidae, Paracalanidae, Sapphirinidae). Only four zooplankton taxa showed no substratum preference. This suggests that reduced availability of branching corals at the high-CO₂ sites, and increased presence of massive bouldering corals, contributed to the reduction of several zooplankton taxa at the high-CO₂ sites.
Figure 4. Influences of CO\textsubscript{2}, Reef, Date, and substratum on dominant zooplankton taxa from emergence traps. Substrata are percent cover of: CR = coral rubble, BC = branching coral, MC = massive (bouldering) coral, SA = sand, MA = macroalgae, and TA = turf algae. ‘**’ indicates <0.001 significance, ‘*’ indicates <0.05 significance, and empty boxes indicate ‘none significance’. For CO\textsubscript{2} and the substrata, green and purple boxes indicate positive and negative relationships, respectively.

Other causes for abundance loss

Altered habitat quality is one explanation for reduced zooplankton abundance, however other direct and indirect causes also likely contribute. Phytoplankton is food for herbivorous and omnivorous taxa (e.g. Acartiidae, Centropagidae, Harpacticoida, Oithoniidae, Oncaeidae, Paracalanidae, Pontellidae, Gastropoda larvae, and Polychaeta). However, total organic carbon, total nitrogen, chlorophyll a, and phaeophytin concentrations did not differ between the high-CO\textsubscript{2} and control sites (p > 0.05 for all measured parameters). Appendix II, Supplementary Tables 2 and 3 show mean phytoplankton biomass values, and the significance of CO\textsubscript{2}, reef, and
time (day versus night) on phytoplankton biomass. This suggests that food limitation did not control the abundances of the herbivorous and omnivorous taxa. Changes in density or nutritional quality of phytoplankton in response to high-CO$_2$ (ref 27) are unlikely due to the short residency time, although elevated CO$_2$ can promote phytoplankton production$^{28}$. The observed reductions in herbivorous and omnivorous zooplankton suggest that per capita phytoplankton availability may even increase. In contrast, carnivorous zooplankton (e.g. Arietellidae, Corycaeidae, Sapphirinidae, Amphipoda, Decapoda larvae, Isopoda, Ostracoda, Chaetognatha, and fish larvae) are likely to experience diminished food abundances, with potential flow-on effects on their abundances.

The impact of ocean acidification on zooplankton swimming behavior has not been studied. Zooplankton motility is a requisite for feeding, avoiding predators, and vertical migration. Our finding that migration behavior was unaffected by high-CO$_2$ levels at the high-CO$_2$ sites suggests their ability to access resources and evade predation appears to remain intact. Nevertheless, behavioral responses of individual taxa to high-CO$_2$ cannot be excluded as a contributing mechanism. For example, high-CO$_2$ disrupts discriminatory and swimming behaviors in response to olfactory cues in some tropical reef fish species$^{29,30}$, and similarly unexpected results are possible for some zooplankton taxa.

Zooplankton migration against vertical currents can enrich zooplankton near reefs$^{31}$. Although the horizontal tows were not conducted directly over the bubble streams, gas bubbles at the seep sites should have enhanced vertical currents and, hence, zooplankton densities particularly for the fast-swimming larger zooplankton. For all taxa, the consistently lower zooplankton densities near the seep sites suggest that vertical currents played no major role for explaining the observed differences in zooplankton biomass between high-CO$_2$ and control sites.

**Consequences for coral reef ecosystems**

Reduced zooplankton abundances may have far-reaching consequences for marine ecosystems and fisheries. In coral reefs, planktivores are an important trophic guild that includes many reef associated adult and larval fish and the reef building corals. Corals rely on heterotrophy for essential nutrients not acquired
through their symbionts for tissue and skeletal growth\textsuperscript{32,33,34}. Increasing heterotrophy is one mechanism for some coral species to compensate for the increased energy demand for calcification under ocean acidification\textsuperscript{35,36}, and yet this option may be diminished if zooplankton abundances are reduced. Note that we only investigated macrozooplankton abundances, not microzooplankton or detritus in the water column. Thus, corals that feed on smaller organisms or those few coral species that also feed during the day and not just at night\textsuperscript{37} may still fare well under reduced abundances of macrozooplankton\textsuperscript{38,39}. This may be the case for the massive bouldering corals, since their abundances are not negatively affected by the documented reduction in macrozooplankton abundances.

We showed that reduced abundances of demersal zooplankton were in part related to indirect ecological effects of ocean acidification, including changes in their day-time habitat, as branching corals were replaced by massive bouldering corals at high-CO\textsubscript{2}. This indirect effect is specific to reef-associated zooplankton and does not apply to oceanic plankton. However, ecological changes of habitat quality and food web structures due to ocean acidification may also alter demersal zooplankton communities in other coastal marine ecosystems.

In addition to acidification, increased atmospheric CO\textsubscript{2} is warming the oceans\textsuperscript{40}, driving some zooplankton species poleward\textsuperscript{41}, enlarging oxygen minimum zones, and restricting vertical migration and distribution of some zooplankton taxa\textsuperscript{42,43}. Seawater stratification is becoming more pronounced, suppressing vertical mixing and prompting up-welled waters to shoal, which through reductions in nutrients and production can also reduce zooplankton by as much as 80\%\textsuperscript{44}. Our findings shed new light on how zooplankton can be affected by ocean acidification and reveal that coral reefs and other coastal ecosystems may be more vulnerable than expected as rising CO\textsubscript{2} can diminish the very basis of their food webs.

**Methods**

**Zooplankton Sampling and Laboratory analysis**

Zooplankton biomass, abundances and community composition were compared between two CO\textsubscript{2} regimes (control and high-CO\textsubscript{2} sites), each at two reefs (Dobu and Upa-Upasina; Milne Bay Province, Papua New Guinea), and for three expeditions (1,2,3). Samples were collected at night (2100-0200 hours local time)
and mid-day (1200-1400 hours) for a total of 24 days during three separate expeditions (17 to 27 January 2013, 24 May to 9 June 2013, 22 March to 2 April 2014), using a 100 µm Nansen plankton net (aperture: 70 cm). Horizontal tows were conducted along 30 m transects at both CO2 sites and reefs, both over the reef (2-3 m water depth) and offshore (50-70 m water depth). At the high-CO2 sites, transects were located along the edge of the seeps but not in the bubble streams to prevent sampling where zooplankton might be disturbed by the bubbles, and to not fill the net with gas bubbles. A hand-held GPS and a HydroBios flowmeter recorded tow distance to determine the volume of water filtered. Three replicate transects were collected at each location. Bulk zooplankton from additional net tows were frozen at -80°C, and analyzed for their fatty acid composition using gas chromatography\textsuperscript{45,46}.

To compare diurnal patterns, horizontal tows were additionally conducted over a 24-hour period at the high-CO2 and control sites of Upa-Upasina during the third expedition once per week for four weeks, with tows every three hours during daylight hours and every two hours during the night.

Daytime habitat preference for three dominant substrata (branching coral, coral rubble, and massive bouldering coral) was tested with emergence traps. The traps consisted of nine custom made pyramid-shaped tents (100 µm mesh net, $L\times W\times H$: 1 m x 1 m x 0.75 m) with detachable cod-ends that had light (3 lumens) fixed inside to attract zooplankton. Three traps were placed over each of the three types of substrata (>50% branching coral, coral rubble, or massive bouldering coral). The habitat preference experiment was conducted during the third expedition, only at Upa-Upasina reef from 8-17 April 2014. Over the course of 10 days, the 9 traps were placed in random locations over the different substratum types alternating between the high-CO2 and the control site. The high-CO2 site and the control sites were both sampled 5 days each. A photo was first taken of the 1.0 m$^2$ quadrat of substratum before the trap was placed over it. Emergence traps were tethered unsealed to the reef substrata with nylon string. Contamination from external zooplankton was expected to be low (a few organisms per trap per night), since demersal zooplankton emerge upward and are unlikely to crawl under a physical barrier, i.e. the trap. Emergence traps were deployed during daylight hours (1300 hours) before zooplankton emerged into the water column, and the cod-ends were retrieved 3-4 hours after dark (2100-2200 hours).

From both the horizontal tows and the emergence traps, the contents of the
cod-ends were stored in a 4% formaldehyde-seawater solution. Later, replicate subsamples were analyzed in the laboratory. Copepods were identified to family level, and non-copepods were identified to class or order. After identification, samples were split in half with a Folsom splitter and half of the sample was placed onto pre-weighed and pre-combusted GF/F 47 mm filters and Aluminum tins. Samples were dried at 60°C for 24 hours before weighing to obtain biomass data (mg dry weight m⁻³).

Seawater Chemistry

The seawater chemistry at Upa-Upasina and Dobu reefs has been documented previously²¹,²⁵. The pH at the total scale (pHT) averaged 8.0 at the control sites and 7.8 at the high-CO₂ sites. The control sites are exposed to a relatively stable pH₇ level whereas the high-CO₂ sites experience more variable pH₇ levels. Water samples were collected during the expeditions and fixed with mercuric chloride solution and later analyzed for their dissolved inorganic carbon (DIC) and total alkalinity (A₇) using a Versatile Instrument for the Determination of Total Inorganic Carbon and Titration Alkalinity (VINDTA 3C). DIC and A₇ were used to calculate other seawater parameters (Appendix II, Supplementary Table 1), including pH at total scale (pHT), partial pressure of carbon dioxide (pCO₂: µatm), bicarbonate (HCO₃⁻: µmol kg⁻¹), and carbonate (CO₃²⁻: µmol kg⁻¹) using the Excel macro CO2SYS⁴⁷ under the constraints set by Dickson and Millero⁴⁸.

Phytoplankton in the water column

Phytoplankton quantity in the water column were compared between control and high-CO₂ sites at Dobu and Upa-Upasina reefs to determine the amount of food available to herbivorous zooplankton. Water samples were collected at midnight (0000 hour) and midday (1200 hour) using a Niskin bottle. Onboard the M/V Chertan, 3 L of water was immediately filtered through pre-combusted 47 mm GF/F filters and stored in liquid nitrogen. Later in the laboratory, pigments were measured for the quantity of chlorophyll a (µg L⁻¹), and phaeophytin (µg L⁻¹) with a fluorometer after dark-extraction in 100% acetone. Replicate samples were analyzed for total organic carbon (TOC, µg L⁻¹) and total nitrogen (TN, µg L⁻¹) with a Shimadzu TOC and TN Analyzer (Shimadzu Corporation). Mean TOC, TN, chl a, and phaeophytin values are presented in Appendix II, Supplementary Table 2. Generalized linear models
(GLMs) were used to determine the statistical significance of environmental factors (CO₂, reef, time, and interaction terms) on concentrations (Appendix II, Supplementary Table 3).

**Statistical Analysis**

Abundance data (individuals m⁻³) were averaged across replicate transects (or emergence traps) within CO₂ levels, reefs and nights. Log abundance ratios for each zooplankton taxon were estimated with generalized additive mixed models (GAMM) with log link function and quasipoisson distribution using the predictors CO₂ (high-CO₂, control), reef (Upa-Upasina, Dobu), and expedition (1,2,3). Log abundance ratios were then back-transformed to obtain the abundance ratio (high-CO₂/control) of each taxon. GAMMs tested the effects of environmental parameters (CO₂, reef, and expedition) on the abundance of each zooplankton taxa and the results can be found in Appendix II, Supplementary Table 4.

Redundancy analysis (RDA) was used to assess the relationship between zooplankton communities and environmental variables (CO₂, reef, and expedition). Zooplankton abundances were 4th-root transformed. Permutation tests were used to determine the statistical significances of the environmental variables between the zooplankton communities.

To determine substratum preference of each zooplankton taxon, the photos were digitally adjusted for tilt and size. The percent coverage was estimated for the targeted substrata (coral rubble, branching coral and massive bouldering coral), as well as for other co-existing groups including sand, macroalgae, and turf algae. The influence of the percent coverage of each substratum category, CO₂, reef, and expedition on the abundance of each zooplankton taxon was evaluated using generalized linear models (GLMs) using a log link function and quasipoisson distribution.

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Note

Chapter 2 provides an overview of the broad-scale patterns occurring within zooplankton communities as a result of long-term ocean acidification exposure. Further detailed information on the zooplankton communities can be found in Appendix III, containing unpublished results that examine the zooplankton community with respect to two objectives:

1. Confirming that the zooplankton communities sampled were residential to the reef and not just the transient zooplankton that may flush through the open system of the high-CO₂ seeps. Unlike transient zooplankton, residential zooplankton would be exposed to the high-CO₂ conditions for extensive time periods, making ocean acidification research on zooplankton relevant for the CO₂ seep sites.

2. Investigating the taxonomic composition and fatty acid composition of the zooplankton under ocean acidification. Changes in either taxonomic composition or fatty acid content of the zooplankton would have nutritional implications for zooplanktivores.

The results shown in this study (Appendix III) confirm that the zooplankton are in fact demersal and residential to the coral reefs in our study sites. Furthermore, although the zooplankton abundances are reduced under-CO₂, detailed investigations reveal that the taxonomic composition and fatty acid content is unaffected by ocean acidification.

Please refer to Appendix III for more information on research objectives, methods, preliminary results, and conclusions that address these two topics.
A Case Study:
Pontellid Copepods
CHAPTER 3

Neustonic copepods (*Labidocera* spp.) discovered thriving as demersal zooplankton in coral reefs

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JNS and AC developed the concept, JNS and KF did the fieldwork, JNS and AC completed the laboratory work, JNS did the statistical analysis and wrote the manuscript, all authors contributed to edited and revising the manuscript.

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89
Abstract

Pontellid copepods are archetypical representatives of the neuston – the highly specialized community living in the very skin of the ocean surface. Their deep blue pigmentation and large eyes are unique adaptations to surface irradiation and predation but poor prerequisites to survival in the transparent waters beneath the sea-surface. Here, we report the discovery of three demersal (i.e. sea-floor associated) representatives of this group, Labidocera bataviae, L. pavo, and Labidocera sp., residential to coral reefs. We (1) document the presence of Labidocera spp. for two separate coral reefs on two expeditions to Papua New Guinea, (2) describe their migration behavior and substrate preference, and (3) quantify the effects of benthic reef community composition on their abundance. All life stages of Labidocera spp. were 43 to 94 times more abundant at the reef sites compared to offshore waters. Although pontellids are generally considered non-migrators, Labidocera spp. showed discernable diel vertical migrations: living in the substrata during the day, emerging into the water column at night (sometimes more than once), and re-emerging into the substrata at dawn. Labidocera spp. showed a pronounced substrata preference for coral rubble, macro algae, and turf, over branching coral, bouldering coral, and sand. In spite of its remarkable behavioral plasticity, changes in reef community composition caused by ocean acidification or bleaching or other human-induced shifts may have profound effects on Labidocera spp. populations residential to coral reefs.

Introduction

Copepods are microscopic crustaceans that constitute the bulk of zooplankton. Most copepods drift with the currents, however, some copepods are demersal. Thus, they live residential to specific benthic environments and emerge only temporarily into the water column, typically during the night\(^1\), taking advantage of the sheltering darkness to forage for food while avoiding visual predators\(^2\). During the day these copepods may live in or above the substrata\(^4\), swarm in the hyperbenthic layer above the seafloor\(^5\), or hide in crevices\(^7\). Different copepod
species are associated with sand flats, kelp beds, sea grass beds, lagoons, mangroves, and coral reefs.

Relatively little is known about the behavior and life histories of copepods living in coral reefs even though they are pertinent for coral health, fisheries production, and nutrient cycling within reefs. Some copepod species can readily be defined as either reef-associated or oceanic, while for other species the division is less clear. Some holoplanktonic groups (i.e. pelagic throughout their life) include representatives also known to inhabit coral reef environments and behave like typical reef zooplankton. Taxa with such high behavioral flexibility include members of the genera Acartia and Oithona. For neustonic copepods, living in the top centimeters of the sea surface, such behavioral plasticity is so far unknown.

Little is known about the family Pontellidae and their role in coral reefs. Of the seven genera of pontellid copepods, Calanopia are known to live within reefs. Most other pontellid genera are considered either oceanic or neritic and also neustonic. Their morphology is adapted for surface dwelling as they are highly pigmented, an adaptation to reduce the effects of damaging ultraviolet radiation and to hide from surface predators. Labidocera represents the largest genus in the family, with several species distributed throughout the Indo-Pacific. These neustonic copepods are often used as indicator species of different water masses, inshore-offshore boundaries, biogeographical boundaries, and seasons. Despite its obvious physical adaptations to live near the surface, the present study shows three Labidocera species to live within coral reefs. The objectives of this study are to (1) document the presence of Labidocera spp. in two Papua New Guinea coral reefs, (2) compare Labidocera spp. abundances between reef and offshore waters, (3) assess life stage composition (copepodites C2, C3, C4, C5 and adult males and females) at two separate reefs and for two expeditions, (4) determine migration patterns and substrata preferences, and (5) examine the impacts of reef composition on their abundance.
Materials and Methods

Study Site

Pontellid copepods were collected from tropical coral reefs that fringe the two sites Dobu and Upa-Upasina and in adjacent offshore waters approximately 500 m from the reef sites in Milne Bay Province, Papua New Guinea. Dobu and Upa-Upasina reef sites are 10.7 km apart from each other and separated by the large Normanby Island and Dobu Island (Figure 1). Both sites were sampled on two expeditions (24 May – 5 June 2013 and 22 March – 20 April 2014) while onboard the MV Chertan. During all collection times, the currents were longshore and weak (<0.03 m s\(^{-1}\)) and wave heights were 0.1 – 0.45 m. The two sites are located near natural CO\(_2\) seep sites described in ocean acidification studies on marine communities elsewhere\(^{33-35}\). In the present study, however, copepods were collected from the control coral reefs away from the seep sites, unaffected by CO\(_2\). Our primary objective was to document typically neustonic pontellid copepods living in healthy reefs unaffected by potential future environmental threats.

![Map of Study Sites](image)

**Figure 1.** Map of two reefs and offshore sites

Field Sampling

Abundances of *Labidocera* were compared to the abundances of other pontellid genera present (*Calanopia* and *Pontella*). Abundances were further compared between offshore and reef sites via horizontal net tows using a Nansen net (70-cm aperture diameter, 100-µm mesh size). Each horizontal net tow was conducted along a shore-parallel transect of approximately 30 m in length at a speed of ~1 knot, with the exact volume of water recorded with a Hydro-Bios digital
flowmeter attached to the center of the Nansen net aperture. Three replicate horizontal net tows were collected at an offshore site and reef site between 2100-0200 hrs on several consecutive nights of two separate expeditions (8 nights at Upa-Upasina and 2 nights at Dobu in 2013 and 6 nights at Upa-Upasina and 3 nights at Dobu in 2014). The reef sites were in shallow (2-3 m) waters with the net towed approximately 0.5 – 1.0 m above the coral and approximately 1.5 m below the sea surface. The offshore sites were also towed approximately 1.5 m below the sea surface but at a seafloor depth of 50-70 m.

During the second expedition, horizontal night tows were additionally collected over the course of 24-hour cycles to observe the migration patterns of *Labidocera* spp. Samples were collected over the reef at Upa-Upasina every two hours during dark hours (between 0630 and 0630 hrs), and every three hours during daylight hours. Four separate 24-cycles were collected over the course of a month with approximately one cycle per week (25-26 March 2014, 4-5 April 2014, 13-14 April 2014, and 18-19 April 2014). Sunset, sunrise, moonset, and moonrise times, along with percent moon illumination, were retrieved from open source data provided by the Astronomical Applications Department, U.S. Naval Observatory (http://aa.usno.navy.mil/data/).

In an attempt to fully understand the substrate preference of *Labidocera* spp., emergence tents of 100-μm mesh size were deployed over areas dominated by different substrata types at Upa-Upasina Reef for 5 nights during the second expedition. Tent dimensions were 1m x 1m x 1m (length x width x height) with a pyramidal design similar to Porter and Porter (1977)\(^{36}\). The detachable codends had a light (3 lumens) fixed inside to attract zooplankton. The emergence tents (nine per night) were deployed during daylight hours (between 1500-1700 hrs) and the codends were retrieved after nightfall once the plankton had time to emerge (between 2000 to 2100 hrs). Emergence tents were placed over patches of reef substrata dominated by coral rubble, branching coral, or bouldering coral (three emergence tents per substrata type). To be defined as any one of the main substrata categories, the base of the emergence tent, *i.e.* the quadrat, had to be dominated by at least 50% of that particular substratum. The quadrat was never 100% covered by any one category, so a photo was taken of each quadrat and post-field image analysis later calculated the percent coverage of different substrata types including
coral rubble, branching coral, bouldering coral, and with additional categories of sand, macro algae, and turf.

All samples collected from each method were preserved in 4% formalin buffered with sodium borate and stored for further analysis.

Laboratory Analysis

All samples collected were subsampled with a Folsom plankton splitter and half of the original samples were counted microscopically for pontellid copepods. Pontellidae were categorized into the dominant genera (Labidocera, Calanopia, Pontella, and Other Pontellidae). Labidocera specimens were predominant within the reef, thus they were identified to species and life stage. Life stages were recorded for copepodite stages C2, C3, C4, C5 female, C5 male, and adult males and females. Nauplii and copepodite stage CI were too small to be collected by the plankton net.

Labidocera spp. specimens were identified according to the descriptions of Scott (1909)\textsuperscript{37}, Mulyadi (2002)\textsuperscript{38}, and Hirabayashi and Ohtsuka (2014)\textsuperscript{39}. Labidocera bataviae constituted 70% of the Labidocera genus group, with scattered occurrences of L. laevidentata, L. pavo, and a species possibly new to science (Labidocera sp.). While L. laevidentata was easily recognizable due to its cephalic hooks\textsuperscript{38}, they contributed to less than 1% of the Labidocera abundance and were removed from further analysis. The other three species were of the same size and morphologically closely related, with the adults only differing in the shape of the 5th swimming leg and the structure of the urosome\textsuperscript{28}. Photographs of the copepod, urosome, and 5th swimming leg of adult female L. bataviae, L. sp. and L. pavo can be found in Figure 2. Note: prior to preservation all copepods in Figure 2 were dark blue in color. The two described species, L. bataviae and L. pavo, have both been documented as neritic, but have also been found in surface waters between 10-40 km of tropical Pacific Islands, (<1% of present pontellids)\textsuperscript{30}. They belong to the pavo species group within the L. detruncata species complex and are therefore closely related. The unidentified species also shows the characteristics of the pavo group. Thus, we assume that their behavior in the reef is very similar and we based our results on the combined abundances of the three species.

94
Statistics

All statistical analyses were computed in R, version 3.2.2 (R Development Core Team, 2015). Generalized linear models (GLMs) were used to determine if there were differences in abundance between offshore and reef, expedition (one vs. two), or sites (Upa-Upasina vs. Dobu) for *Labidocera* spp., and for the other present pontellid genera (*Calanopia* sp., *Pontella* sp., or Other Pontellidae). GLMs were also used to determine if *Labidocera* spp. abundance was correlated to percent cover of the different substrata types (coral rubble, branching coral, bouldering coral, sand, macro algae, and turf) and date. Data distributions were chosen for each GLM and diagnostics of model stability (leverage, Cook’s and dfbetas) were calculated\(^4\). All model stability checks indicated that no influential cases or outliers existed in the data. ANOVAs were applied to the optimal GLMs.

![Figure 2. Photos of the three *Labidocera* species – *Labidocera bataviae* (female): a habitus, b uroscope, c swimming leg 5 (P5); *Labidocera* sp. (female): d habitus, e uroscope, f P5; *Labidocera pavo* (female): g habitus, h uroscope, and i P5. Scale bars - A,D,G: 500 µm (lower right); B,C,E,F,H,I: 100 µm (upper right)](image-url)
Results

Comparison of reef versus offshore abundances

Very few pontellid copepods were found offshore. In contrast, pontellids of the genus *Labidocera* occurred in high abundance over the reefs at both Upa-Upasina and Dobu, and those of the genus *Calanopia* were also present at both reefs, albeit in lower abundances (Figure 3). Although abundance varied between dates and expeditions, there were consistently more *Labidocera* spp. and *Calanopia* sp. present over the reef compared to offshore (Figure 4). Results from the GLM indicate that *Labidocera* and *Calanopia* were both more abundant over the reef and varied in abundance depending on the expedition, with *Labidocera* abundances also differing between sites; meanwhile, none of the factors (reef vs. offshore, expedition, or site) affected the abundances of *Pontella* or 'Other Pontellids' (Table 1). For all pontellid genera, abundances were not significantly different ($p > 0.05$) for the two-way and three-way interactions between the factors.

![Box plot of pontellid abundances](image)

**Figure 3.** Abundance of pontellid genera at offshore and onshore sampling locations at two separate coral reefs in Papua New Guinea.
Table I. Results from generalized linear models examining the differences in abundance of various pontellid genera in response to reef (reef vs. offshore), expedition (1 vs. 2), or site (Upa-Upasina vs. Dobu). Two-way and three-way interactions did not significantly affect the abundance of any pontellid genera ($p > 0.05$). Df=1 for all analysis.

<table>
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<tr>
<th>Pontellid Genus</th>
<th>Reef-Offshore</th>
<th></th>
<th>Expedition</th>
<th></th>
<th>Site</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$X^2$</td>
<td>$p$</td>
<td>$X^2$</td>
<td>$p$</td>
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<td>$p$</td>
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<tr>
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<td>63.3</td>
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<td>2.74</td>
<td>0.102</td>
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<tr>
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<td>0.14</td>
<td>0.711</td>
<td>0.19</td>
<td>0.668</td>
</tr>
</tbody>
</table>

Figure 4. Copepod abundances over the reef (circle) are compared to offshore (triangle) for the two pontellid genera *Labidocera* and *Calanopia* at Upa-Upasina and Dobu over 19 nights from two expeditions.
Due to the noticeable dominance of *Labidocera* spp. amongst the pontellid copepods, additional information on their life stages was also determined during the second expedition. All life stages were found more abundant living over the reef. Also, there was no one particular life stage that seemed drastically more likely to be advected offshore (Figure 5).

**Figure 5.** The a abundance (individuals m$^{-3}$) and b percent composition of each life stage of *Labidocera* spp. collected from nocturnal horizontal tows at Upa-Upasina and Dobu study sites during the second expedition. Stage composition is compared between samples collected over coral reefs (dark grey) and several hundred meters offshore from the fringing reef crests (light grey). Life stages exclude the nauplii stages and the first copepodite stage (C1), but include copepodite stages C2, C3, and C5 and the adults.
Nightly migration patterns

Diurnal nightly migration patterns were observed for Labidocera spp. at Upa-Upasina over the reef for four separate 24-hr cycles within a one-month period (Figure 6). Labidocera spp. remained amongst the substrata during the day and emerged into the water column after dusk, re-entering into the substrate at dawn. A second emergence often occurred in the middle of the night (between 0000-0200 hrs). The exact migration patterns and abundances differed between the four cycles and there was no distinct pattern that coincided with moonlight level.

Figure 6. Nocturnal migration patterns of Labidocera spp. abundance over Upa-Upasina reef collected via horizontal tows

Substrate preferences

Substrate preference was only analyzed for Labidocera spp. living at Upa-Upasina on the reef. Results from a multi-factor GLM reveal that Labidocera spp. abundance, collected via emergence tents placed over patches of coral reef substrata, were significantly influenced by the percent cover of coral rubble (ANOVA, \( F_{(1,36)} = 4.45; p = 0.04 \)), macro algae (ANOVA, \( F_{(1,32)} = 7.2; p = 0.04 \)), and turf (ANOVA, \( F_{(1,31)} = 12.2; p = 0.009 \)). The cover of branching coral (ANOVA, \( F_{(1,35)} = 3.27; p = 0.08 \)), bouldering coral (ANOVA, \( F_{(1,34)} = 3.72; p = 0.06 \)), and sand (ANOVA, \( F_{(1,33)} = 3.64; p = 0.07 \)) did not significantly affect Labidocera spp. abundances (Figure 7). Although macro algae and turf never dominated a quadrat (<20% of cover), they proved a suitable substratum for Labidocera spp. to reside within. When separated into the three dominant substratum categories, coral rubble appears to be the substrate of choice for all life stages of Labidocera spp. at Upa-Upasina Reef compared to branching coral and bouldering coral (Figure 8). Additionally, the number of Labidocera spp. present was significantly different between sampling days during the substrata preference collection period (ANOVA, \( F_{(1,27)} = 33.6; p < 0.001 \)).
Figure 7. *Labidocera* spp. abundance at Upa-Upasina Reef as a function of percent cover of six substrate types: coral rubble, branching coral, bouldering coral, sand, macro algae, and turf. A single multi-factor generalized linear model (GLM) revealed which substrata types had an influence on *Labidocera* spp. abundance and the GLM results ($F_{(1,30)}$, and p values) are included in each plot. The solid line represents the linear regression between percent cover of each substrata and *Labidocera* spp. abundance, while the dashed lines mark the 95% confidence intervals.
Discussion

In the Indo-Pacific region, Labidocera bataviae and L. pavo are pontellid copepods known to be both neritic and neustonic\textsuperscript{26}, but this study provides evidence that at least some populations of Labidocera spp. are able to reside within coral reefs. All life stages of Labidocera spp. were consistently more abundant over the reef compared to offshore samples at two coral reefs in Papua New Guinea and over two expeditions (Figure 4 and 5). Although far less abundant than Labidocera spp., the pontellid copepod Calanopia sp. was also more abundant living within coral reefs (Figure 3 and 4), confirming earlier observations for this genus\textsuperscript{21,22}.

Labidocera species are known to live continuously at the sea surface and are non-migrants\textsuperscript{41}. Labidocera spp. populations living in coral reefs, alternatively, live amongst coral rubble, macro algae, and turf during the day and then at dusk they
migrate into the water column, sometimes emerging for a second time in the middle of the night, before returning to the substratum at dawn (Figure 6). Diurnal migration is a common behavior for demersal zooplankton that live within coral reefs\(^1,3\). Different species of zooplankton may migrate in slightly different patterns\(^1\), but in general *Labidocera* spp. mimic the behavior of the demersal zooplankton around them and emerge into the water column at night to forage and escape predation from nocturnal planktivorous fish and heterotrophic corals that extend their polyps at night\(^42,43\). At dawn they return to the substrata to avoid visual detection from fish\(^44\).

*Labidocera* spp. emergence patterns depend on changes in diurnal light, although other factors are likely a cue. From other studies in coral reefs, demersal zooplankton emergence patterns depend in part on circadian rhythms and the lunar cycle\(^1,45–47\), although the lunar cycle has no obvious effect on *Labidocera* spp. migration patterns (Figure 6). In estuarine areas, certain copepod species will migrate when tides shift and currents are low allowing them to maintain their position in a bay and not be swept away\(^48,49\). This may explain why *Labidocera* spp. sometimes emerge in the middle of the night even though there is no immediate change in light. Although *Labidocera* generally do not diurnally migrate and instead remain at the surface, diurnal migration has been observed for the species *Labidocera euchaecta*, *Labidocera jaafari*, and *Labidocera pectinata* in a mangrove estuary in Malaysia\(^50\), showing the ability of some *Labidocera* species to change their behavior within a mangrove. *L. pavo* has also been observed swarming and nocturnally migrating, sometimes with the tide, in subtropical waters near Japan\(^51–53\). As seen in this study, *Labidocera* spp. also begin to exhibit diurnal migration behavior within a coral reef ecosystem.

*Labidocera* spp. abundance varied throughout the night and also between each of the four cycles (Figure 6). The difference in diurnal patterns and the number of copepods that underwent the patterns may explain why there are such high variances in the abundances between days sampled and between expeditions, with order of magnitude differences between samplings (Figure 4). Samples were collected for several hours throughout the night (2100-0200 hrs), over several days, and for two expeditions. *Labidocera* spp. were likely collected throughout different parts of their migration pattern, thus explaining why abundances vary so much between the days and expeditions. Furthermore, Upa-Upasina reef notably had more *Labidocera* spp. present compared to Dobu (Figure 3 and 4). The reasons why Upa-
Upasina reef had greater abundances of *Labidocera* spp. are unknown but could be due to many possible reasons, like differences in food availability or the number of predators in the surrounding area. Despite variation in abundance across space and time, the pattern remained consistent that more *Labidocera* spp. lived residential to coral reefs compared to offshore and they altered their behavior to diurnally migrate within the reef.

Once sunlight dawned and *Labidocera* spp. return to the substrata, they preferred to live in association with coral rubble, macro algae, and turf (Figure 7), even though macro algae and turf only covered a small percentage of the area within the emergence tents. Macro algae and turf are home to many harpacticoid copepods,$^{54,55}$ and are an important link to providing food for coral reef fishes.$^{54,56}$ However, less is known about calanoid copepods living within macro algae. Furthermore, calanoid copepods are known to inhabit coral rubble, but often have a greater preference for living near branching coral in parts of the Great Barrier Reef.$^5$

Percent cover of branching coral did not influence *Labidocera* spp. abundance within the Papua New Guinea reefs. In some coral reefs, branching corals have a higher number of zooplankton associated with them because the increased structural complexity offers more hiding places.$^4,36$ On the contrary, branching corals have a larger surface area of stinging tentacles that can capture copepods and other zooplankton.$^{57}$

Percent cover of bouldering coral also did not have an impact on *Labidocera* spp. abundance. Other calanoid copepods like *Acartia* have been observed swarming around bouldering corals, sometimes even mimicking the shape of the coral rock as a means to avoid predators and also to maintain their position within reefs by hiding from currents.$^{58}$ Different copepod species prefer different substrata to seek refuge, and although other copepod species may like to hide around branching coral or bouldering coral, *Labidocera* spp. did not like either and instead prefer to live within the coral rubble, and when present, in macro algae and turf.

In order for *Labidocera* spp. to make behavioral changes in their migration patterns and living preferences, there must be some advantages compared to remaining non-migratory and oceanic, including higher food availability. The advantage of increased food certainly comes at an expense since living in the reef means living with additional predators not found in the open ocean like planktivorous reef fish, corals, and other benthic planktivores.$^{15,16,47}$ Increased flexibility in plankton
behavior are a reflection that these copepods are highly evolved, and yet there are several unknowns about reef-dwelling copepods. For example, the origin, time of arrival, and site fidelity of reef-dwelling Labidocera spp. are unknown. Labidocera development is temperature dependent and in tropical waters the growth from nauplii to adulthood is between 14-15 days\textsuperscript{59}. Within one year several generations of Labidocera spp. may have lived in association with coral reefs at both Upa-Upasina and Dobu. All life stages captured in the net tows and emergence tents (copepodite stage C2 through adulthood) are more abundant in the reef and within coral rubble (Figure 5 and 8), with connectivity between the two reefs fairly minimal considering so few copepods are swept offshore. Interestingly, no life stage seemed more vulnerable to export. Juvenile copepods are weaker swimmers than adults\textsuperscript{60}, and yet the juvenile Labidocera spp. are not disproportionately swept away from the reef, although nothing is known about the nauplii stages or copepodite stage C1 since they were too small to be caught by the 100-\textmu m mesh nets.

Despite the knowledge gaps, all evidence suggests that Labidocera spp. has adapted its lifestyle to live residential within coral reefs and its abundance suggests an important food source sustaining reef trophodynamics. Corals consume zooplankton and acquire essential nutrients like nitrogen and phosphorus otherwise unattained from their carbon-only diet procured from symbiotic zooxanthellae living in their tissue\textsuperscript{15,57}. These nutrients are necessary for zooxanthellae regulation\textsuperscript{61}, reproduction\textsuperscript{62,63}, and tissue and skeletal growth\textsuperscript{64}. Without heterotrophy coral health may be compromised. Furthermore, as the dominant taxonomic group in zooplankton, copepods also help sustain reef fisheries. Planktivorous fish form ‘walls of mouths’ at the upstream reef margin\textsuperscript{16} where they feed voraciously on copepods, sometimes with different fish species preferring different copepod species\textsuperscript{65,66}. Growth rates and survivorship of larval fish in part depend on copepod and other zooplankton availability\textsuperscript{67}. Any changes in copepod abundances may impact larval fish populations\textsuperscript{17}, and any mechanism that affects the planktonic larval stage of many large growing reef fish will potentially affect reef fisheries.

One co-factor that might influence Labidocera spp. abundances is a change in the reef’s benthic community composition since these copepods have a preferred habitat to live in. Changes in the reef benthos are expected for future coral reefs impacted by ocean acidification, bleaching, and other human-induced shifts in the reef. For example, coral reefs exposed to long-term ocean acidification conditions
show a shift from complex, structural corals to more massive bouldering corals\textsuperscript{33}, macroalgae dominated communities\textsuperscript{66}, or soft-corall communities\textsuperscript{69}. In the first possibility, *Labidocera* spp. abundance would decline as they were shown to be negatively correlated with bouldering corals. In a macroalgae environment they would thrive, and in a soft-coral community it is unknown how their abundances would change. Overall, changes in the reef benthic community would likely impact *Labidocera* spp. abundances with consequences for the rest of the plankton community, triggering further changes in the coral reef ecosystem.

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CHAPTER 4
Pontellid copepods, *Labidocera* spp., affected by ocean acidification:
A field study at natural CO$_2$ seeps

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All authors developed the research question, JNS and KF conducted the fieldwork, JNS and AC completed the laboratory work, JNS completed the statistical analysis and wrote the paper, all authors contributed to editing and revising the manuscript.

This manuscript has been submitted to *PLoS ONE*. 
Abstract

Natural CO₂ seeps in coral reefs were used as natural laboratories to study the impacts of ocean acidification on the pontellid copepod, *Labidocera* spp. Pontellid abundances were reduced by ~70% under high-CO₂ conditions. Physiological parameters and substratum preferences of the copepods were explored to determine the underlying causes of such reduced abundances. Stage- and sex-specific copepod lengths, feeding ability, and egg development were unaffected by ocean acidification, thus changes in these physiological parameters were not the driving factor for reduced abundances under high-CO₂ exposure. *Labidocera* spp. are demersal copepods, hence they live amongst reef substrata during the day and emerge into the water column at night. Deployments of emergence traps showed that their preferred reef substrata at control sites were coral rubble, macro algae, and turf algae. However, under high-CO₂ conditions they no longer had an association with any specific substrata. Results from this study indicate that even though the physiology of a copepod might be unaffected by high-CO₂, *Labidocera* spp. are highly vulnerable to ocean acidification, possibly due to their impaired ability for substratum selection within the reef.

Introduction

Copepods are microscopic crustaceans that dominate most freshwater and seawater zooplankton communities¹,², from the tropics to the poles³. They have a wide range of morphologies and behaviors⁴, and play an important ecological role in aquatic food chains. Within the marine realm, copepods are also vital to the microbial loop, remineralization of nutrients, and the biological pump⁵,⁶. Because copepods are a crucial link between phytoplankton primary producers and higher trophic levels, any changes in copepod populations may disseminate throughout entire marine ecosystems.

Anthropogenic carbon dioxide emitted into the atmosphere gets absorbed by surface waters in the ocean and changes its chemistry⁷,⁸. The addition of carbon dioxide limits the amount of available carbonate ions in the water column and reduces seawater pH, in a process called ocean acidification (OA)⁹–¹¹. Lowered aragonite and calcite saturation states under OA reduce calcification⁸,¹²,¹³, thus initial OA research on plankton primarily focused on calcifying taxa like coccolithophores.
and pteropods. In recent years, effort has been extended to also understanding OA impacts on copepods. The exoskeletons of copepods are composed of chitin, a modified polysaccharide containing nitrogen. Chitin contains no calcium carbonate and is therefore considered unresponsive to OA. Nonetheless, the sheer abundance and importance of copepods to global ocean ecosystems makes understanding their reaction to changes in seawater chemistry indispensable.

To date, the effect of OA on planktonic copepod species worldwide is poorly understood. In part this is due to the high diversity of marine copepods (>2,000 species described to date), with various species likely responding differently to the same stress. The initial consensus was that copepods are mostly tolerant to OA, although recent evidence has begun to challenge this viewpoint.

Multigenerational studies on copepods under OA conditions suggest that naupliar production declines, juveniles are often more sensitive than the adults, metabolic costs increase, and reproductive success becomes limited. Copepods exposed for short experimental periods to OA conditions are often more negatively impacted than copepods that have been exposed to OA for a second generation. The ability of copepods to tolerate changes in seawater pH is also highly associated with the natural range of environmental conditions they live in. Additional research indicates that OA may alter the nutritional quality of copepod prey, which has negative consequences for copepod somatic growth and egg production. Furthermore, changes in nutritional quality can reduce the trophic transfer efficiency of carbon from phytoplankton to copepods, although changes in the phytoplankton caused by OA do not always have a negative impact on copepods. Combining all the research on how copepods may cope with OA shows that the answer is quite complex. Responses are likely species-specific, with several species expected to fare well under OA, and both direct and indirect impacts affecting copepods simultaneously.

Most studies thus far on copepods have been conducted in the laboratory and on generalist species that are naturally tolerant to a wide range in environmental parameters and laboratory conditions. Laboratory experiments provide valuable information on understanding the underlying mechanisms of how OA affects the copepods, however few copepod species have been studied to date, and no single species has been studied for its response to OA in its natural environment. The study presented here is novel for two reasons: first, this is the first study to examine
OA effects on a copepod species in its natural environment. Second, we investigated non-generalist copepods adapted to a narrow range of environmental conditions under the assumption it may be less tolerant to change, including OA, than generalist species that live in a wide range of conditions. We conducted this field study at natural CO$_2$ seep sites in coral reefs where copepods live residential within their natural habitat, and have been exposed to OA for their entire lifetime and likely for multiple generations.

*Labidocera* spp. were studied because these pontellid copepods were found to be highly reduced at these seep sites$^{28}$. Although *Labidocera* spp. are traditionally considered as neustonic species, some species live residentially within coral reefs$^{39}$. Due to their apparent sensitivity to OA, we chose to study *Labidocera* spp. in greater detail to understand the effects of OA on their biology. This study had the following objectives: 1.) Determine the effects of OA on total abundances as well as for each life stage for copepodites C2-C5 and adults in *Labidocera* spp., 2.) Determine if aspects of their physiology, specifically copepod length, feeding, and egg development, were affected by OA, and 3.) Determine if their associations with daytime reef substrata were affected by OA.

**Methods**

**Study Site**

The effects of ocean acidification on *Labidocera* spp. were examined at two separate CO$_2$ seeps and adjacent control sites (Dobu and Upa-Upasina) in Milne Bay Province, Papua New Guinea. The seeps release ~99% CO$_2$ gas into fringing coral reefs, locally reducing seawater pH. The higher $p$CO$_2$ and associated changes in the carbonate chemistry parameters are the only differences in seawater chemistry between the seeps and the adjacent control sites$^{40}$. Water temperature (27-29°C) and salinity (~34.5 psu) are similar along the CO$_2$ gradients, and so are geomorphology and oceanographic parameters of the study sites, with water depths between 2-3 m and slow long-shore currents < 5 cm s$^{-1}$. Copepods were collected and compared between control (averaged pH$_{cal}$ = 8.0) and high-CO$_2$ sites (averaged pH$_{cal}$ = 7.8) at these two separate seeps and their associated control reefs, and for
two expeditions (24 May - 9 June 2013, and 22 March – 17 April 2014) while onboard the M/V Chertan.

Sample Collection

Copepods were collected at night using horizontal net tows and emergence traps. Three replicate horizontal net tows were collected per night at both the control and high-CO2 sites between 2100-0200 hours over several consecutive nights at both seeps and during both expeditions. Each tow was along a 30 m transect parallel to the shoreline using a Nansen net (70 cm aperture diameter, 100 µm mesh size) at a speed of approximately 1 knot. The tows were conducted in shallow water (2-3 m depth) with the plankton net approximately 1 m above the reef. A Hydro-Bios digital flowmeter was attached to the center of the net aperture to record the exact volume of the water sampled.

Emergence traps were deployed during the second expedition only at the Upa-Upasina reef. The pyramid-shaped 1 m tall emergence traps were made of 100 µm plankton mesh attached to a 1x1 m² quadrat, following the design of Porter and Porter (1977)⁴¹. Detachable cod-ends that contained a weak light (3 lumens) were attached to the top of the pyramid. The traps were deployed during the day between 1500-1700 hours when few zooplankton were present in the water column. Cod-ends were collected at night between 2000-2100 hours, after the demersal copepods emerged into the water column after dusk (~18:30). Emergence traps were placed over three dominant substrata types: coral rubble, branching coral, and massive bouldering coral, where 'dominant' was defined as >50% cover by the given type of substratum. Since no quadrat was covered 100% by any one substratum type, photos were taken of each quadrat and the percent coverage of the three dominant and non-dominant substrata (sand, fleshy macro algae, and turf algae) were estimated. Nine tents were deployed per night (3 replicates per 3 dominant substratum type) at either the control site or the high-CO2 site, alternating between two CO2 sites.

All samples were preserved in 4% formalin buffered with sodium borate and stored for further analysis.
Laboratory Analysis

Samples from both the horizontal tows and emergence traps were divided in half using a Folsom splitter, and *Labidocera* spp. abundances were counted in half of the original sample using microscopy. Additionally, *Labidocera* spp. collected during the second expedition were enumerated by life stage (copepodite stages 2 – 5 [C2-C5] and adults). Males and females were identified separately for copepodite C5 and adults. The youngest life stages were not counted since they were too small to be caught with the 100 µm mesh of the plankton net.

Total length was measured for subsamples of *Labidocera* spp. to determine if size differences may occur under OA. For 248 females from the horizontal tows, the gut fullness and the maturity of the oocytes were also examined. The gonad morphology of *Labidocera* spp. matched the description of the *Acartia*-type gonad\(^4\), where all oocyte developmental stages are present. Only the mature oocytes were counted in the adult females.

To compare feeding ability, the guts of the 248 female specimens were dissected. It was noted whether the guts of the female copepods were empty, 1/3 full, 2/3 full, or completely full. Compact fecal pellets were only rarely observed.

Statistical Analysis

All statistical analyses were computed in R version 3.2.2 (ref 43). Generalized linear models (GLMs) with a quasipoisson distribution and log link function were used to determine the effects of CO\(_2\), reef, and expedition on *Labidocera* spp. abundance on total abundances, abundances of each life stage, and the number of mature oocytes inside the adult females. GLMs with a gaussian distribution were used to determine effects of CO\(_2\) and reef on total length for each life stage. GLMs with a quasibinomial distribution were used to determine the effects of CO\(_2\) and reef on gut fullness. Model assumptions of independence, homogeneity of variance, and normality of error were evaluated through diagnostic tests of leverage, Cook's distance, and dfbetas\(^4\). Checks for all GLMs indicated that no influential data points or outliers existed in the data and model assumptions were met.
Results

Four species of *Labidocera* were present in the samples, with a strong dominance by *L. bataviae* (~70% of *Labidocera* specimens). *L. pavo*, *Labidocera* sp. (a yet un-described new species), and *L. laevidentata* were the other species identified. The latter was morphologically different from the other three species\(^4^5\), rare (<1%) and, was therefore excluded from further analysis. However, *L. bataviae*, *L. pavo*, and *Labidocera* sp. are closely related and belong to the *pavo* species group within the *L. detruncate* species complex\(^4^6\). These three species are considered to have a similar lifestyle and have the same size ranges; they are morphologically nearly identical, except that the shape of the 5th swimming leg and the urosome is different in the adult stage\(^4^6\). Thus, for this study *Labidocera* sp. represents the three species *L. bataviae*, *L. pavo*, and the un-described species *Labidocera* sp.

*Reduced abundances for later life stages under high-CO₂ conditions*

Total abundances of *Labidocera* spp. were highly reduced at the high-CO₂ sites (*F*(1,112) = 76.8, *p* < 0.001; Figure 1), in spite of the also significant differences in abundance between reefs (*F*(1,111) = 15.4, *p* < 0.001), expeditions (*F*(1,110) = 10.2, *p* = 0.002), and the interaction between reef and expedition (*F*(1,107) = 5.1, *p* = 0.027). Two-way interaction terms (CO₂: reef and CO₂: expedition) had no significant influence on total *Labidocera* spp. abundance (*F*(1,109) = 0.69, *p* = 0.410 and *F*(1,108) = 0.13, *p* = 0.714), and the three-way interaction term (CO₂: reef: expedition) was also non-significant (*F*(1,106) = 1.2, *p* = 0.274).

*Labidocera* spp. abundances were significantly higher at the high-CO₂ reefs (10.6 ± 2.2 SE copepods m⁻³) compared to offshore waters (3.2 ± 1.2; *p* = 0.004) where water depth was 50-70 m and reefs were absent. This difference in abundance confirmed a large proportion of the *Labidocera* spp. were still resident to the seeps.

The abundance of each life stage was examined in the samples from the second expedition. Results showed that most life stages were significantly reduced under ocean acidification at both reefs, with a few life stages responding differently to high-CO₂ between the two reefs (Table 1, Figure 2A). There was no difference in abundance between control and high-CO₂ sites for copepodite C2 (*F*(1,16) = 2.8, *p* = 0.119), which were quite rare in the samples (2% of individuals). Furthermore, there
were no differences in the percent composition of each life stage within the total *Labidocera* spp. community between CO₂ levels or reefs (Figure 2B). The ratio between copepodites to adults was not different between CO₂ levels ($F_{(1,16)} = 0.9$, $p = 0.368$), or the interaction between CO₂ and reefs ($F_{(1,14)} = 0.1$, $p = 0.819$), but it was different between reefs ($F_{(1,15)} = 7.0$, $p = 0.019$). Also, the ratio of males to females was unaffected by all parameters, CO₂ ($F_{(1,16)} = 0.01$, $p = 0.937$), reef ($F_{(1,15)} = 0.4$, $p = 0.531$), and the interaction between the two ($F_{(1,14)} = 0.6$, $p = 0.443$).

**Figure 1.** Differences in *Labidocera* spp. abundance between CO₂ sites and reefs.

**Table 1.** Effects of CO₂ sites, reefs, and their interaction on abundances of *Labidocera* spp. life stages.

<table>
<thead>
<tr>
<th>Life Stage</th>
<th>CO₂</th>
<th>Reef</th>
<th>CO₂:Reef</th>
</tr>
</thead>
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<tr>
<td></td>
<td>$F_{(1,16)}$</td>
<td>$p$</td>
<td>$F_{(1,16)}$</td>
</tr>
<tr>
<td>C2</td>
<td>2.8</td>
<td>0.119</td>
<td>0.9</td>
</tr>
<tr>
<td>C3</td>
<td>6.2</td>
<td>0.026</td>
<td>0.5</td>
</tr>
<tr>
<td>C4</td>
<td>11.8</td>
<td>0.004</td>
<td>8.4</td>
</tr>
<tr>
<td>C5 female</td>
<td>31.0</td>
<td>&lt;0.001</td>
<td>20.3</td>
</tr>
<tr>
<td>C5 male</td>
<td>27.0</td>
<td>&lt;0.001</td>
<td>23.4</td>
</tr>
<tr>
<td>adult female</td>
<td>6.4</td>
<td>0.024</td>
<td>0.1</td>
</tr>
<tr>
<td>adult male</td>
<td>5.8</td>
<td>0.031</td>
<td>0.1</td>
</tr>
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</table>
Figure 2. *Labidocera* spp. (a) abundance and (b) percent composition for life stages compared between CO$_2$ levels.
Copepod physiology unaffected by ocean acidification

a.) Copepod lengths unaffected by OA

Since the life stages have the same size ranges across the three Labidocera species, we combined the lengths of all three species for all length analyses. There was no difference in copepod lengths between CO₂ levels for any of the life stages (Figure 3). Males and females are dimorphic, with females being larger than the males, and this is evident beginning with the last copepodite stage (C5) and into adulthood. There was no difference in lengths between high-CO₂ and control sites for either the adult males, the adult females, or the copepodite life stages.

Figure 3. Difference in length of life stages of Labidocera spp. between CO₂ levels.

b.) No difference in gut fullness under OA

There was no difference in the gut fullness of adult female copepods between CO₂ sites ($\chi^2 = 114, \text{ df } = 152, p = 0.20$), but gut fullness differed between reefs (greater gut fullness at Upa-Upasina then at Dobu reef ($\chi^2 = 356, \text{ df } = 151, p = 0.02$).
c.) *No difference in the number of oocytes under OA*

Immature oocytes in the ovaries and the diverticula were present in all females, but not all females had mature gonads. Thus, only the occurrence and number of mature oocytes were noted in adult females. The number of mature oocytes in the adult females copepods was not different between CO₂ levels ($\chi^2 = 20, \text{ df} = 152, p = 0.18$), but copepods at Upa-Upasina had more mature oocytes than those at Dobu ($\chi^2 = 614, \text{ df} = 151, p < 0.01$).

*No substrate association under ocean acidification*

Emergence traps data showed a reduction in *Labidocera* spp. abundance at the high-CO₂ site over all types of substrata. At the control reef, their abundances were significantly associated with the cover of coral rubble, macro algae, and turf algae (Figure 4). In contrast, at the high-CO₂ site, their abundances were not correlated with any specific substratum (Figure 4; Table 2). Instead, their numbers were consistently low for all substrata, suggesting a CO₂-related loss in substrata preference capacity.

![Figure 4](image-url)  
*Figure 4.* *Labidocera* spp. abundances and substrata cover regressions at high CO₂ and control sites of Upa-Upasina.
Table 2. Effect of date and substrata cover on *Labidocera* spp. abundance at control and high-CO₂ sites.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th></th>
<th></th>
<th>High-CO₂</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(df,df)</td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Date</td>
<td>4,26</td>
<td>22.6</td>
<td>&lt;0.001</td>
<td>2.7</td>
<td>0.053</td>
</tr>
<tr>
<td>% Coral Rubble</td>
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<td>4.4</td>
<td>0.044</td>
<td>0.03</td>
<td>0.872</td>
</tr>
<tr>
<td>% Branching Coral</td>
<td>1,34</td>
<td>3.3</td>
<td>0.082</td>
<td>2.2</td>
<td>0.147</td>
</tr>
<tr>
<td>% Massive Bouldering Coral</td>
<td>1,33</td>
<td>3.7</td>
<td>0.064</td>
<td>0.15</td>
<td>0.699</td>
</tr>
<tr>
<td>% Sand</td>
<td>1,32</td>
<td>3.6</td>
<td>0.067</td>
<td>0.13</td>
<td>0.719</td>
</tr>
<tr>
<td>% Macro Algae</td>
<td>1,31</td>
<td>4.7</td>
<td>0.040</td>
<td>1.3</td>
<td>0.258</td>
</tr>
<tr>
<td>% Turf</td>
<td>1,30</td>
<td>7.9</td>
<td>0.009</td>
<td>0.09</td>
<td>0.755</td>
</tr>
</tbody>
</table>

Discussion

Our field study examining the effects of ocean acidification on the pontellid copepod *Labidocera* spp. showed reductions in total abundances and in the abundances of in most life stages and in both sexes (copepodite C3-C5 and adult life stages). Volcanic CO₂ seeps create conditions to study *in situ* changes to OA for fully acclimatized groups of organisms in their natural habitat, i.e., under natural levels of food and substratum availability, predation, currents, temperature and light, and unaltered capacity for nocturnal migration. Our results were consistent across two separate seep sites, and over two expeditions. We have shown before that total abundances of zooplankton residing in coral reefs may be reduced in response to OA, with some species-specific differences in the severity of responses between taxa^{28}. Here we show that for the pontellid copepods, all life stages were reduced under high-CO₂ at Upa-Upasina, while only adults but not younger life stages were reduced at Dobu. We also show that reductions in *Labidocera* spp. abundances were not due to changes in stage-specific sizes, feeding (gut fullness), or reproduction (oocyte numbers). In contrast, our data suggest that under future OA conditions, these copepods no longer associate with a specific habitat type.

For the parameters measured, copepod physiology was unaffected by ocean acidification. Growth is often measured on individual copepods from start to end of an experiment, or from length-weight ratios^{47}; both methods were not suitable for this field study as it is unknown how copepod weights compare between CO₂ levels and
we did not measure feeding within a specific time frame to calculate feeding rates. Instead, we measured copepod lengths for hundreds of individuals from both the control and high-CO₂ sites for all life stages, and found that copepod lengths were similar across CO₂ levels for all life stages measured (Figure 3).

Gut fullness is an indicator of feeding, food assimilation and egestion. The similar levels of gut fullness between the control and high-CO₂ sites suggested that feeding ability was unaffected by OA. Most laboratory experiments examine feeding rates between a start and end time point under exposure to different CO₂ levels. Laboratory experiments on copepod feeding rates under ocean acidification have shown mixed results, with grazing of some species unaffected by high-CO₂ and other species increasing their feeding rates. Logistic constraints precluded the execution of incubation experiments to measure feeding rates. However, we measured gut fullness, which is an estimate of their ability to feed, and for Labidocera spp. their feeding seemed unimpaired by OA. Thus, bottom-up constraints from consuming different quantities of food are unlikely to explain the reduced abundances found at the high-CO₂ reefs.

The quantity of food found in the guts of Labidocera spp. remained unaltered under OA, but perhaps changes in their diet may have contributed to their reduced abundances. Labidocera spp. are omnivorous, consuming phytoplankton and small zooplankton. Phytoplankton biomass did not differ between the control and high-CO₂ sites, and the quality of phytoplankton is also assumed to be similar between CO₂ levels. However, the abundance of other zooplankton taxa, including smaller copepods like Paracalanidae that Labidocera spp. may feed on, is reduced at the high-CO₂ seeps sites. Thus, Labidocera spp. may rely more on phytoplankton for food if copepod prey density is reduced. The repercussions of such changes in their diet should be explored to further understand possible causes for their reduced abundances.

Multigenerational studies suggest that egg production can be either suppressed or unaffected by elevated CO₂ (ref 30), depending on the copepod species. As Labidocera spp. are residential to the reef, they are assumed to be exposed to ocean OA conditions for the majority of their lifetime, and likely for multiple generations. Isolated islands often have endemic species of coastal zooplankton suggesting they have successful retention mechanisms; nonetheless, nothing is known about the connectivity of these copepods between reefs, or
whether these copepods self-recruit as do some demersal marine organisms56, or if they disperse as nauplii. Thus, total exposure time to high-CO2 conditions is unknown, but all life stages starting from copepodite C2 through to adults were consistently found more abundantly over the reef and not offshore, suggesting that most of their lives are spent residential to the reef and exposed to ocean acidification conditions near the seeps. Despite the long-term exposure to high-CO2, we observed that OA did not have an apparent effect on the number of oocytes produced within the oviducts of the adult females. However, nothing is known about hatching success rates or the quality of the oocytes (i.e. yolk formation, of which Labidocera copepods have three distinct forms of endogenous yolk57).

Results are mixed as to whether juvenile copepods are more affected by ocean acidification compared to the adults. Some show no affect on juvenile copepods26,58, while others reveal naupliar production is reduced and the juvenile are less likely to survive than the adults19,21. Although we did not collect the nauplii stages and copepodite stage I (C1), we could compare the ratios between copepodites (stages C2-C5) to adult abundances, and the ratio was unaffected by high-CO2 conditions. Nearly all ocean acidification experiments conducted in the laboratory use females, thus very little is known of how the males react to CO2 stress compared to the females20,26,58, however, in our study we could compare the ratio of male to female abundances across CO2 levels to see if one sex was more negatively impacted than the other. The ratio between males and females remained unaffected by CO2, therefore both sexes were likely equivocally impacted by ocean acidification.

Although there were no differences in copepod length, gut fullness, and oocyte production between high-CO2 and control sites, these measures all differed between reefs. Lengths of each stage were slightly larger at Upa-Upasina than at Dobi, and female adults had more food in their stomachs and a larger number of mature oocytes at Upa-Upasina compared to Dobi. Increased feeding at Upa-Upasina likely explains why the copepods at Upa-Upasina reef were slightly larger and had more energy available to generate oocytes than at Dobi.

Changes in habitat from branching coral to more massive bouldering coral explains why some zooplankton taxa are reduced at these seep sites28. That does not seem to be the case for Labidocera spp. whose preferred day-time habitat is coral rubble, macro algae, and turf algae, but all three substrata types have similar percent cover across the high-CO2 and control sites, with coral rubble and macro
algae covering ~3% and ~5%, respectively. Turf algae has a slightly higher percent cover at ~38%. For this genus of copepods, they lose their association with specific substrata. At the high-CO₂ sites, *Labidocera* *spp.* abundances were low at all types of substrata, and unrelated to the percent coverage of each substratum.

How or why *Labidocera* *spp.* lose their ability to select a substratum type is unknown, but perhaps OA affects the chemical sensory ability of copepods to detect where to live in the reef. Copepods have light receptors, mechanosensory setae, chemosensory sensilla, and bimodal sensilla that are all used to detect physical and chemical cues within their environment⁵⁹. OA disrupts the ability of some tropical coral reef fish species to recognizing reef substrata as home⁶⁰,⁶¹. Nothing is known about copepods’ ability to smell coral reefs, but considering the vital role of olfaction in copepods to detect mates, food, and predators⁶⁹, it is likely that it may also play an important role to help *Labidocera* *spp.* smell a suitable substrate. Similarly, some meroplankton species use smell in addition to other cues (e.g. sound⁶², vision⁶³) to detect and settle on their preferred substrata in coral reefs⁶⁴,⁶⁵. It therefore remains to be explored whether there is a disruption in the sensory capabilities of *Labidocera* *spp.* in high-CO₂ conditions to smell their preferred substrate within the reef.

There are other potential explanations for the observed reduction in abundances, including a potential avoidance of high-CO₂ areas. In a flume laboratory choice experiment, the copepod *Centropages tenuiremis* preferred to stay in seawater of ambient pH 8.15 or slightly reduced pH (7.8), and avoided seawater with low pH levels of 7.6 and 7.0 (ref 66). Note that at a pH of 7.8, which was the same for our high-CO₂ conditions, *C. tenuiremis* did not avoid the CO₂-enriched seawater. The possibility of complete avoidance can be excluded because then there would be no *Labidocera* *spp.* present at the high-CO₂ sites. However, it is unknown whether or not some *Labidocera* *spp.* avoid the high-CO₂ seawater and is worthy of further investigations. Perhaps *Labidocera* *spp.* swimming along the reef simply do not settle because they do not like the taste or smell of the environment. The ability of *Labidocera* *spp.* to smell or taste their preferred substrate, as well as high-CO₂ seawater, should be studied in order to understand the underlying mechanisms behind *Labidocera* *spp.* abundance loss at the reefs under ocean acidification conditions.

The results of this study highlight a few important points relevant to OA research on copepods. First, a dramatic reduction within the community of certain
sensitive species, like *Labidocera* spp., suggests that such species may be indicator species for habitats impacted by ocean acidification. Second, the field results suggest conclusions about OA tolerances derived from laboratory studies may be unsubstantiated. For *Labidocera* spp., the field results indicate that although many aspects of their physiology may be unaffected by OA, their populations are still vulnerable to OA, as their abundances were reduced. Third, this is the first study to suggest that the ability of these copepods to detect their preferred habitat was compromised. This study indicates the importance of supporting field observations with field and laboratory experiments to understand how OA may impact copepods and other marine organisms in a future high CO2 world.

Copepods living in the open ocean where substrata preference is not relevant will not face the same problems, but understanding the mechanisms why *Labidocera* spp. no longer have an association with specific substrata may be relevant for other copepods and should be further investigated. If the chemoreception of copepods was compromised under OA, this could also impact oceanic copepods, which too use smell for a number of important biological purposes. Laboratory experiments should therefore be conducted on *Labidocera* spp. to determine why they are not found associated with their preferred reef substrata at near-future levels of elevated CO2.

Acknowledgments

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Ecosystem Impacts
CHAPTER 5
Reduced heterotrophy in the stony coral 
Galaxea fascicularis after life-long exposure to elevated carbon dioxide

Joy N. Smith, Julia Strahl, Sam H.C. Noonan, Gertraud Schmidt, Claudio Richter, Katharina E. Fabricius

JNS and JS conducted a pilot study which later led to the development of the current study, which was designed by JNS, JS, GMS, CR, KEF. JNS, JS, SHCN, KEF conducted the fieldwork. JNS did the laboratory work and data analysis. JNS led the writing with significant contribution from KEF. All authors reviewed and edited the manuscript.

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Abstract

Ocean acidification imposes many physiological, energetic, structural and ecological challenges to stony corals. While some corals may increase autotrophy under ocean acidification, another potential mechanism to alleviate some of the adverse effects on their physiology is to increase heterotrophy. We compared the feeding rates of *Galaxea fascicularis* colonies that have lived their entire lives under ocean acidification conditions at natural carbon dioxide (CO₂) seeps with colonies living under present-day CO₂ conditions. When provided with the same quantity and composition of zooplankton as food, corals acclimatized to high CO₂ showed 2.8 to 4.8 times depressed rates of zooplankton feeding. Results were consistent over four experiments, from two expeditions and both in field and chamber measurements. Unless replenished by other sources, reduced zooplankton uptake in *G. fascicularis* acclimatized to ocean acidification is likely to entail a shortage of vital nutrients, potentially jeopardizing their health and survival in future oceans.

Introduction

Corals evolved in oligotrophic waters to be mixotrophs, i.e. both auto- and heterotrophs. Autotrophy is the more studied component of the two nutritional modes. However, heterotrophy is just as important, even though its role in coral health is often ignored or underestimated. In addition to supplementing the organic carbon supplied by endosymbiotic zooxanthellae living within their tissue, heterotrophy provides corals with essential micro- and macronutrients that are not attained through autotrophy. These nutrients, including nitrogen and phosphorus, are needed for tissue growth, zooxanthellae regulation, and reproduction. Corals obtain these nutrients by the uptake of dissolved organic matter, detrital particulates suspended in the water column, bacteria, and zooplankton. Some species of corals increase their reliance on heterotrophy when under stress due to high turbidity, increased seawater temperatures that lead to the loss of their endosymbionts (coral bleaching), and short-term exposure to elevated carbon dioxide (CO₂) concentrations. As environmental stressors from anthropogenic causes continue to increase, heterotrophy may become more relevant in the future to maintain coral health. Here, we explore coral heterotrophy with respect to one of
the biggest environmental threats of all, ocean acidification. The term ‘ocean acidification’ describes the shift in seawater carbonate chemistry as anthropogenic CO₂ is absorbed by the oceans¹⁷. Under ocean acidification, seawater pH and calcium carbonate saturation states are both reduced. The reduced concentration of carbonate ions increases energy demands to maintain rates of calcification and growth, and triggers other physiological and energetic changes¹⁸.

The number of studies is limited, but some suggest that coral heterotrophy may reduce the impacts caused by ocean acidification¹⁵,¹⁶,¹⁹. Several laboratory experiments show that adult and juvenile corals can maintain calcification rates with heterotrophy under ocean acidification¹⁵,¹⁹,²⁰. Other studies found that feeding or nutrient loading did not offset the impacts to coral calcification by increased CO₂ (ref 21,22). For example, calcification rates of *Porites rus* reduced during short-term high CO₂ exposure but were unaffected by the provision of food²³. It also remains unresolved whether coral heterotrophy may be affected by ocean acidification, and any underlying mechanisms explaining those changes. Previous studies that have investigated the effects of elevated CO₂ on coral heterotrophy have shown mixed results. For example, *Porites lutea* expanded its polyps more in high CO₂ waters, perhaps in an attempt to feed more and ameliorate the negative effects of ocean acidification²⁴. Also, the corals *Acropora cervicornis* and *Porites rus* displayed increased rates of heterotrophy under elevated CO₂ (ref 15,16), mitigating the adverse effects of elevated CO₂ on calcification, while *Stylophora pistillata* had reduced rates under laboratory conditions²⁵.

In this study, we investigated the impact of ocean acidification on zooplankton capture rates in a coral species known for its voracity in feeding, *Galaxea fascicularis*. This coral feeds on zooplankton by extending mesenterial filaments through the polyp mouth, capturing particles, and then either ingesting them or digesting them externally, outside the coelenteron²⁶,²⁷. Our study was based on four complementary field and laboratory experiments. They were conducted during two expeditions to fringing reefs in Papua New Guinea where CO₂ seeps create natural pH gradients. We compared the morphology, behavior and feeding rates of *G. fascicularis* colonies grown in seawater with elevated CO₂ (pH₇ (total scale) = 7.8, pCO₂ ~ 760 µatm) against those grown at control CO₂ (pH₇ = 8.1, pCO₂ ~ 420 µatm). We tested the following hypotheses: Colonies acclimatized to elevated CO₂ (1) have smaller polyps due to energetic constraints for calcification, (2) expand their
polyps further, and (3) have increased rates of heterotrophy. We also tested for (4) food selectivity in *G. fascicularis* as a function of CO$_2$ levels, and (5) whether the neurotransmitter receptor GABA$_A$ was involved in the observed changes in the feeding ability of *G. fascicularis* under ocean acidification.

GABA (gamma-aminobutyric acid) is one of many neurotransmitters within the central nervous system of cnidarians that helps regulate circadian rhythms in corals$^{28,29}$ and modulates feeding responses in the cnidian *Hydra vulgaris*.$^{30-32}$ Two receptors are associated with the neurotransmitter GABA: GABA$_A$ and GABA$_B$. There are multiple binding sites on each receptor with various possible agonists (a chemical that activates a biological response) and antagonist (a chemical which blocks the action of any agonist), which can attach to the binding site. The functioning of the GABA receptor GABA$_A$ is of particular interest within ocean acidification research and has been linked to interference of neurotransmitter functioning in fish, mollusks, and other marine organisms.$^{33,34}$ Sensory and behavioral impairment of these organisms can effectively be reversed with one of the antagonist to the GABA$_A$ receptor, gabazine, although it has never been tested in corals. *G. fascicularis* under ocean acidification were treated with gabazine to determine its possible influences on heterotrophy.

*G. fascicularis* fragments used in this study have been exposed to high CO$_2$ conditions their entire life; therefore, all observations of feeding behavior of *G. fascicularis* reflect heterotrophy of corals with life-long acclimation to ocean acidification.

**Results**

**Feeding rates**

*G. fascicularis* colonies acclimatized to high CO$_2$ conditions (average pH$_T$ of 7.8) consumed less zooplankton compared to colonies under control conditions (pH$_T$ 8.1; Figure 1). This result was consistent for all experiments across methods and expeditions. The difference in the total number of zooplankton consumed per surface area was statistically different between CO$_2$ levels, but not between methods (i.e. field versus chamber), or between expeditions (Table 1). The interaction between method and expedition had a significant influence on the total number of
zooplankton consumed, although there was no difference for the main effect variables of method and expedition (Table 1).

Figure 1. Rates of heterotrophy in the coral *Galaxea fascicularis* in all experiments from two methods (field and chamber), two expeditions, and two CO2 levels (control and high CO2).

Table 1. Results of a generalized linear model regression of coral feeding rates in response to method, expedition, CO2, and their interaction terms.

<table>
<thead>
<tr>
<th>Factors and Interactions</th>
<th>F(6,44)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>F(1,61) = 0.46</td>
<td>0.50</td>
</tr>
<tr>
<td>Expedition</td>
<td>F(1,60) = 1.9</td>
<td>0.18</td>
</tr>
<tr>
<td>CO2</td>
<td>F(1,62) = 51.9</td>
<td>&lt; 0.001 *</td>
</tr>
<tr>
<td>Method: Expedition</td>
<td>F(1,57) = 9.4</td>
<td>0.003 *</td>
</tr>
<tr>
<td>Method: CO2</td>
<td>F(1,59) = 0.39</td>
<td>0.53</td>
</tr>
<tr>
<td>Expedition: CO2</td>
<td>F(1,58) = 0.25</td>
<td>0.62</td>
</tr>
<tr>
<td>Method: Expedition: CO2</td>
<td>F(1,58) = 0.48</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Following the observation of reduced feeding rates during the first expedition, we assessed in the second expedition whether the reduced heterotrophy was caused by CO2-induced impairment of neurotransmitters. The addition of gabazine during the chamber experiment from expedition 2 had no significant impact on the feeding rates (one-way ANOVA: F(2,22) = 0.51; P = 0.48). Thus, heterotrophy rates under high CO2 were not restored by the treatment with gabazine, the GABA$_A$ receptor antagonist.
Composition of consumed food and selective feeding

Although the total number of zooplankton consumed was different between CO₂ levels, the types of zooplankton consumed by *G. fascicularis* were not different between CO₂ levels. Taxonomic richness of the zooplankton prey consumed was not different between CO₂ levels (three-way ANOVA: $F_{(1,13)} = 2.74; P = 0.10$), although it was higher in the chamber experiments compared to the field experiments ($F_{(1,15)} = 20.2; P < 0.001$), and higher in expedition 2 compared to expedition 1 ($F_{(1,15)} = 8.17; P = 0.006$). Multivariate community analyses on the prey consumed by corals supported these results and indicated that the zooplankton community consumed was also not different between CO₂ levels (three-way ANOVA: $F_{(1,56)} = 1.45; P = 0.14$; Figure 2; Appendix IV, Supplementary Information Figure S2), although differed between methods ($F_{(1,56)} = 2.86; P = 0.003$), expeditions ($F_{(1,56)} = 14.5; P = 0.001$), and the interaction across the two variables ($F_{(1,56)} = 2.95; P = 0.005$).

![Figure 2](image)

*Figure 2.* Community analysis of zooplankton consumed under contrasting CO₂ regimes. Ordination plot from a canonical correlation analysis (CCA).

The types of prey identified in the coelenteron of dissected corals had much lower taxonomic richness than the plankton available in the water column: corals contained only 11-17 zooplankton taxa of the 26-33 taxa present in the water. Corals preferentially ingested some zooplankton taxa, including *Pontellidae* and *Paracalanidae* copepods, decapods, amphipods, and chaetognaths, whereas
*Oithonidae* copepods that are abundant in the water column were scarce in the food consumed (Figure 3).

**Figure 3.** The percent composition of the top available and consumed zooplankton taxa is shown for both expeditions, methods, and between CO2 levels. Plots for the 16 most commonly consumed zooplankton taxa compare the percent of each taxon consumed by the coral represented in the coelenteron (blue symbols) to the percent of the community that each zooplankton is available in the water column (red symbols). Each zooplankton taxon has two rows, with the top row (circles) representing the control site and the bottom row (triangles) representing the elevated CO2 site. Each panel represents a separate experiment (two expeditions and two methods). Asterisks indicate a significant difference between the percent consumed and percent available in the water column (t-tests, p-value < 0.05).

Results from logistic regressions that examined the effects of elevated CO2, expedition, and method, on the probability that each zooplankton taxon may be consumed indicated slight variation in the rates of consumption of the various taxa in response to these factors (Table 2). There was no difference in selectivity between high CO2 and control corals for the most available and most frequently consumed zooplankton taxa. However, the rare *Acartidae* copepodites, *Harpacticoida*, *Isopoda*,
Ostracoda, and Polychaeta appeared preferentially consumed at the control CO2 level. These taxa all represent a small proportion of the plankton available and consumed (<2 %). Furthermore, consumption rates of several zooplankton taxa differed between expeditions and methods. For example, Tortanidae copepods were rarely consumed during the first expedition, and yet during the second expedition they constituted on average 30.2% of the coral diet in the field experiment and 22.6% in the chamber experiment. Similarly, uptake rates of decapods and chaetognaths were relatively high during the second expedition.

Corallite size and polyp expansion between CO2 levels

No difference was observed in the size of G. fascicularis corallites between colonies originating at the seep and control sites (1-way ANOVA: $F_{(1,62)} = 2.7, P = 0.11$). Elevated CO2 also had no effect on polyp expansion of G. fascicularis at the seep and control sites, neither in the field nor in the chamber experiments. While coral polyps were not expanded more under elevated CO2 compared to control CO2 levels (4-way ANOVA: $F_{(1,124)} = 1.1; P = 0.29$), they were expanded significantly more in the field compared to the chamber experiments ($F_{(1,120)} = 22.0; P < 0.001$), and in expedition 2 compared to expedition 1 ($F_{(1,125)} = 12.2; P < 0.001$; see Appendix IV, Supplementary Table S1). Furthermore, corals expanded their polyps more at the end of each experiment compared to the beginning ($F_{(1,123)} = 6.3; P = 0.013$).
Table 2. Probability for each of the 16 most common zooplankton taxon to be consumed by *Galaxea fascicularis*, as a function of CO₂ (seep vs. control), expedition (one vs. two), method (field vs. chamber), and the interactions of these parameters (three-way interactions were non-significant for all taxa and are not shown). χ² (with df = 1 for all parameters) and p-values from the logistic regression analysis are presented, with bold print indicating significances at p < 0.05.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>CO₂</th>
<th>Expedition</th>
<th>Method</th>
<th>CO₂</th>
<th>Expedition</th>
<th>Method</th>
<th>CO₂</th>
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<tr>
<td>Acartiidae (adults)</td>
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<td>2.44</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
<td>1.58</td>
<td>&lt;0.001</td>
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<td>0.81</td>
<td>0.907</td>
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<td>0.001</td>
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<tr>
<td>Oithonidae</td>
<td>5.01</td>
<td>0.464</td>
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<td>&lt;0.001</td>
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<td>Oincaidae</td>
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<td>6.00</td>
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<td>8.70</td>
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145
Discussion

The observed effects of ocean acidification on heterotrophy in the stony coral *Galaxea fascicularis* contradicted our initial hypothesis. We expected corals to ingest more zooplankton under high CO₂. Instead, we found that food consumption rates were reduced under elevated CO₂, both in the field and in chamber experiments, and during two expeditions. Since the colonies in our high and ambient CO₂ treatments had been subjected to life-long exposure to their respective CO₂ environments, this study presents the first investigation of heterotrophy in corals that were fully acclimatized to elevated CO₂ throughout their entire post-settlement lives.

The taxonomic composition of the zooplankton consumed by *G. fascicularis* was different compared to the zooplankton community available to the corals. Such selectivity is known for corals¹². Selectivity may be indicative of plankton behavior; for example, some zooplankton taxa swim more slowly or clumsily making it easier to capture them, while some taxa have chemical defenses that make them unpalatable to corals²⁶. Whether it is from their own choosing or more from the behavior or chemical defenses of the zooplankton, there was strong selection for certain zooplankton, and this selectivity appeared to be largely unaffected by CO₂ treatments, with the exception of only a few uncommon taxa (Table 2).

Selectivity results may be slightly biased towards larger zooplankton taxa since smaller groups digest faster than larger zooplankton³⁶. However, the feeding time in this study was purposely chosen to be one hour so that complete digestion could be avoided. Complete digestion takes hours to days, and even small nauplii are still recognizable after only 60 minutes in the coelenteron¹²,²⁶,³⁶,³⁷. Furthermore, since the mesh size of the plankton net was 100 µm, the smallest zooplankton types were excluded from the experiment. In fact, most zooplankton consumed were easily identifiable to species level even when partially digested, hence the category ‘unidentified consumed zooplankton’ represented only 13% of the items retrieved from the coelenteron.

*G. fascicularis* consumed less zooplankton in the high CO₂ water despite having the same access to food, the same state of polyp expansion, and the same corallite sizes between CO₂ treatments. The reasons for the observed reduction in feeding rates could be many, however our study negated several potential causes. Reduced heterotrophy was not caused by a reduction in corallite size since *G.*
*fascicularis* corallites were the same size between CO₂ levels, even though exposure to elevated CO₂ reduces corallite sizes in some other coral species. For example, the temperate coral *Oculina patagonica* showed smaller corallites at elevated CO₂ due to high energetic costs for calcification; however, after one month of acidic conditions the skeleton completely dissolved and polyp sizes increased when calcification ceased and the resulting free energy was channeled into somatic growth. With respect to *G. fascicularis*, it is possible that net calcification rates may change under ocean acidification conditions despite the morphology of the corallites remaining similar for both CO₂ levels.

Reduced heterotrophy was also not caused by a difference in polyp expansion, which remained unaffected by ocean acidification for *G. fascicularis*. In contrast, another study observed that polyps from the coral *P. lutea* extended further under high CO₂ conditions. During the second expedition, however, *G. fascicularis* polyps were expanded more, which happened to occur during a new moon compared to the first expedition that had a full moon. Corals are known to feed differently with the lunar cycle, coinciding with lunar effects on zooplankton migration patterns. Also, polyps were expanded more in the field experiments compared to the chamber experiment, probably because the corals were undisturbed in the field.

A deficiency in the functioning of GABA<sub>A</sub> neurotransmitter receptors in *G. fascicularis* was also not a likely cause for the observed reduction in heterotrophy. Gabazine plays a role in *Hydra vulgaris* feeding response, therefore we expected it to also influence coral feeding behavior of *G. fascicularis* since both of these cnidarians share similar nervous systems. Despite our predictions, the treatment of *G. fascicularis* with gabazine yielded no change in coral heterotrophy. The effect of ocean acidification on coral neurotransmitters cannot be completely excluded, however, because different chemicals besides gabazine may bind to the neurotransmitter receptors (e.g. the agonist muscimol and the antagonist bicuculline). To thoroughly understand the effect of ocean acidification on neurotransmitters of *G. fascicularis*, the reactions of other receptor antagonists and agonists to elevated CO₂ need to be evaluated.

Additional experiments are needed to reveal the underlying mechanisms responsible for the reduced feeding rates in *G. fascicularis*. Potential causes or contributors that deserve further study include reduced particle retention, changes in cellular homeostasis of the tentacle cells, reduced nematocyst functioning, altered
mucus production, physiological stress that makes them less capable to feed, an increase in autotrophy, and potential changes in plankton behavior, as briefly outlined here. G. fascicularis exerted similar effort to capture zooplankton between CO₂ levels by extending their polyps to the same level. That they ingested fewer food particles in ocean acidification conditions may reflect upon the polyps’ ability to capture food. Food retention may be reduced if the functionality of their stinging cells (nematocysts) is disrupted⁴¹,⁴². Nematocyst performance may be vulnerable to changes in pH since the acid-base balance in cells corresponds to the intracellular concentration of free H⁺ ions. A study on the jellyfish Pelagia noctiluca indicated that the cell homeostasis of nematocysts is profoundly compromised by acidification of the surrounding seawater impairing the cells’ discharge capability⁴³. Although cellular homeostasis in nematocysts may vary between jellyfish and corals, nematocyst functioning may be impaired for corals under ocean acidification and merits further investigation.

Another possible cause for the observed reduced feeding rates could be that the polyps themselves lose their ability to retain food particles. Food particles may be stung or killed, but the mucosal or tentacular action of the polyps may not trap the particles, resulting in the loss of prey items²⁶. Mucus enhances coral heterotrophy⁴⁴, therefore heterotrophy will likely be vulnerable to any changes in mucus production, but nothing is known about how ocean acidification may affect coral mucus.

It is perceiveable that G. fascicularis may also have reduced rates of heterotrophy in response to a reduced energy demand. Elevated CO₂ enhances the photosynthetic-derived energy supply in some coral species, and this energy is available to support critical functions like calcification. Coral calcification is generally considered to decline with elevated CO₂ levels⁴⁵, although some studies report parabolic and even positive calcification responses to ocean acidification conditions⁴⁶,⁴⁷. However, corals are more nutrient limited than carbon limited in oligotrophic and shallow (high-light) environments². Furthermore, feeding rates of corals only reach saturation when food concentrations are high, with heterotrophy generally more efficient in oligotrophic habitats⁴⁸. Considering that G. fascicularis from the CO₂ seep sites live in a nutrient-poor and high-light environment, it is highly unlikely that feeding becomes saturated and their need for essential nutrients not attained from photosynthesis would still be prevalent. Therefore, G. fascicularis
would likely continue to feed on zooplankton at the CO2 seep sites if they were still capable even under an increased carbon supply from photosynthesis.

Regardless of the underlying mechanisms, reduced heterotrophy under elevated CO2 will have biological impacts on corals. Growth, reproduction, zooxanthellae maintenance49, and other metabolic processes depend on nitrogen, phosphorus, and other essential trace elements, which are exclusively attained through heterotrophy6,50–52. We are only starting to understand the long-term impacts of ocean acidification on tissue growth, phototrophy, respiration, heterotrophy, and their energetic interdependencies, in selected species of coral. Many but not all coral species increase their rates of photosynthesis at higher pCO2 levels53. Reduced heterotrophy may also impact coral lipid content and fatty acid composition, since they are co-determined by zooplankton consumption54. Furthermore, lower feeding rates may slow skeletal and tissue growth considering that growth is positively correlated with rates of heterotrophy for several coral species6,52, so lower feeding rates may slow growth. Heterotrophy is certainly beneficial to corals and yet clearly heterotrophy declines for G. fascicularis under elevated CO2. Any potential impact to their basic biology warrants further research.

Despite the remaining knowledge gaps, decreased heterotrophy will have important implications for the health and resilience of corals. As ocean conditions increasingly become unfavorable for many coral species, their ability to react to such stress will become imperative to their survival. Some coral species will persist while others will not, and our data show that some G. fascicularis colonies are able to survive under high CO2 in the field, despite their lifetime exposure to elevated CO2 conditions and associated reduced zooplankton feeding rates. However, it was beyond the scope of this study to measure their physiology (tissue biomass, lipid content, calcification rates, or other biophysical parameters indicative of their overall health). Such measurements should be conducted to better understand coral long-term survivability under ocean acidification.

Methods

Study site

The feeding experiments were conducted at Upa-Upasina Reef, a fringing reef in Milne Bay Province, Papua New Guinea, where a natural volcanic CO2 seep
provides gradients in seawater pH. A spatial map of the seawater carbonate chemistry, along with a detailed description of the Upa-Upasina high CO₂ and control site can be found in Fabricius et al (2011 & 2015). G. fascicularis colonies were collected near the seep site where seawater approximates 7.8 pHₜ (total scale), and from a control site with control CO₂ at ~8.1 pHₜ. The chamber feeding experiments were conducted aboard the back deck of the ship while moored near Upa-Upasina Reef, with G. fascicularis fragments that were freshly collected from the reef. The field and chamber experiments were conducted during two ship expeditions to the site (12-14 April 2014 and 18-20 November 2014).

**Seawater carbonate chemistry**

The carbonate chemistry for the field sites varied through time and long-term measurements have been reported in previous literature. Additionally, seawater pH at total scale (pHₜ) was recorded at the control and elevated CO₂ sites for several days surrounding the commencement of the feeding experiments using SeaFET pH sensors (Appendix IV, Supplementary Information Figure S3). pHₜ values had similar ranges compared to previous expeditions. Water samples were also collected, fixed with saturated mercuric chloride solution (HgCl₂), and later analyzed for their dissolved inorganic carbon (DIC: µmol kg⁻¹) and total alkalinity (Aₜ: µmol kg⁻¹) using the Versatile Instrument for the Determination of Total Inorganic Carbon and Titration Alkalinity (VINDTA 3C).

Carbonate chemistry was also measured for the seawater used for the chamber experiments and water temperature (°C) was recorded on site. Water samples saturated with HgCl₂ were stored and later measured for DIC and Aₜ. The water temperature was 25°C at the time the samples were analyzed in the laboratory for its carbonate chemistry using the VINDTA 3C. DIC and Aₜ were used to calculate other seawater parameters (Table 3), including pH at total scale (pHₜ), partial pressure of carbon dioxide (pCO₂: µatm), bicarbonate (HCO₃⁻: µmol kg⁻¹), carbonate (CO₃²⁻: µmol kg⁻¹), aqueous carbon dioxide (CO₂(aq): µmol kg⁻¹), the saturation state of calcite (Ω₇₅), and the saturation state of aragonite (Ω₉₈), using the Excel macro CO2SYS under the constraints set by Dickson and Millero (1987).
Table 3. Seawater carbonate chemistry of the chamber experiments with dissolved inorganic carbon (DIC) and total alkalinity (AT) measured from water samples fixed with saturated mercuric chloride solution (HgCl₂). DIC and AT were inputted into the Excel macro CO2SYS and used to calculate pH at total scale (pHT), partial pressure of carbon dioxide (pCO₂), bicarbonate (HCO₃⁻), carbonate (CO₃²⁻), aqueous carbon dioxide (CO₂(aq)), the saturation state of calcite (ΩCA), and the saturation state of aragonite (ΩAR).

<table>
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<th>Expedition</th>
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<th>pHT</th>
<th>AT (μmol kg⁻¹)</th>
<th>DIC (μmol kg⁻¹)</th>
<th>pCO₂ (atm)</th>
<th>HCO₃⁻ (μmol kg⁻¹)</th>
<th>CO₃²⁻ (μmol kg⁻¹)</th>
<th>CO₂(aq) (μmol kg⁻¹)</th>
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**Food collection**

Zooplankton were freshly collected via plankton net tows from the control site at approximately 9 pm, i.e. 2 - 3 h after sunset, and shortly before the start of the field and the chamber experiments. Each net tow was very slow to minimize stress to the zooplankton. Live samples were handled with care and only living zooplankton were used as food for corals (i.e. zooplankton still suspended in the water column and actively swimming. No zooplankton that had settled at the bottom of the collection container were used). Three to six zooplankton samples were preserved in 4% formalin and kept as references to determine variation in the number and taxonomic composition of zooplankton between samples.

**Field feeding experiment**

Tents of 100 μm plankton mesh and 25 cm base diameter (approximately 8 L volume) were used to contain zooplankton close to corals for the duration of the feeding experiment. Five tents were placed over separate *G. fascicularis* colonies each at the high CO₂ and the control sites. To prevent corals from consuming zooplankton that are naturally in the water column, the tents were deployed during daylight when zooplankton numbers are low and demersal zooplankton have not emerged into the water column yet. At approximately 9 pm, SCUBA divers injected three 60 ml syringes of freshly collected and concentrated zooplankton into each tent. Polyp expansion (25, 50, 75 or 100 percent expanded) was recorded at the
beginning and end of the feeding period. After approximately one hour, the tents were removed and a fragment of each colony was extracted with a hammer and chisel and preserved in 4% formalin. The field experiments were conducted once during expedition 1 (three replicate coral colonies per CO2 level), and twice on two consecutive nights during expedition 2 (five replicate coral colonies per CO2 level for both nights). The plankton fed to the corals during the second expedition had similar composition and concentration in the two consecutive nights, so the results from both nights were pooled together and considered one experiment.

**Chamber feeding experiment**

*G. fascicularis* fragments were collected from both the high CO2 site and the control site. They were placed in flow-through aquaria for four days to recover. The aquaria consisted of two 60 L bins with an outboard pump supplying a constant inflow of fresh seawater. For 12 hours prior to the feeding experiment, 100 µm mesh was placed over the input valve to starve *G. fascicularis*, allowing any previously consumed food to be digested. Three hours prior to the feeding experiment, each coral fragment was transferred onto a raised grid platform in individual cylindrical incubation chambers (89 mm diameter, 106 mm height, 637 ml volume) without exposing them to air. Corals collected from the seeps were placed in the chambers filled with seawater from the seep site, while those from the control site were placed in chambers filled with seawater from the control site (seawater carbonate chemistry for chamber experiments found in Table 3).

Chambers were 80% immersed in a water bath. Airspace in the chamber and a hole in its upper lid facilitated gas exchange. To generate a current within the chamber, a battery driven pulley system activated magnetic stirrer bars underneath the grid. *G. fascicularis* were fed at around 9 pm. Taking care to supply only living zooplankton, concentrated zooplankton was injected through a hole in the top lid of the chamber with a volumetric pipette. The zooplankton concentration was lower during the second expedition compared to the first, so a larger volume of plankton solution was inserted into the chamber during the second (30 ml) compared to the first expedition (20 ml). An additional three samples of the food were preserved in 4% formalin and kept as references. The feeding experiment was conducted in the dark, although red light was used for a few minutes at the commencement and cessation of the experiment to assess their state of polyp expansion. *G. fascicularis*
fed for approximately one hour and then each coral piece was removed and immediately stored in 4% formalin. The chamber experiment was conducted once through an initial pilot study during expedition 1 (7 replicate coral colonies per CO₂ level), and repeated during expedition 2 with additional replicates (12 replicate coral colonies per CO₂ level).

To determine if elevated CO₂ interferes with neurotransmitter receptor functioning, six of the coral fragments per CO₂ treatment were exposed to gabazine (SR-95531, Sigma-Aldrich) at a concentration of 4 mg L⁻¹ seawater for 30 min (chamber experiment, second expedition). Coral fragments were gently washed and transferred into their chambers filled with gabazine-free seawater. The other six colonies per CO₂ treatment were exposed to the same handling procedure, but their 30 min transfer was into a container without gabazine. Experiments were then conducted as outlined above.

Food samples for corals

Food samples given to corals were compared within and between experiments. Food samples given to each replicate coral fragment were similar in quantity and composition within each experiment, and they were not different between high CO₂ (7.8 pH₁) and control treatments (8.1 pH₁) and replicates. However, food samples varied in quantity and composition between the four field and chamber experiments. Details about the analysis of food samples are in Appendix IV, Supplementary Information, including Figure S1.

Laboratory analysis

Coral consumption was measured through coelenteron content analysis. G. fascicularis fragments were removed from formalin and placed in freshwater. Every polyp coelenteron was probed using a tungsten needle and dissecting forceps. Extracted zooplankton were identified to their major taxonomic groups. Total corallite number and corallites containing food particles were enumerated. Each coral fragment was photographed and the surface area calculated within the image-processing program, ImageJ. Corallite size was calculated by dividing the surface area of each coral fragment by the number of corallites.
Statistics

All statistical analyses were computed in R, version 3.2.2 (R Development Core Team, 2015). Generalized linear models (GLMs) were used to determine if: (1) the number of zooplankton consumed (standardized by surface area) differed across CO2 regimes (seep vs. control), expedition (one vs. two), or methods (field vs. chamber), (2) species richness (Shannon-diversity index) of the zooplankton taxa consumed by corals differed between CO2 regimes, expedition, or methods, (3) gabazine affected coral feeding rates, (4) zooplankton concentration in the food samples was different between each of the experimental runs, (5) corallite sizes were different between corals originating from seep and control sites, and (6) polyp expansion differed across CO2 levels, methods, seasons, or from the beginning to the end of the experiment. Appropriate data distributions and link functions were chosen for each GLM. Model assumptions of independence, homogeneity of variance, and normality of error were evaluated through diagnostic tests of leverage, Cook's distance, and dfbetas\(^59\). Checks for all GLMs indicated that no influential data points or outliers existed in the data and model assumptions were met. ANOVAS (Type II) were used to determine the minimal adequate GLM with the ‘Anova’ function in the R library ‘car’ (version 2.1-1)\(^60\). The effects of the explanatory variables on the response variables were then reported based on these GLMs.

Canonical correspondence analysis (CCA) was used to determine if the zooplankton community composition of the food available to the corals, and the food consumed by the corals, differed in relation to the explanatory variables (CO2, expedition, method). To account for many zeros in the data where some zooplankton taxonomic groups were rarely present or rarely consumed, the community data was standardized using the Hellinger (square root) method within the decostand function of the vegan package in R\(^81\). A Monte-Carlo permutation test was used to determine the optimal CCA model and to assess the significance of the variation in species composition attributable to the explanatory variables (CO2, expedition, method).

For each zooplankton taxon, its percent representation in the coral coelenteron content was compared against its percent in the available food using two-tailed t-tests, assuming unequal variances between samples. Logistic regressions were used to model the response of each zooplankton taxon contained in the corals to the explanatory variables of CO2, expedition, and method. Logistic regressions use binary data of ‘successes’ and ‘failures’. In this example, ‘success’
equals the probability of each taxon being consumed (p), and ‘failure’ equals the probability of not being consumed (1-p). Logistic regressions are within the framework of GLMs and use log-odd-ratios, defined by the logit link function, to estimate the (log) odds of each taxon being consumed under each independent variable. GLMs with a binary data distribution and logit link function were checked for overdispersion. Overdispersion (residual deviance greater than the residual degrees of freedom) existed, so the data distribution was changed to quasibinomial. Anovas with a Chi-square test were applied to the results of each GLM for each zooplankton taxon.

Acknowledgments

We thank the community at Upa-Upasina for their permission to study the corals on their reef, as well as the crew of the M.V. Chertan, especially Obedi Daniel and Robin (‘Lei’) Luke, for their support during the fieldwork. We additionally thank Johanna Arndt and Meliha Bademci for helping hands in the laboratory, and Glenn De’ath and Karin Boos for statistical advice. This project was funded in part by the Erasmus Mundus funded joint doctoral program MARES (FPA 2011-0016), the Great Barrier Reef Foundation’s ‘Resilient Coral Reefs Successfully Adapting to Climate Change’ Program in collaboration with the Australian Government, the BIOACID Phase II Programme of the German Science Ministry BMBF (Grant 03F0655B), and the Australian Institute of Marine Science. The authors also acknowledge the contribution by J. Ries and an anonymous referee, whose helpful reviews improved the manuscript.

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DISCUSSION
CHAPTER 6
Discussion

Ocean acidification is an ongoing and calamitous process that has already commenced in the world’s oceans, as seawater pH has already declined by a global average of 0.1 units (ref 1). Changes in certain regions like urbanized estuaries²,³, polar regions⁴,⁵, and coral reefs⁶ can be amplified beyond 0.1 pH units. Due to the gravity of the issue, ocean acidification research has been propelled to the forefront of research topics in marine science. Governments have recognized the influence that ocean acidification might have on politics, economics and the environment. The topic is now also included in the latest reports by the Intergovernmental Panel on Climate Change (IPCC)⁷. Thus, ocean acidification impacts on marine communities are of paramount interest not only to marine ecologists, but global leaders as well.

The topic of my PhD addresses key questions within this emerging multidisciplinary field. My work has focused on the effects of ocean acidification on microscopic organisms that are the basis of food webs, zooplankton. The novelty of this PhD research lies in the fact that ocean acidification impacts were observed on zooplankton communities living in their natural environment. Thus far, most ocean acidification research has been conducted in the laboratory, Contrary to field work, laboratory experiments have the advantage of controlling for all environmental parameters; however, the conditions of the organisms’ natural environment are altered so the animals may not behave normally. Natural CO₂ seeps were used as windows into the future to document differences in zooplankton communities that live in ambient CO₂ conditions to communities that live in high-CO₂ conditions. Samples were collected and experiments conducted over three expeditions to two seep sites in Papua New Guinea, ensuring that trends were consistent in space and time. Overall, the thesis sought out to examine how ocean acidification affects zooplankton abundance, migration behavior, and some aspects of their biochemical signature. Once changes were observed in the zooplankton, the mechanisms behind the alterations were investigated. Specifically, shifts in habitat caused by ocean acidification were explored for their potential influences on the demersal zooplankton. A genus of copepods (Labidocera spp.), found to be highly sensitive to ocean acidification, was further investigated in more detail for possible changes in
their physiology and habitat preference. This genus of copepods was previously not known to reside in reefs. The discovery that a copepod considered to be neustonic was indeed residing in reefs accentuates our minimal understanding of tropical and reef associated zooplankton.

This discussion serves to highlight major findings of this study that challenge previous perceptions of how ocean acidification may impact zooplankton. Also, the validity of using seeps for studying zooplankton is discussed. Results are also put into perspective of their ecological relevance. The discussion concludes with an outlook into the fate of zooplankton communities and coral reef ecosystems in future oceans.

**Challenged perceptions**

Our perception and understanding of the biological effects of ocean acidification are often first theoretical, followed by empirically derived data. Some studies produce unexpected results and challenge us to revise our initial comprehension. For example, chemistry principles of ocean acidification suggests that all marine calcifiers will reduce their calcification rates, and yet extensive research now reveals that calcification reactions are species-specific with some organisms able to maintain their calcification rates by up-regulating pH at their site of calcification\(^9,9\). Similarly unexpected results have been encountered in zooplankton research. Over the past several years, tests on ocean acidification effects on zooplankton have begun to challenge initial consensus, and results from this PhD also challenge current perceptions.

Leading zooplankton biologists convened at The 5\(^{th}\) International Zooplankton Production Symposium and held a workshop entitled “Impacts of ocean acidification on zooplankton.” The general consensus reached at the end of the workshop was that CO\(_2\) had no discernable effect on most zooplankton (excluding calcifying species), although the consensus recognized the many knowledge gaps yet to be investigated\(^10\). Certainly some evidence supports this statement. For example, microcosm experiments show no changes in zooplankton communities under short-term high-CO\(_2\) exposure\(^11-13\). Also, a field study tried to relate long-term trends in pH and calcifying plankton in the North Sea and found no relationship (although there was some uncertainty related to the historical pH data), suggesting that any potential
ocean acidification effects on calcifying species were possibly masked by climatic, chemical, and biotic drivers\textsuperscript{14}. However, the results from this thesis challenge the concept that zooplankton communities will be tolerant to future change. They show that for reef-associated demersal plankton, abundances are three-fold reduced. Neither percent composition nor biochemical signature of the zooplankton community was affected by ocean acidification, but still, such a substantial loss in total abundance will have consequences for the higher trophic levels that rely on zooplankton for nutrition. Reduced abundances appeared to be partly due to indirect effects of ocean acidification, including habitat loss. The repercussions of ocean acidification are complex, thus studying zooplankton in their natural environment portrays a more realistic view of what their communities may actually be like in the future, since all the effects related to ocean acidification, both direct and indirect, are collectively molding the community.

The consensus that states that zooplankton will be tolerant to ocean acidification\textsuperscript{10} is also partly based on laboratory experiments conducted on copepod physiology. Several species have been shown to be physiologically tolerant to high-CO\textsubscript{2} except under extreme levels that far extend the levels predicted for the coming centuries\textsuperscript{15,16}. Most of the copepod species studied are generalist species, and are hence predisposed to coping with a wide range of environmental conditions. In the case of the pontellid copepods (\textit{Labidocera} spp.) living at the reefs in Papua New Guinea, physiological parameters remained unaffected by high-CO\textsubscript{2}, although their abundances were \textasciitilde 70\% reduced at the CO\textsubscript{2} seeps. When examining the entire zooplankton community, habitat loss from branching coral to massive bouldering coral, appeared to be the main driving factor for overall reductions in zooplankton abundances. However, \textit{Labidocera} spp. did not lose their preferred habitat (coral rubble, macro algae, and turf algae), since these substrates were of equal coverage at both the control and high-CO\textsubscript{2} reefs. Instead, abundances of \textit{Labidocera} spp. were no longer associated with these substratum types under high-CO\textsubscript{2} conditions, and the reasons why remain to be determined. Nonetheless, the complexities of multiple driving forces reflect the sum of species-specific responses, which will collectively contribute to losses in total abundances.

Another perception challenged by this thesis was the response of coral heterotrophy to ocean acidification. For some species of coral the reliance on heterotrophy increases under stress, whether that stress originates from a bleaching
event\textsuperscript{17}, increased turbidity\textsuperscript{18}, or ocean acidification\textsuperscript{19}. Contrary to this expectation, the stony coral \textit{Galaxea fascicularis} consumed significantly less zooplankton under high-CO\textsubscript{2} conditions. Despite attempts to understand why feeding rates were reduced, we found no obvious explanation, as the reduced feeding rates were not attributable to differences in feeding effort, polyp size, or a disruption in the communication of their neurotransmitters. The coral feeding experiment was the first to show that corals consume less zooplankton when exposed to life-long ocean acidification. Heterotrophic responses of other zooplanktivores under high-CO\textsubscript{2} are worth investigating for other species living at the seeps.

\textbf{CO\textsubscript{2} seeps as natural laboratories for zooplankton research}

Carbon dioxide seeps are open systems, and only organisms that are sedentary or territorial are guaranteed to be exposed to ocean acidification conditions for prolonged periods of time. Thus, CO\textsubscript{2} seeps can only be used to study the effects of ocean acidification on residential zooplankton, and not holoplankton or phytoplankton that merely drift through the high-CO\textsubscript{2} area. Not all CO\textsubscript{2} seep sites that exist around the world would be suitable for studying zooplankton. Currents must be low so that the zooplankton are not swept away. In low current conditions, zooplankton can swim faster than the current and are able to maintain their position within reefs. Such was the case at the CO\textsubscript{2} seeps in Papua New Guinea.

The presence of reef-associated zooplankton was confirmed by comparing communities between reef and offshore waters, and between day and night (refer to results in Appendix III for evidence). Not only was there much more zooplankton living over the reef than offshore, but abundances also greatly increased at night over the reef but not offshore. Furthermore, the community composition was completely different over the reef compared to offshore waters, and between day and night. We are therefore confident that the nocturnal zooplankton, which was the focus of this study, were residential to the reefs and were exposed to high-CO\textsubscript{2} conditions for presumably most of their life, and some possibly even for multiple generations.

The carbon dioxide seeps in Papua New Guinea lead to coral reef community shifts from structurally complex corals to massive bouldering corals. However, CO\textsubscript{2} seeps also exist elsewhere around coral reefs. A seep off the coast of Iwotorishima
Island, Japan shows a shift from hard coral reefs to soft corals\textsuperscript{20}, and another CO\textsubscript{2} seep off the coast of Maug, part of the Commonwealth of the Northern Mariana Islands, shifts from reef corals to macro algae\textsuperscript{21}. Shifts from structurally complex hard corals to massive bouldering corals, soft corals, or macro algae are all possible regimes changes for coral reefs in future oceans, all of which would induce a different habitat-driven response in the demersal zooplankton. To fully understand potential shifts in the zooplankton community, the response of demersal zooplankton should also be investigated at other CO\textsubscript{2} seep sites.

Several limitations of using carbon dioxide seeps as natural laboratories exist and they are briefly discussed here. For example, pH at the seeps fluctuates more than in natural coral reefs due to wave mixing, tidal dependent residence times, and fluctuating CO\textsubscript{2} emissions out of the seafloor. Typically, more gas is released during low tide than high tide (tidal range was \textasciitilde0.9 m). The zooplankton at the high-CO\textsubscript{2} sites are therefore exposed to sometimes large fluctuations in CO\textsubscript{2} levels with an average pH of 7.8, whereas the zooplankton at the control sites are in seawater that exhibits much smaller daily fluctuations in pH with an average of 8.1.

Another factor likely different compared to future real-life scenarios is the exposure of phytoplankton, the food for several zooplankton species, to high-CO\textsubscript{2} conditions. Phytoplankton quantity was similar between high-CO\textsubscript{2} and control sites. Since phytoplankton drift through the open system their exposure time is too short to respond to high CO\textsubscript{2} at the seeps. However in the future, the entire oceans, not just in localized regions around seeps, will be CO\textsubscript{2}-enriched. For some phytoplankton species this changes their fatty acid concentration and content, and thus their nutritional value as a food source, with potentially deleterious effects on the copepods that consume them\textsuperscript{22}. However, studies that relate phytoplankton quality to trophic constrains on copepods under ocean acidification remain ambivalent, with mixed results showing some but not all phytoplankton affected\textsuperscript{22,23}, and some but not all copepods affected\textsuperscript{22,24}. At the Papua New Guinea seep sites, the fatty acid content of the bulk zooplankton remained unaffected by ocean acidification due to the lack of change in their taxonomic composition. One of our major findings was that zooplankton as a food source was reduced in quantity, but maintained a similar taxonomic composition and fatty acid content. The conclusion that fatty acid composition remains similar may potentially be different in future oceans. Thus, the
community response for zooplankton in response to phytoplankton communities exposed to ocean acidification is unknown.

Despite some limitations of CO₂ seeps, they are the best option for studying long-term impacts of ocean acidification on zooplankton communities. As opposed to laboratory or mesocosm experiments, nothing about their environment has been manipulated and they exist in the complex ecosystem as they would naturally. Ideally laboratory and field experiments should compliment each other, and laboratory experiments should be designed in a way that maximizes their relevance for the organisms living in the field.

Ecological relevance

The overall zooplankton community was reduced in abundance, with no regime shifts or changes in taxonomic composition or fatty acid content. A habitat shift from structurally complex corals to massive bouldering corals meant that for several of the zooplankton taxa, their preferred habitat was diminished. Habitat loss is but one mechanism contributing to abundance loss, since nearly all of the zooplankton taxa were reduced but not all of them prefer to live among branching coral.

Coral rubble was one of the preferred substrata for the pontellid copepods, *Labidocera* spp., but it only exists in small amounts (~3% cover) at both the high-CO₂ and control sites. Although coral rubble coverage did not change between CO₂ levels, pontellid abundances were still highly reduced. In fact, pontellid copepods were one of the most sensitive taxonomic groups within the zooplankton community to ocean acidification. A closer investigation revealed that their reduced abundances at high CO₂ were not due to habitat loss, which was the case for the overall zooplankton, but instead their abundances were no longer associated with any substrata. Alternatively, they occurred in low numbers equally in all substratum types. This highlights that different ecological, behavioral, and physiological mechanisms can act separately on the different taxonomic groups, and in combination will lead to the observed losses in the whole zooplankton community.

Unrelated to ocean acidification research, the fact that the pontellid copepods had a preferred habitat in the healthy (ambient CO₂) reefs was a discovery on its own, considering they were previously considered neustonic. This highlights how
little is known about zooplankton living residential to coral reefs. Many of the species living demersal to the reefs are also found living in the open ocean (e.g. Acartia, Oithonidae copepods), and yet how and when they alter their behavior to live residential in coral reefs is unknown. Despite morphological traits in pontellid copepods that have evolved for living life at the sea-surface\textsuperscript{25,26}, they were discovered living within the demersal zooplankton community, where they contribute to coral reef trophodynamics. These copepods are large in size and have a higher percent lipid content than smaller copepods\textsuperscript{27}. They were also a favorite type of food for the stony coral \textit{Galaxea fascicularis}.

We also found that \textit{G. fascicularis} consumed less zooplankton in high-CO\textsubscript{2} conditions. Since we do not know the underlying mechanisms as to why \textit{G. fascicularis} consumed less zooplankton, we cannot predict what other reef associated organisms might be vulnerable. For example, if heterotrophy is reduced because the stinging cell of the polyp is impaired, this would have possible impacts on not just corals, but other cnidarians as well, including jellyfish. Jellyfish are one of the largest zooplankton members in size and are expected to proliferate in warming oceans\textsuperscript{28}, but if their stinging cells were impaired then the impacts from ocean acidification may counteract that from warming. Regardless, corals that consume fewer food particles will have less nutrients required for tissue growth, reproduction, and calcification\textsuperscript{29}. For some coral species reduced feeding rates affect skeleton growth (linear extension rate, buoyant weight, density, and calcification)\textsuperscript{29,30}. Reduced skeletal density would weaken the coral, compounding weakness attributable to ocean acidification. Weak coral skeletal structure makes the reef as a whole more vulnerable to storm damage, dissolution and bioerosion, which destabilizes the very matrix of coral reefs\textsuperscript{31}.

Reduced reef associated zooplankton also means diminished food for nocturnal zooplankton feeders, altering ecosystem functions and potentially decreasing the economic value of fisheries. Most planktivorous fish forage during the day and consume transient zooplankton\textsuperscript{34}, and therefore will not be affected by losses in nocturnal zooplankton. However, several planktivorous reef fish forage on large demersal zooplankton between dusk and dawn, e.g. several Apogonidae and Holocentridae species\textsuperscript{35}. Such taxa may be food limited at the high-CO\textsubscript{2} sites. Fish diversity and community structure were unaffected by ocean acidification at these same CO\textsubscript{2} seeps in Papua New Guinea\textsuperscript{33}. That is partly attributable to the fishes’
ability to swim in and out of the high-CO₂ areas and access food from areas unaffected by CO₂. If fish were contained within the high-CO₂ sites, their communities may react differently to permanent exposure to ocean acidification conditions. This is of concern as fish provide the main protein source for many coastal developing countries, and is also a consumed by developed nations³².

Not only fish, but also macroinvertebrates (e.g. crabs, molluscs) are harvested for human consumption. Macroinvertebrates as adults are reduced at the CO₂ seeps³⁶, and this is reflected in the meroplankton. Meroplankton constitute the taxa that are only plankton as larvae but not as adults and include decapods, shrimp larvae, and bivalve larvae, which were all reduced in the zooplankton community. For example, adult decapod crustaceans were reduced at the high-CO₂ sites by 22% (ref 36), while decapod larvae in the meroplankton were reduced by 33%. Meroplankton are often dispersed great distances from reefs, although survival is low and chances of encountering land and a suitable reef are estimated at 1 in 3,300 for the South Pacific Islands³⁷. Multiple retention mechanisms also retain some meroplankton in coastal waters via physical factors and larval behavior³⁸–⁴⁰. Some decapod larvae vertically migrate and are able to avoid being swept away by tidal currents in order to maintain their position near coastal regions³⁹,⁴¹. Decapod larvae were more abundant over the reef compared to offshore waters, which may reflect that the larvae are being produced at the reef, but also might indicate that some are retained in the reef. A reduced adult population of decapods under high-CO₂ conditions subsequently produces fewer larvae observed in the meroplankton. Since the larvae are reduced more than the adults under ocean acidification (33% compared to 22%), perhaps the difference is due to the loss of those dispersed by currents, and perhaps fewer of the larvae survive in high-CO₂ conditions. Crustaceans are reduced due to habitat loss³⁶, and this is reflected in the meroplankton.

Reductions in zooplankton abundance will likely induce bottom-up controls on the food web structure, with changes in the zooplankton community dynamics possibly altering ecosystem functioning. Less zooplankton also means less nutrients recycled and may impact biogeochemical cycling within the reefs. With coral reefs considered nutrient limited, a reduction in resources may disturb the entire nutrient cycle within coral reef ecosystems.
Amidst the negative connotations associated with reduced zooplankton abundances, there are still some positive implications from the results. For example, there was no loss in diversity amongst the demersal zooplankton under ocean acidification. Although all taxonomic groups were reduced in abundance, they were still present at the high-CO\textsubscript{2} sites, thus they are physiologically capable to survive pH conditions of 7.8. Unlike other communities (e.g. coral communities, rocky shore communities) where certain species are no longer present under high-CO\textsubscript{2} conditions and regime shifts occur\textsuperscript{20,21,42,43}, that is not the case with the zooplankton community. The possible flow-on effects on the rest of the ecosystem will be less severe than if zooplankton were not only reduced in abundance but also changed in community composition.

The short generation times of zooplankton could imply faster evolution than for larger organisms, potentially increasing their ability to adapt to ocean acidification\textsuperscript{44,45}. However, ocean acidification is not the only anthropogenic factor impacting zooplankton. Ocean warming, pollution, and hypoxia are also progressively increasing, further influencing zooplankton communities. The ability of zooplankton communities to cope with all the stressors combined will determine their fate in future oceans and the role they will play in coral reef ecosystems.

Conclusions

The incessant progression of ocean acidification does not bode well for many marine ecosystems and their associated organisms. Coral reefs are especially in jeopardy due to direct and indirect effects of ocean acidification. The results from this thesis reveal yet another mechanism that may contribute to the degradation of coral reefs, since the very basis of their food webs, demersal zooplankton, was reduced in abundance under high-CO\textsubscript{2} conditions. Different drivers influence different zooplankton taxa and collectively contribute to an overall decline in the demersal zooplankton community. Habitat loss was the primary cause for zooplankton abundance loss, but it was not the only cause, as seen with the pontellid copepods that were no longer associated with any type of substrata under high-CO\textsubscript{2} conditions. The loss in zooplankton abundance will have implications for their predators, including corals. A case study on heterotrophy for the stony coral
Galaxea fascicularis revealed, unexpectedly, that they consumed less zooplankton under ocean acidification. Fewer zooplankton particles available in the water column combined with depressed feeding rates suggests that corals may become nutrient limited, with likely consequences for their overall health. Decreased zooplankton quantities will have repercussions on the ecosystem that may be felt socially and economically.

References


21. Enochs, I. C. *et al.* Shift from coral to macroalgae dominance on a volcanically


34. Hamner, W. M., Jones, M. S., Carleton, J. H., Hauri, I. R. & Williams, D. M. Zooplankton, planktivorous fish, and water currents on a windward reef face:


# APPENDIX I
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AABW</td>
<td>Antarctic Bottom Water</td>
</tr>
<tr>
<td>AAIW</td>
<td>Antarctic intermediate water</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AT</td>
<td>Total alkalinity</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>CCA</td>
<td>Canonical correspondence analysis</td>
</tr>
<tr>
<td>Chl a</td>
<td>Chlorophyll a</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CO₂(eq)</td>
<td>Aqueous carbon dioxide</td>
</tr>
<tr>
<td>pCO₂</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>CO₃²⁻</td>
<td>Carbonate</td>
</tr>
<tr>
<td>DIC</td>
<td>Dissolved inorganic matter</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>GAMM</td>
<td>Generalized additive mixed model</td>
</tr>
<tr>
<td>GLM</td>
<td>Generalized linear model</td>
</tr>
<tr>
<td>H⁺</td>
<td>Hydrogen ion</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>Bicarbonate</td>
</tr>
<tr>
<td>H₂CO₃</td>
<td>Carbonic acid</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>Mercuric chloride solution</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HNLC</td>
<td>High nutrient low chlorophyll</td>
</tr>
<tr>
<td>IPCC</td>
<td>Intergovernmental Panel on Climate Change</td>
</tr>
<tr>
<td>NADW</td>
<td>North Atlantic deep water</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>OA</td>
<td>Ocean acidification</td>
</tr>
<tr>
<td>pHₜ</td>
<td>pH at total scale</td>
</tr>
<tr>
<td>POC</td>
<td>Particulate organic matter</td>
</tr>
<tr>
<td>RDA</td>
<td>Redundancy analysis</td>
</tr>
<tr>
<td>SCUBA</td>
<td>Self-contained underwater breathing apparatus</td>
</tr>
<tr>
<td>VINDTA</td>
<td>Versatile Instrument for the Determination of Total Inorganic Carbon</td>
</tr>
<tr>
<td>Ωₘₐₙ</td>
<td>Saturation state of aragonite</td>
</tr>
<tr>
<td>Ωₙₜₐₜ</td>
<td>Saturation state of calcite</td>
</tr>
</tbody>
</table>
APPENDIX II
Supplemental Information for Chapter 2: Ocean acidification reduces demersal zooplankton that reside in tropical coral reefs

Supplementary Table 1. Mean and SE for total pH (pHT), total alkalinity (AT), dissolved inorganic carbon (DIC), partial pressure of carbon dioxide (pCO₂), bicarbonate (HCO₃⁻), and carbonate (CO₃²⁻) of the seawater at two reefs (Dobu and Upa-Upasina), two CO₂ levels (control and high-CO₂), and three expeditions (1,2,3).

<table>
<thead>
<tr>
<th>Expedition</th>
<th>Reef</th>
<th>CO₂</th>
<th>pHT</th>
<th>AT (µmol kg⁻¹)</th>
<th>DIC (µmol kg⁻¹)</th>
<th>pCO₂ (µatm)</th>
<th>HCO₃⁻ (µmol kg⁻¹)</th>
<th>CO₃²⁻ (µmol kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dobu</td>
<td>control</td>
<td>8.0 ± 0.04</td>
<td>2250 ± 3</td>
<td>1963 ± 13</td>
<td>486 ± 28</td>
<td>1745 ± 19</td>
<td>206 ± 7</td>
</tr>
<tr>
<td>2</td>
<td>Dobu</td>
<td>control</td>
<td>8.0 ± 0.004</td>
<td>2222 ± 3</td>
<td>1956 ± 5</td>
<td>483 ± 6</td>
<td>1753 ± 6</td>
<td>191 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>Dobu</td>
<td>control</td>
<td>8.0 ± 0.01</td>
<td>2287 ± 19</td>
<td>1899 ± 5</td>
<td>423 ± 14</td>
<td>1672 ± 8</td>
<td>216 ± 11</td>
</tr>
<tr>
<td>1</td>
<td>Dobu</td>
<td>high-CO₂</td>
<td>7.8 ± 0.05</td>
<td>2262 ± 4</td>
<td>2064 ± 21</td>
<td>863 ± 128</td>
<td>1891 ± 32</td>
<td>151 ± 13</td>
</tr>
<tr>
<td>2</td>
<td>Dobu</td>
<td>high-CO₂</td>
<td>7.8 ± 0.04</td>
<td>2260 ± 2</td>
<td>2055 ± 15</td>
<td>789 ± 132</td>
<td>1880 ± 19</td>
<td>155 ± 7</td>
</tr>
<tr>
<td>3</td>
<td>Dobu</td>
<td>high-CO₂</td>
<td>7.8 ± 0.01</td>
<td>2208 ± 7</td>
<td>2055 ± 70</td>
<td>767 ± 221</td>
<td>1876 ± 96</td>
<td>146 ± 8</td>
</tr>
<tr>
<td>1</td>
<td>Upa</td>
<td>control</td>
<td>8.0 ± 0.02</td>
<td>2222 ± 1</td>
<td>1952 ± 11</td>
<td>515 ± 25</td>
<td>1745 ± 16</td>
<td>194 ± 6</td>
</tr>
<tr>
<td>2</td>
<td>Upa</td>
<td>control</td>
<td>8.0 ± 0.02</td>
<td>2224 ± 7</td>
<td>1946 ± 16</td>
<td>463 ± 28</td>
<td>1736 ± 21</td>
<td>198 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>Upa</td>
<td>control</td>
<td>8.0 ± 0.03</td>
<td>2203 ± 10</td>
<td>1896 ± 27</td>
<td>428 ± 42</td>
<td>1669 ± 37</td>
<td>217 ± 5</td>
</tr>
<tr>
<td>1</td>
<td>Upa</td>
<td>high-CO₂</td>
<td>7.8 ± 0.03</td>
<td>2261 ± 6</td>
<td>2081 ± 16</td>
<td>910 ± 59</td>
<td>1919 ± 21</td>
<td>139 ± 6</td>
</tr>
<tr>
<td>2</td>
<td>Upa</td>
<td>high-CO₂</td>
<td>7.8 ± 0.03</td>
<td>2264 ± 5</td>
<td>2089 ± 18</td>
<td>894 ± 71</td>
<td>1929 ± 24</td>
<td>137 ± 8</td>
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<tr>
<td>3</td>
<td>Upa</td>
<td>high-CO₂</td>
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<td>2068 ± 9</td>
<td>816 ± 59</td>
<td>1902 ± 16</td>
<td>160 ± 31</td>
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**Supplementary Table 2.** Mean values ± SE of total organic carbon (TOC, µg L⁻¹), total nitrogen (TN, µg L⁻¹), chlorophyll a (µg L⁻¹), and phaeophytin (µg L⁻¹) during the day and night, at two reefs, and for two CO₂ levels.

<table>
<thead>
<tr>
<th>Time</th>
<th>Reef</th>
<th>Day</th>
<th>Night</th>
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<tbody>
<tr>
<td>CO₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level</td>
<td></td>
<td>Control</td>
<td>High-CO₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dobu</td>
<td>Upa-Upasina</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Organic</td>
<td>206 ± 64</td>
<td>212 ± 98</td>
</tr>
<tr>
<td></td>
<td>Carbon</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>32 ± 1</td>
<td>29 ± 7</td>
</tr>
<tr>
<td></td>
<td>Nitrogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Phaeophytin</td>
<td>0.17 ± 0.1</td>
<td>0.18 ± 0.1</td>
<td>0.18 ± 0.1</td>
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</tbody>
</table>

**Supplementary Table 3.** Generalized linear model (GLM) results (F and p values) determining the significance of environmental variables (CO₂, reef, and time) on phytoplankton biomass (mg m⁻³). For all parameters, \((df,df) = (1,22)\).

<table>
<thead>
<tr>
<th>Environmental parameter</th>
<th>Total Organic Carbon (µg L⁻¹)</th>
<th>Total Nitrogen (µg L⁻¹)</th>
<th>Chlorophyll a (µg L⁻¹)</th>
<th>Phaeophytin (µg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>CO₂</td>
<td>1.00</td>
<td>0.329</td>
<td>0.17</td>
<td>0.686</td>
</tr>
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Supplementary Table 4. Generalized additive mixed model (GAMM) results (df, F and p values) determining the significance of environmental variables (CO2, reef, and expedition) on the abundance (individuals m⁻³) for each zooplankton taxa.

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APPENDIX III
Unpublished data on zooplankton community response to ocean acidification

This appendix chapter provides more detail on the changes in the zooplankton community under ocean acidification.

Collaborators include: Claudio Richter, Katharina Fabricius, Holger Auel

Objectives:

1. Confirm that zooplankton are residential to the reefs
2. Investigate the effects of ocean acidification on the 'quality' of zooplankton as a food source, with quality being determined based on:
   a.) changes in taxonomic composition, and
   b.) fatty acid composition of zooplankton communities
Zooplankton confirmed living residential to coral reefs

Justification

In order to have confidence that the zooplankton at the seeps are indeed exposed to long-term ocean acidification condition, we must first confirm that they are residential zooplankton and not just the open-ocean species that are flushed through the system. Zooplankton living in coral reefs are generally composed of two distinct communities, those that originate from coastal and open oceans and transit through the coral reef system, called holoplankton, and those that are residential to the reef and live within or swarm above the substrate during the day and migrate into the water column at night, called demersal plankton\(^1,2\). The amount of zooplankton derived from either community depends on the physical environment of a particular reef, which is generally controlled by currents, tides, and exposure to the open ocean\(^3\)–\(^5\). Here, we first compared the zooplankton community composition between offshore and reef sites, and compared the community composition between day and night, to determine how much of the zooplankton at the seep sites are residential to the reefs.

Methods

Zooplankton samples were collected from two separate CO\(_2\) seeps, Dobu and Upa-Upasina reefs, in Papua New Guinea using horizontal net tows (refer to Methods section of Chapter 2 for more detail on collection process). For offshore comparisons, additional sites were sampled. Samples were collected from four sites at each seep location: control, offshore from the control, high-CO\(_2\), and offshore from the high-CO\(_2\). Reef sites were located in fringing, shallow water (2-3 m) reefs, while the offshore sites were located in deeper water (50-70 m), approximately 500 m away from the coastline. Samples were collected over three separate expeditions (17-27 January 2013, 24 May-5 June 2013, and 22 March–20 April 2014). To determine if zooplankton were residential to the reefs, zooplankton communities were compared between reef and offshore sites, and compared between day and night (since demersal zooplankton emerge into the water column at night).
Result Highlights

**Reef v. Offshore Community Comparisons**

*a.) Greater zooplankton abundance over reefs compared to offshore*

Zooplankton abundances were greater at the control sites than the offshore sites ($F_{(3,65)} = 45.6, p < 0.001$). In contrast, abundances at the high-CO$_2$ sites were similar to those at the offshore sites (Figure 1).

![Zooplankton Abundance Chart](image)

**Figure 1.** Nocturnal zooplankton abundance (individuals m$^{-3}$) over reefs and offshore from those reefs, at both control and high-CO$_2$ sites at two CO$_2$ vents (Dobu and Upa-Upasina).

*b.) Different taxonomic composition between reef and offshore sites*

To confirm that the zooplankton living at the high-CO$_2$ sites was not just the open-ocean zooplankton floating through the reef, the community composition of the zooplankton at the high-CO$_2$ sites was compared to that offshore from the high-CO$_2$ sites with a canonical correspondence analysis (CCA). This analysis demonstrated
strong differences in community composition between the offshore and high-CO₂ reef sites (permanova: $P = 0.001$; Figure 2a). The community composition of the control reef and the offshore control were also different ($P = 0.001$; Figure 2b). Hence, zooplankton residing both at the high-CO₂ reefs or the control reefs, were different communities compared to those residing in offshore waters.

**Figure 2.** CCA biplot comparing community composition (% taxonomic composition) between: (a) high-CO₂ site and offshore-high-CO₂ site, and (b) control site and offshore-control site. Both reefs combined (Dobu and Upa-Upasina).

**Day v. Night Community Comparisons**

a.) Greater abundance during the night than during the day

During the day, only transient holoplankton are present in the water column while residential zooplankton are hiding in the reef substrata. We therefore compared the day samples to the nighttime samples to quantify the amount of residential zooplankton that emerge. Zooplankton abundances were significantly different between reefs ($F_{(1,0.2)} = 4.8$, $p = 0.04$), CO₂ levels ($F_{(1,0.88)} = 19.6$, $p = 0.0001$), as well as between day and night ($F_{(1,25.3)} = 564.3$, $p < 2.2e^{-16}$). The difference in zooplankton abundance was greater between day and night compared to the difference at night between the control and high-CO₂ sites (Figure 3), with the average zooplankton abundance 30.4, 19.1, 47.0, and 23.7 times higher at night.
than during the day at the control and high-CO$_2$ sites at Dobu, and the control and high-CO$_2$ sites at Upa-Upasina, respectively.

**Figure 3.** Difference in zooplankton abundance (individuals m$^{-3}$) between day and night at the two study reefs.

b.) *Different taxonomic composition between day and night*

Not only were there differences in abundance between day and night, but also the percent composition of the zooplankton community was different between day and night (permanova: $p = 0.001$; Figure 4).

**Figure 4.** CCA biplot comparing community composition (% taxonomic composition) between daytime and nighttime samples. Both reefs combined (Dobu and Upa-Upasina).
Conclusions

• Zooplankton are more abundant and have a different taxonomic composition over reefs compared to offshore; therefore, the zooplankton must reside at the reef.

• Zooplankton are more abundant and have a different taxonomic composition at night than during the day; therefore, the zooplankton collected must be the demersal zooplankton that nocturnally migrate into the water column.

• Zooplankton collected are in fact demersal and reside within the reef and CO₂ seeps; thus, we can have confidence that the zooplankton in these studies are in fact exposed to ocean acidification conditions and are not merely the transient zooplankton passing through the open system of the CO₂ seeps.
Taxonomic composition and fatty acid content unaffected by ocean acidification

Justification

The quality of zooplankton as a food source for reef planktivores has implications for the health of individual consumers, which collectively can contribute to the overall health of the reef and its food webs. The quality of zooplankton can be measured in several ways. For example, community composition can be indicative of particle size distribution. They can also be indicative of the nutrient and energy content of food, since different zooplankton taxa constitute varying levels of carbohydrates, nutrients, proteins, and total caloric values. Furthermore, zooplankton quality can be measured through the biochemical signature of the zooplankton, such as its fatty acid composition. Here, both taxonomic composition and fatty acid content are compared between the control and high-CO2 sites.

Methods

A more detailed description of the collection and sampling methods can be found in the Methods section of Chapter 2. Community composition for samples was calculated by first subsampling each original sample with a Folsom splitter. Zooplankton were identified with microscopy to the Family level for copepods, and Order for non-copepods. Later the percent composition was calculated from the abundance data.

Frozen samples were evaluated for their fatty acid content of the bulk zooplankton following the procedure of Folch et al (1956) and Hagen (2000). Lipids were extracted from bulk zooplankton samples and dissolved in chloroform and methanol solution in the ratio 2:1 (by volume). Samples were then homogenized using a Potter homogenizer (Braun, Potter S), followed by an ultrasonic cell disrupter (Bandelin electronic, UW 2070), and afterwards the lipid extracts were washed with aqueous KCl solution and centrifuged to separate the different phases. Subsamples of the extracted lipids were prepared for gas-chromatography using the methods of Kattner and Fricke (1986), and fatty acids and fatty alcohols were then analyzed with a gas chromatograph (Agilent Technologies 7890 A).
Result Highlights

Percent composition under ocean acidification

The zooplankton community varied across days and expeditions (Figure 5). Additionally, the taxonomic composition of the zooplankton differed between reefs, but there was no substantial shift in the composition between CO2 levels. All the same taxonomic groups present in the control reefs were present in the high-CO2 reefs, with generally slightly higher percentages of copepods at the high-CO2 sites compared to the control sites (Figure 5).

Figure 5. Percent composition of zooplankton taxa in samples from control and high-CO2 sites at two reefs (Dobu and Upa-Upasina) and for three expeditions.
Since copepods dominated the zooplankton community, comparisons were also made within the copepod community alone. Again, there were no dramatic shifts in the copepod community between CO\textsubscript{2} levels, despite slight temporal variations (Figure 6). For both the overall zooplankton community and the copepod community, there was no major difference in percent composition between CO\textsubscript{2} levels, only slight differences daily, between expeditions, and between Dobu and Upa-Upasina.

![Diagram showing temporal variation in percent composition of copepod families compared between control and high-CO\textsubscript{2} sites at two reefs (Dobu and Upa-Upasina) and for three expeditions.](image)

**Figure 6.** Temporal variation in percent composition of copepod families compared between control and high-CO\textsubscript{2} sites at two reefs (Dobu and Upa-Upasina) and for three expeditions.
**Fatty acid composition of bulk zooplankton**

Very few individual fatty alcohols and fatty acids were significantly different in percentage between high-CO₂ and control sites (Table 1). There was no difference in fatty acid composition across CO₂ regimes (permanova: p = 0.440), but there was a difference in fatty acid composition between reefs (p = 0.001; Figure 7).

Table 1. Mean and standard deviation of the percent of each fatty alcohol and fatty acid within the bulk zooplankton samples. Additional, results from generalized linear models (GLMs) indicate which fatty alcohols or fatty acids were significantly different between CO₂ levels (control or high-CO₂) or reefs (Dobu or Upa-Upasina) or between the interaction of the two variables. Significant differences (p < 0.05) are highlighted in bold font.

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Figure 7. CCA biplot comparing the fatty acid composition between (a) control and high-CO$_2$ sites, and (b) reefs (Dobu and Upa-Upasina).

Conclusions

- Taxonomic composition of overall zooplankton community and copepod community were unaffected by ocean acidification.
- Fatty acid content of bulk zooplankton was unaffected by ocean acidification.
- Zooplankton composition (both taxonomic and fatty acid content) will likely be similar in future oceans under ocean acidification; thus, zooplanktivores will have less zooplankton available zooplankton for them to consume but it will be of similar composition.

References

3. Hamner, W. M., Jones, M. S., Carleton, J. H., Hauri, I. R. & Williams, D. M.


Supplemental Information for Chapter 5: Reduced heterotrophy in the stony coral *Galaxea fascicularis* after life-long exposure to elevated carbon dioxide

Supplementary Figures

Supplementary Figure S1. Food samples given to corals.

(a) The food concentration of zooplankton in the water was greater in expedition 1 compared to expedition 2, and greater in the chamber experiments compared to the field experiments.
(b) Results from a canonical correspondence analysis (CCA) reveal that the composition of food samples given to corals differed between expedition 1 and expedition 2.
Supplementary Figure S2. Zooplankton community consumed by corals vary between expedition and method.
Supplementary Figure S3. Time series of seawater pH$_T$ for the field site during both expeditions.
Supplementary Table 1S. Results of GLM regression for polyp expansion

<table>
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**Supplementary Text**

*Food samples for corals*

To determine the variance between food samples between replicates, treatments, field and chamber experiments, and the two expeditions, the coefficient of variation (CV) was calculated for each zooplankton taxonomic group, as well as for the total number of zooplankton. Food samples given to corals were similar in quantity and composition within each experiment. When comparing food samples across replicates within the same experiment, coefficient of variance (CV) values for the total number of zooplankton and for all dominant taxonomic groups were always <1. In other words, the food samples had similar food concentrations in each replicate syringe for each experiment. Only rare taxonomic groups (<1% of the entire community) had high variation between replicate food samples, i.e. CV>1.

Generalized linear models were used to compare zooplankton quantity between experiments and canonical correspondence analyses were used to compare the composition of zooplankton in the food samples between experiments. Zooplankton quantity of the food samples was different between each experiment and the composition of the food samples differed between expeditions (Supplementary Figure S1). More specifically, food concentrations were significantly different between the chamber and field experiments (3-way ANOVA: $F_{(1,16)} = 102; P < 0.001$) and between the two expeditions ($F_{(1,15)} = 311; P < 0.001$). There was no difference in food concentrations between the two field experiments conducted on consecutive nights during the second expedition ($F_{(1,14)} = 1.9; P=0.19$); therefore, those experiments were grouped together for all further analysis. Food concentrations were higher for expedition 1 compared to expedition 2, and greater for the chamber experiments compared to the field experiments. The mean food concentrations (number of zooplankton L$^{-1} \pm$SE) for each experiments were: expedition 1 - chamber, 2063.5±23.5; expedition 1- field, 1342.7±26.3; expedition 2 - chamber, 894.3±172.1; and expedition 2-field, 276.8±52.4. Despite lower food concentrations in expedition 2, species richness was actually significantly higher in expedition 2 compared to expedition 1 (two-way ANOVA: $F_{(1,16)} = 9, P < 0.001$), with an average ± SE of available prey types in expedition 2 being 26±2.4 and 33±0.6 in expedition 1. Species richness of available food types was not different between methods (two-way ANOVA: $F_{(1,15)} = 9, P = 0.06$). A community analysis of the food
samples confirms that the zooplankton communities were significantly different between expeditions (two-way ANOVA applied to CCA results: $F_{(1,14)} = 12.1; P = 0.001$), but not methods ($F_{(1,14)} = 12.1; P = 0.62$). The quantity and composition of zooplankton available to *Galaxea fascicularis* varied between experiments, but they were similar within each experiment and across the CO$_2$ treatments, thus ocean acidification affects on coral feeding behavior can still be evaluated.

Community analysis of zooplankton consumed by corals for different expeditions and methods

Although the community consumed by *G. fascicularis* did not differ across CO$_2$ levels (Figure 2 from main text), it did differ depending on the expedition and method (chamber versus field experiments; Supplementary Figure 2).

Results from generalized linear models (GLM): effects of method, expedition, and CO$_2$ on polyp expansion

Polyp expansion of corals was different across methods, expedition, and from the beginning of the experiment to the end. However, polyp expansion did not differ across CO$_2$ regimes or any of the interaction terms (Supplementary Table S1).

pH of seawater for field experiments

Seawater pH at total scale (pH$_T$) was recorded for several days around the commencement of the feeding experiments. Measurements were collected at the control and elevated CO$_2$ sites using SeaFET pH sensors and the data can be found in Supplementary Figure S3.
Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

*The effects of ocean acidification on zooplankton: using natural CO₂-seeps as windows into the future*

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

______________________________
(Unterschrift)