

Coping with a changing world: adaptive mechanisms in
marine three-spined stickleback *Gasterosteus aculeatus*

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1 Summary

Due to ongoing increases in carbon dioxide (CO₂) concentrations in the atmosphere, the world's climate is changing at an unprecedented rate, leading to rising global mean air and water temperatures. When atmospheric CO₂ dissolves in seawater, ocean pH declines, causing an acidification of the ocean. These altered environmental conditions are highly probable to have severe impacts on marine organisms by affecting their performance and survival. When environmental stress is increasing, some species can migrate to habitats with more favourable conditions, others have to adapt to changing conditions. Whether species will be able to adapt fast enough to keep pace with changing environments will be one decisive factor for population persistence.

In this thesis, a prime model organism, the three-spined stickleback *Gasterosteus aculeatus*, was used to study ecological and evolutionary effects of rising water temperatures and ocean acidification on marine fish populations.

Rising temperatures are likely to stress marine species, dependent on their thermal tolerance. Thermal stress can alter immune functions of organisms, thereby increasing the susceptibility to infectious diseases. By combining the effects of elevated temperature and bacterial infection in a common garden experiment, the influence of thermal stress on evolutionary trajectories of disease resistance in three-spined stickleback populations could be investigated (chapter II). Environmental stress negatively impacted life-history traits and pathogen resistance of sticklebacks. Furthermore, thermal stress reduced genetic differentiation between populations by releasing cryptic within-population variation. While life-history traits showed positive genetic correlations between temperatures and genotype by environment interactions (GxE), thermal stress led to negative genetic correlations in disease resistance, showing that evolutionary responses in altered environments can be hard to predict from prevailing conditions.

Rising temperatures, on the other hand, promote the development of many parasites and pathogenic bacteria, increasing the risk of infection and diseases during summer. To understand infection patterns in relation to water temperature, the parasite and bacterial communities of marine fish species were investigated over a period of two

years (chapter I). Temporarily elevated water temperatures resulted in increased macroparasite and bacterial diversities in marine fish species. In addition, some parasite groups showed a forward shift and extension in infection peaks, following warmer spring seasons. This shows that even subtle changes in seasonal temperatures can have an effect on the epidemiology and phenology of parasites as well as opportunistic pathogens. Increased virulence of pathogens at higher temperatures in combination with immune-compromised hosts can have far fetching consequences for marine ecosystems.

Next to rising ocean temperatures, marine organisms have to cope with ocean acidification. It has been shown, that elevated CO₂ concentrations negatively impacted fish development and survival, particularly in early developmental stages. A powerful mechanism to mediate the effects of global change is transgenerational acclimation. By acclimating parents and offspring to different CO₂ concentrations, within- and transgenerational effects of ocean acidification on life-history traits of marine sticklebacks were studied (chapter III). Exposure to elevated CO₂ concentrations led to an increase in clutch size in adults as well as increased juvenile survival and growth rates. Transgenerational effects could be found for juvenile growth and for otolith characteristics, suggesting that parental acclimation can modify ocean acidification effects.

To summarize, three-spined sticklebacks cope better with ocean acidification than with rising ocean temperatures. Transgenerational acclimation enables sticklebacks to respond quickly to environmental changes and provides time for genetic evolution, as long-term adaptive mechanism, to catch up. The high tolerance to fluctuations in water chemistry and temperature as well as the substantial amount of standing genetic variation suggest that stickleback populations can adapt fast enough to changing environmental conditions.

2 Zusammenfassung

Durch stetig ansteigende Kohlenstoffdioxid-Konzentrationen (CO₂) ändert sich das weltweite Klima mit rasanter Geschwindigkeit und Luft- und Wassertemperaturen steigen. Wenn sich atmosphärisches CO₂ im Meerwasser löst, sinkt der pH-Gehalt des Meeres und führt zu einer Versauerung des Ozeans. Diese veränderten Umweltbedingungen können schwerwiegende Folgen für die Leistungsfähigkeit und das Überleben von Meeresbewohner haben. Wenn der Umweltstress ansteigt, können einige Arten in Habitate mit geeigneteren Bedingungen ausweichen, andere wiederum müssen sich an die veränderten Umweltbedingungen anpassen. Ob Lebewesen in der Lage sind, sich schnell genug an die sich ändernde Umwelten anzupassen, wird ein entscheidender Faktor für das Bestehen von Populationen sein.

Um ökologische und evolutionäre Effekte des globalen Wandels auf marine Fischpopulationen zu untersuchen, wurde ein hervorragend geeigneter Modellorganismus, der Dreistachlige Stichling *Gasterosteus aculeatus*, für diese Arbeit verwendet.

Abhängig von der thermalen Toleranz, können ansteigende Temperaturen Stress in marinen Lebewesen hervorrufen. Thermaler Stress kann Immunfunktionen von Organismen beeinflussen und dadurch die Anfälligkeit für Infektionskrankheiten erhöhen. Durch die kombinierten Effekte von erhöhter Temperatur und bakterieller Infektion in einem „common garden“ Experiment, konnte der Einfluss von thermalem Stress auf evolutionäre Mechanismen der Pathogenresistenz bei Stichlingspopulationen untersucht werden (Kapitel II). Umweltstress beeinträchtigte Fitnesskomponenten und Pathogenresistenzen bei Stichlingen. Zudem führte thermaler Stress zu reduzierter genetischer Differenzierung zwischen Populationen. Während Fitnesskomponenten eine positive genetische Korrelation zwischen Temperaturen zeigten, führte thermaler Stress zu einer negativen genetischen Korrelation für Krankheitsresistenz. Ausgehend von vorherrschenden Bedingungen scheinen evolutionäre Antworten auf veränderte Umwelten schwer vorhersagbar.

Ansteigende Temperaturen begünstigen andererseits die Entwicklung von vielen Parasiten und pathogenen Bakterien und erhöhen dadurch das Risiko für Infektionen und Krankheiten während des Sommers. Um temperaturbedingte Infektionsmuster

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zu verstehen, wurden die Parasiten- und Bakteriengemeinschaften von marinen Fischarten in einem Zeitraum von zwei Jahren untersucht. Kurzzeitig ansteigende Wassertemperaturen verursachten erhöhte Makroparasiten- und Bakteriendiversitäten in marinen Fischarten. Zusätzlich zeigten einige Parasitengruppen eine Verschiebung und Ausdehnung des Infektionshöhepunkts durch wärmere Frühlingsmonate. Bereits kleine Änderungen in saisonalen Temperaturen können die Epidemiologie und die Phänologie von Parasiten, als auch von opportunistischen Pathogenen, beeinflussen. Erhöhte Pathogenvirulenz durch ansteigende Temperaturen kombiniert mit immungeschwächten Wirten kann zu weitreichenden Konsequenzen für marine Ökosysteme führen.

Neben ansteigenden Wassertemperaturen, müssen marine Lebewesen auch mit Ozeanversauerung umgehen. Es wurde bewiesen, dass erhöhte CO₂ Konzentrationen die Entwicklung und das Überleben von Fischen beeinträchtigt. Ein wirkungsvoller Mechanismus, um die Effekte des globalen Wandels zu mildern, ist transgenerationale Akklimationisierung. Durch das Akklimationisieren von Eltern und Nachwuchs an verschiedene CO₂ - Konzentrationen, konnten inner- und transgenerationale Effekte von Ozeanversauerung auf marine Stichlinge untersucht werden (Kapitel III). Erhöhte CO₂ - Konzentrationen führten zu vergrößerten Eigelegen bei den Eltern, als auch zu erhöhten Überlebens- und Wachstumsraten bei den Juvenilen. Transgenerationale Effekte konnten für juveniles Wachstum und für Otolithencharakteristiken bestätigt werden und lassen darauf schließen, dass elterliche Akklimationisierung die Auswirkungen von Ozeanversauerung verändern können.

Zusammenfassend ist zu erwähnen, dass der Dreistachlige Stichling mit Ozeanversauerung besser umgehen kann, als mit ansteigenden Meerestemperaturen. Zudem ermöglicht transgenerationale Akklimationisierung, Stichlingen auf Umweltveränderungen schnell zu reagieren, bevor genetische Evolution, als Langzeitanpassungsmechanismus, übergreift. Die hohe Toleranz für schwankende Wasserchemie und Temperatur, als auch die erhebliche Menge an dauerhafter genetischer Varianz ermöglicht Stichlingspopulationen sich schnell genug an die sich verändernde Umweltbedingungen anzupassen.

3 Introduction

3.1 Global change

Since the industrial revolution, anthropogenic activities such as extensive burning of fossil fuel, cement production or deforestation have mainly contributed to increasing concentrations of greenhouse gases (Siegenthaler et al. 2005; Peters et al. 2012). An important greenhouse gas is carbon dioxide (CO_2), which is constantly increasing in the atmosphere since the 19th century, going from the pre-industrial level of about 280 parts per million (ppm) to the current level of about 390 ppm (Tans 2009). Climate models predict a further increase of atmospheric CO_2 concentrations up to 700 to 1000 ppm by the end of this century (IPCC 2012). These large changes in the earth atmosphere composition are associated with an increase in global mean air and water temperature of 1.5 to 4°C until the year 2100 (IPCC 2012). Closely related with global warming is also the occurrence of extreme events such as heat waves that are expected to rise in frequency and intensity, leading to unexpected high summer temperatures (Stott et al. 2004; Smith et al. 2009).

The world's oceans play an essential role in the exchange of CO_2 with the atmosphere. By absorbing approximately one third of the constantly increasing atmospheric carbon dioxide (Sabine et al. 2004), the carbonate system has been modified (figure 1).

When atmospheric CO_2 dissolves in seawater, it forms carbonic acid (H_2CO_3), which immediately dissociates into bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and hydrogen ions (H^+). The excess of H^+ causes a decline in ocean pH, leading to an acidification of the ocean (Caldeira and Wickett 2005; Peters et al. 2012). During the last 150 years, the average oceanic pH has decreased by 0.1 units (Doney 2010) and is projected to decline another 0.3 to 0.4 units until the year 2100 (IPCC 2012). Next to more acidic seawater, hydrogen ions combine with free carbonate ions leading to an ongoing reduction of available carbonate ions in the ocean (Fabry et al. 2008).

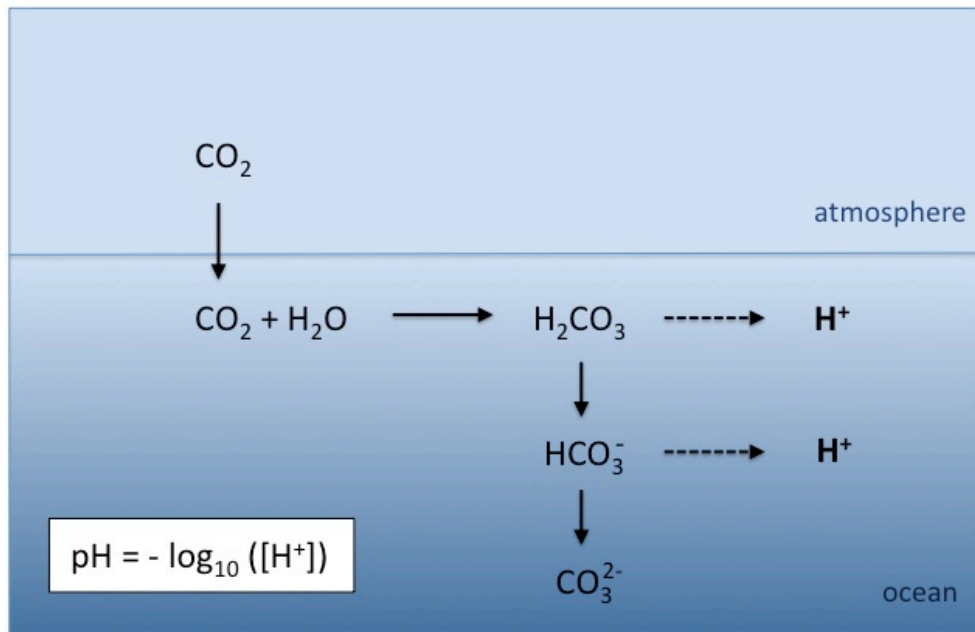


Figure 1 The chemical processes of ocean acidification.

3.2 Impacts of global change on marine species

These altered environmental conditions are highly probable to have profound impacts on marine ecosystems, yet a comprehensive understanding of how global change is affecting them has been poorly developed. Earth system models are purely physical models, projecting abiotic changes, but do not consider biological responses in the ocean (Mooney 1991; Heavens et al. 2013). There is an ongoing debate about whether the rise of water temperature or ocean acidification will have more severe consequences for the performance and survival of marine organisms and which species are more sensitive to these changes (Harley et al. 2006; Munday et al. 2012; Bijma et al. 2013).

Ocean warming

The sensitivity of species to ocean warming will strongly depend on their thermal tolerance and their capacity for physiological responses (Harley et al. 2006; Portner and Peck 2010). Many marine organisms live already close to their upper thermal limit and continually rising temperatures are likely to affect their biochemistry, physiology

and behaviour, which in turn might result in reduced fitness and survival (Beitinger et al. 2000; Dominguez et al. 2004).

Species sensitivity to rising temperatures varies within and among species. It is known that larval stages are more susceptible to thermal stress than adults (Brauner 2008; Ishimatsu et al. 2008), and that less active or sessile organisms are more vulnerable to ocean warming than species that can escape to locations with more favourable environmental conditions (Hughes et al. 2003; Bijma et al. 2013). Reef-building corals, for example, are highly sensitive to increases in ocean temperatures and warming led to widespread coral bleaching and mortality events (McWilliams et al. 2005; Carpenter et al. 2008).

However, warmer temperatures accelerate biochemical reactions, permitting higher activity, increased growth rates as well as enhanced reproduction. Increased metabolism is energetically costly and requires higher food consumption rates to maintain a positive energy balance (Lafferty 2009). A negative energy balance is likely to cause thermal stress in species, altering, for instance, immune functions by weakening first-line defences and leading to increased host susceptibility to infectious diseases (Harvell et al. 2002; Lafferty et al. 2004; Alborali 2006). Under these specific conditions, opportunistic pathogens, that are normally benign and non-pathogenic, are likely to harm host species (Griffiths 1991; Martin et al. 2002).

In general, many pathogenic bacteria and parasites are favoured in warmer waters (Harvell et al. 2002; Lafferty et al. 2004; Cerrano and Bavestrello 2009). Rising water temperatures lead to faster development and increased transmission stages per generation, resulting in higher virulence and increased disease outbreaks during summer (Vezzulli et al. 2010). Elevated ocean temperatures are also likely to expand the range of pathogens and to extend the length of transmission periods by providing prolonged summer conditions (Sankurathri and Holmes 1976; Karvonen et al. 2010). Nevertheless, a subset of pathogenic bacteria and parasites might also decline with warming, depending on their thermal tolerance (Karvonen et al. 2010).

Next to increases in disease prevalence, ocean warming is also altering species distribution and migration behaviour. To escape unfavourable conditions, some species will be driven towards the poles or in the depth (Parmesan and Yohe 2003; Perry et al. 2005). But when dispersal capacities are limited or suitable habitats are unavailable,

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widespread extinctions might arise as well (Thomas et al. 2004; Bijma et al. 2013). On the other hand, when new species are introduced in native ecosystems, a transfer of new parasites and diseases to the local population might occur (Perry et al. 2005). When native species are susceptible to introduced parasites, it may have severe consequences for this ecosystem (Cohen and Carlton 1998; Lafferty and Kuris 1999). Ocean warming is not only likely to alter host-parasite-interactions, but may also shift life cycles of predator and prey, leading to a mismatch of species phenologies. The consequences for population dynamics and food web structures might be tremendous (Adrian et al. 2006; Durant et al. 2007).

Ocean acidification

Ocean warming is only one of several projected impacts of global change, ocean acidification is another. Many studies have focused on calcifying organisms, which seem to be primary affected by ocean acidification (Orr et al. 2005; Gazeau et al. 2007; Turley et al. 2010). For skeletal structures, calcium carbonate (CaCO_3) is formed from calcium (Ca^{2+}) and carbonate ions. The reduced availability of carbonate ions under low pH conditions interferes with the calcifying process (Guinotte and Fabry 2008).

A recently published review by Wittmann and Pörtner (2013) summarizes the sensitivities of different marine taxa to ocean acidification by using physiological performances, such as aerobic scope, fertilization, developmental time, immune response or behavioural changes, as indicators for individual fitness in response to elevated CO_2 exposure. Except for crustaceans, 40-60% of all species of remaining animal taxa were negatively impacted by slightly increased CO_2 levels in the range from 500-650 μatm (figure 2). Within the CO_2 ranges of 651-850 μatm and 851-1370 μatm , reflecting predicted carbon dioxide levels for the year 2100, fish showed surprisingly the highest sensitivities (figure 2).

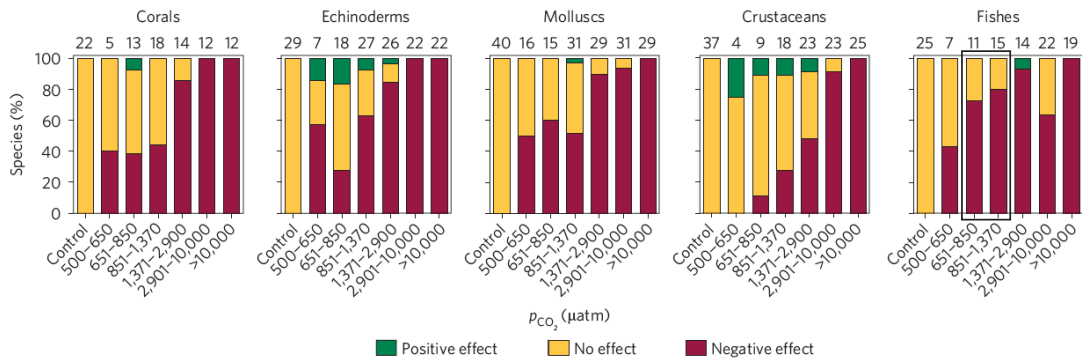


Figure 2 Sensitivities of marine taxa to ocean acidification. Coloured columns show effects of elevated pCO₂ ranges on individual fitness of animal groups. Numbers on top of columns represent considered species numbers. Modified after Wittmann and Pörtner 2013.

Adult fish are considered to be relatively tolerant to changes in ambient CO₂ due to active adjustments of their acid-base balance (Claiborne et al. 2002; Evans et al. 2005; Melzner et al. 2009). But these processes may come at a cost, likely reducing the amount of energy available for physiological performance and survival (Portner et al. 2004; Kroeker et al. 2010; Forsgren et al. 2013).

Contrary to adults, early developmental stages of marine fish species are highly susceptible to increases in CO₂ concentrations. Due to insufficient acid-base regulations, fish eggs and larvae are particularly sensitive to ocean acidification (Ishimatsu et al. 2008; Melzner et al. 2009). Recent studies have been shown negative effects of acidic seawater on survival (Baumann et al. 2012), development (Munday et al. 2009b; Frommel et al. 2012a), RNA/DNA ratios (Franke and Clemmesen 2011), otolith growth (Checkley et al. 2009; Munday et al. 2011) and behaviour (Ferrari et al. 2012; Nilsson et al. 2012) of marine fish larvae.

When dispersal capacities are not limited and suitable habitats are available, fish are able to migrate to locations with more favourable environmental conditions (Bijma et al. 2013; Jones et al. 2013). But what will happen when ocean warming requires a poleward migration, whereas ocean acidification would drive fish to the equator, as colder waters acidify faster?

3.3 Adaptive mechanisms

To cope with a changing world, fish and other marine species have to adapt. But will these organisms be able to adapt fast enough to deal with global change?

In general, adaptation requires standing genetic variation, providing the basis for any evolutionary change (May 1994). When environmental conditions change, the genetic composition of a population is likely to change as well, as a consequence of natural selection. Genotypes that contribute more to mean population fitness will increase in frequency, while other genotypes with lower fitness will decrease (microevolution), leading to a well-adapted population (Gienapp et al. 2008; Visser 2008). Fast genetic adaptation to changing environmental conditions either requires a substantial amount of standing genetic variation within populations or short generation times of species (Poertner and Farrell 2008).

Rapid but non-genetic adaptation (acclimation) to environmental change can work via phenotypic plasticity, which is defined as the ability of a single genotype to alter its phenotype in response to different environmental conditions (Price et al. 2003; Visser 2008). At the population level, different genotypes respond differently in changing environments, leading to genotype-by-environment interactions (GxE) (Pigliucci 2005). For understanding phenotypic plasticity and genotype-by-environment interactions, reaction norms are extremely useful (Conner and Hartl 2004; Gazeau et al. 2007), depicting the phenotypes produced by different genotypes within a population across different environments (figure 3).

Plasticity can also work over multiple generations via transgenerational acclimation (Munday et al. 2013). Non-genetic parental effects can mitigate or even compensate the effects of rising water temperatures and ocean acidification and have been shown to help marine species to cope with rapid environmental change (Donelson et al. 2012; Miller et al. 2012; Parker et al. 2012). These results indicate the importance of long-term studies, because the full potential to cope with a changing world may take several generations to be expressed (Munday et al. 2012; Garrard et al. 2013).

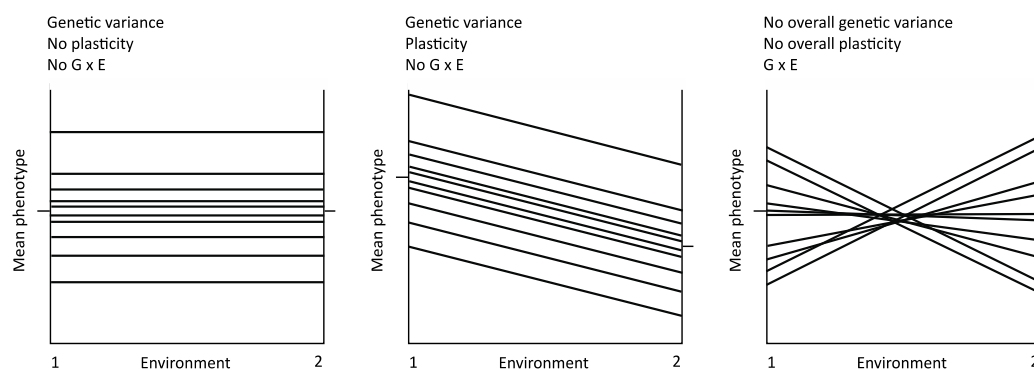


Figure 3 Family-specific reaction norms for three hypothetical situations. Lines represent different family-specific phenotypic means of two different environments. Outer tick marks show overall phenotypic mean within each environment. Modified after Conner & Hartl 2004.

Phenotypic plasticity enables organisms to respond quickly to environmental changes and provides time for genetic adaptation to overtake (Chevin and Lande 2010). Whether species will be able to acclimate or adapt fast enough to keep pace with changing environments will be one decisive factor for population persistence (Hoffmann et al. 1995; Reusch and Wood 2007; Bell and Collins 2008).

3.4 The supermodel three-spined stickleback

Many of global change related studies on teleosts were conducted on tropical fish, which are highly sensitive to changing environmental conditions (Donelson et al. 2011; Munday et al. 2012; Miller et al. 2013). Studies on temperate fish are only slowly increasing, yet many ecological questions remain still unanswered. Moreover, studies estimating the evolutionary potential of marine fish species under global change scenarios are even scarcer (Munday et al. 2013).

Therefore, I used a temperate model organism, the marine three-spined stickleback *Gasterosteus aculeatus*, to study the ecological and evolutionary responses to rising water temperature and ocean acidification (figure 4).

Marine three-spined sticklebacks rapidly and repeatedly colonized freshwater habitats

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throughout the whole northern hemisphere (Bell and Foster 1994) and can be found in a wide range of habitats, from marine and brackish to limnic waters (Östlund-Nilsson et al. 2006). The high tolerance of this species to fluctuations in water chemistry and temperature shows strong physiological plasticity and a large potential for acclimation to environmental stressors (Pottinger et al. 2002; Östlund-Nilsson et al. 2006; Barrett et al. 2011). The extensive intra-specific variation combined with easy maintenance and breeding in the laboratory made the three-spined stickleback to a prime model organism for ecological and evolutionary questions (Barber and Nettleship 2010).



Figure 4 Three-spined stickleback *Gasterosteus aculeatus*.

Faroese stamp (1994) shows male (coloured) and female of three-spined stickleback. Picture provided by Postverk Føroya - Philatelic Office.

Even if highly stress tolerant, some studies revealed negative impacts of elevated water temperature on stickleback development (Guderley and Leroy 2001; Macnab and Barber 2012), survival (Jordan and Garside 1972; Dittmar et al. 2014) and reproduction (Hopkins et al. 2011). Moreover, a temperature increase of 4°C above ambient temperature resulted in a reduced stickleback population biomass by 60% in warmed mesocosms (Moran et al. 2010). Further, an experimental heat wave induced decreased innate immune activity in sticklebacks, which may increase the risk of infection with parasites and pathogenic bacteria in the nearer future (Dittmar et al. 2014).

So far, most studies on three-spined sticklebacks investigated thermal or osmotic stress (Jordan and Garside 1972; Moran et al. 2010; Hopkins et al. 2011; Lefebure et al. 2011), but only one study considered the impact of ocean acidification on

sticklebacks, revealing behavioural defects in juveniles due to elevated CO₂ concentrations (Jutfelt et al. 2013).

Particularly in marine ecosystems, the possible ecological impacts and the population-specific potential for evolutionary responses to changing environments are still less known.

3.5 Thesis outline

This thesis addresses the overall question whether or not marine three-spined sticklebacks can cope with a changing world. To answer this question, it is necessary to reveal possible effects of rising water temperatures and ocean acidification on stickleback populations and to investigate their potential for evolutionary responses and plasticity, as adaptive mechanisms, to mediate the impacts of global change. In order to do this, three projects were designed that are presented in the following chapters.

Chapter I is based on a two-year investigation of the parasite and bacterial communities of four common fish species (three-spined stickleback *Gasterosteus aculeatus*, Atlantic herring *Clupea harengus*, European sprat *Sprattus sprattus* and lesser sand eel *Ammodytes tobianus*) in the northern Wadden Sea. It was found that short periods of elevated water temperatures resulted in increased macroparasite and bacterial diversities. Moreover, some classes of macroparasites showed a shift in infection peaks that followed the water temperature of preceding months. This may show that even subtle changes in seasonal temperatures can have an effect on the epidemiology and phenology of parasites as well as opportunistic pathogens, potentially leading to far fetching consequences for sensitive ecosystems. This field investigation formed a fundamental basis for experimental work by identifying suitable and relevant candidates for laboratory experiments.

In chapter II, I investigated the combined effects of elevated temperature and infection with a pathogenic bacterium on evolutionary trajectories of stickleback populations from coastal North Sea areas. It was found that environmental stress negatively

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impacted life-history traits and pathogen resistance of marine three-spined sticklebacks. In addition, thermal stress reduced genetic differentiation between populations and led to negative genetic correlations in disease resistance, showing that evolutionary responses in altered environments can be hard to predict from prevailing conditions. This study demonstrates the importance of experimental assessment of realistic stress scenarios and thereby highlights the impact of predicted climate change on marine fish populations.

In chapter III, within- and transgenerational effects of ocean acidification on life-history traits of marine sticklebacks were studied by acclimating parents and offspring to different CO₂ concentrations. Exposure to elevated CO₂ concentrations led to an increase in clutch size in adults as well as increased juvenile survival and growth rates between 60 and 90 days post-hatch. Transgenerational effects could be found for juvenile growth and for otolith characteristics, suggesting that parental acclimation can modify ocean acidification effects. Nevertheless, it should be considered that acclimation is energetically costly, which might have severe impacts on other traits, such as longevity or immunity. To my knowledge this is the first study, which examines transgenerational effects of elevated CO₂ concentrations on temperate marine fish.

4 Chapter I

Seasonal variation in parasite infection patterns of marine fish species from the northern Wadden Sea in relation of interannual temperature fluctuations

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Abstract

Changing environments are likely to shift host-parasite interactions. The development of many parasites and pathogenic bacteria is positively correlated with temperatures, increasing the risk of infection and diseases during summer. These shifts may have profound implications, particularly in changing coastal ecosystems, where long-term developments of parasite fauna are largely unexplored.

Therefore, we investigated the parasite and bacterial Vibrionaceae communities of four common small fish species (three-spined stickleback *Gasterosteus aculeatus*, Atlantic herring *Clupea harengus*, European sprat *Sprattus sprattus* and lesser sand eel *Ammodytes tobianus*) in the northern Wadden Sea over a period of two years.

We found that some classes of macroparasites (trematodes, nematodes) showed a shift in infection peaks that followed the water temperatures of preceding months, while other parasite groups showed no effects of temperature differentials on infection parameters. Moreover, relative diversities of both macroparasites and Vibrionaceae followed the temperature differential between the years.

This may show that even subtle changes in seasonal temperatures can have an effect on the extended epidemiology and phenology of parasites as well as opportunistic pathogens that can potentially have far fetching consequences for sensitive ecosystems.

Introduction

Parasites play an intrinsic but hidden role in marine ecosystems (Mouritsen and Poulin 2002), yet little is known about how parasites are influenced by changing environments. As environmental conditions alter, shifts in host-parasite interactions are likely to occur. These changes could affect parasites in a direct or indirect way by influencing their free-living infective stages or their host range. Hence, environmental changes can result in gain or loss of parasite species as well as in increased or decreased host susceptibility (Lafferty 1997; Lafferty et al. 2004). In particular, temperature will drive future ecological change in marine ecosystems (Harley et al. 2006). The mean water temperature and extreme events like heat waves are predicted to increase in the nearer future (Schär et al. 2004; Murray and Ebi 2012) and the development of many parasites and pathogenic bacteria is temperature-dependent (Harvell et al. 2002). Especially many bacterial disease agents are favoured in warmer waters by speeding up its biochemical reactions, which result in faster development and increased transmission stages per generation (Pounds et al. 2006; Lafferty 2009). This can lead to higher parasite fitness and increased disease outbreaks during summer months (Karvonen et al. 2010). Also for macroparasites, such as trematodes, it has been shown that elevated water temperatures may be beneficial, e.g. by extending the length of transmission periods (Sankurathri and Holmes 1976; Karvonen et al. 2010). On the other hand a subset of pathogenic bacteria and parasites might decline with warming, depending on their thermal tolerance (Karvonen et al. 2010).

However, long-term developments of parasite fauna are largely unexplored in changing coastal ecosystems. A highly dynamic coastal ecosystem of global importance is the European Wadden Sea (Vanbeek et al. 1989). As an essential nursery ground for many fish species, altered host-parasite-interactions may have profound consequences for this sensitive ecosystem.

In the past, most parasitological studies in the Wadden Sea focussed on invertebrates (Lauckner 1971; Thieltges 2006; Thieltges et al. 2013) or on commercially important fish species like flatfishes or gadoids (Hilger et al. 1991; Moller and Anders 1992). Macroparasite communities of small-sized fish were only rarely considered (Groenewold et al. 1996; Zander 2005). Nevertheless, macroparasites often utilize

small fish as intermediate host and these fishes play an important role in the food web of the Wadden Sea by transmitting parasite to predacious fish and seabirds (Kühl and Kuipers 1978).

To deepen our understanding of seasonal infection patterns of macroparasites, we investigated the parasite community of four common small fish species (three-spined stickleback *Gasterosteus aculeatus*, Atlantic herring *Clupea harengus*, European sprat *Sprattus sprattus* and lesser sand eel *Ammodytes tobianus*) in the northern Wadden Sea over a period of two years and tried to relate our findings to water temperature, as a potential driver of infection patterns (figure 1).

Knowledge on potential bacterial pathogens (microparasites) in Wadden Sea fish is even scarcer (Wegner et al. 2012; Thieltges et al. 2013). Therefore, we also included potentially pathogenic bacteria of the family Vibrionaceae into our survey. Vibrionaceae include many facultative symbionts and pathogenic strains (Austin and Austin 2007), which are strongly associated with warmer water temperatures and immune-compromised hosts (Vezzulli et al. 2010; Baker-Austin et al. 2013).

The combination of parasite prevalence, abundance and diversity of macro- and potential microparasitic infections in relation to water temperature, as one of the main drivers of epidemiological patterns in aquatic habitats, will help to clarify, how environmental change might impact host-parasite interactions.

Methods

Sampling and parasite screening

Four common fish species (three-spined stickleback *Gasterosteus aculeatus*, Atlantic herring *Clupea harengus*, European sprat *Sprattus sprattus* and lesser sand eel *Ammodytes tobianus*) from the Sylt-Rømø-Bight, North Sea, Germany were chosen as representatives to investigate the parasite community of small fish in the northern Wadden Sea, Germany. Using a seasonal sampling scheme, twelve fish per species ($n_{\text{total}} = 308$) were caught during eight different cruises with the RV “Mya” between 2011 and 2013 (table 1). After sampling, fish were kept in aquaria with flow through until investigation a few days later.

	Date	T	Sal	pH	Fish sampled				Bacteria cultivated				Bacteria sequenced				
					GA	CH	SS	AT	GA	CH	SS	AT	GA	CH	SS	AT	
2011	08.03.11	0.8	25.6	8.13	12	12	4	1	22	10	6	1	22	8	6	1	
	13.07.11	17.3	29.2	8.25	12	12	10	12	5	22	17	13	4	20	15	13	
	26.10.11	8.6	27.2	8.13	12	12	12	12	21	17	3	5	21	17	3	4	
	16.01.12	4.2	28.0	8.20	12	12	12	6	10	19	20	9	10	19	16	9	
2012	26.03.12	7.3	27.3	8.23	12	12	12	11	21	25	21	12	20	24	20	12	
	16.07.12	17.1	29.9	8.17	0	12	0	12	0	22	0	6	0	22	0	6	
	24.10.12	11.1	27.4	8.03	12	12	12	10	7	12	12	7	6	8	9	5	
	2013	05.02.13	2.0	28.0	8.12	12	12	0	2	15	9	0	0	13	8	0	0

Table 1 Details of fish samples. Numbers of sampled fish, cultivated and sequenced bacteria represent sample sizes for three-spined stickleback *Gasterosteus aculeatus* (=GA), Atlantic herring *Clupea harengus* (=CH), European sprat *Sprattus sprattus* (=SS) and lesser sand eel *Ammodytes tobianus* (=AT), respectively. Terms “2011” and “2012” were used to differentiate between sampling years. T= Temperature in °C, Sal = Salinity in ‰

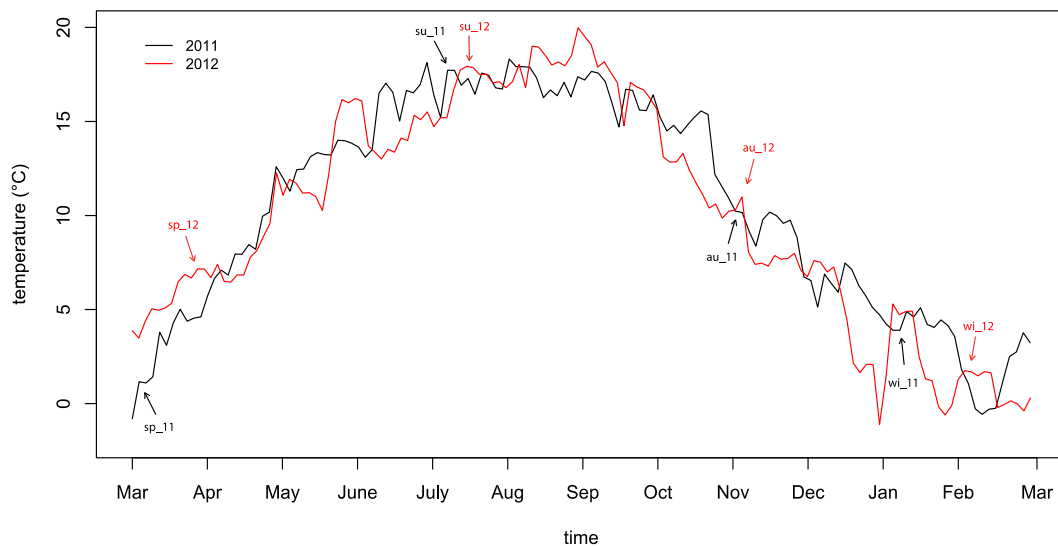


Figure 1 Water temperature in the Sylt-Rømø-Bight, North Sea, in 2011 and 2012. Arrows show sampling days. Data provided from weekly sampling taken by Tatyana Romanova. sp=spring, su=summer, au=autumn, wi=winter

For parasite screening narcotized fish (with MS-222) were measured (total length 0.1 cm) and weighed (0.01 g) and body, fins and gills were screened for ectoparasites. The fish was killed by an overdose of MS-222 and afterwards dissected and screened for endoparasites under a stereomicroscope. A trichinae press was used to detect cysts and parasites in heart, liver, intestines and gills. Macroparasites were photographed under a

stereomicroscope for morphological identification and stored in ethanol for molecular DNA barcoding identification.

Vibrionaceae isolation

To isolate Vibrionaceae species, fish liver, intestine, gills and blood were plated out of selective medium (TCBS agar, Sigma-Aldrich, Steinheim) and incubated at 25°C for 24 hours. A subsample of bacteria colonies was suspended in 2 ml growth medium and incubated at 25°C and with 300 U/min for 24 hours. Vibrionaceae identification was performed by amplifying the 16S rRNA region using PCR universal eubacterial primers 16S-27f (forward: 5'-AGAGTTTGATCMTGGCTCAG-3') and 16S-1392r (reverse: 5'-ACGGGCGGTGTGTGTRC-3'). The 20 µl PCR mixture consisted of 10.75 µl ddH₂O, 4 µl 5x Flexi Buffer, 1.2 µl MgCl₂ (25 mM), 1 µl dNTP (10 mM), 1 µl forward and reverse primers (5 pM/µl), 0.05 µl GoTaq DNA Polymerase (5 U/µl, all PCR chemicals from Promega, Mannheim) and 1 µl template DNA (bacterial liquid culture was heated to 95°C for 5 min, concentrations approximately 10-20 ng/µl). Thermal cycling included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 30 sec at 95°C, 30 sec at 55°C and 90 sec at 72°C and a final extension step at 72°C for 10 min. Sequencing was performed by the institute of clinical molecular biology (IKMB University hospital Kiel).

Parasite barcoding

DNA from macroparasites was extracted using the DNAeasy Blood and Tissue Kit (Qiagen, Hilden) according to the manufacturer's protocol. DNA suspension was kept at -20°C until use. Barcoding was performed by amplifying the 28S rDNA region of nematodes and trematodes using PCR primers D2A (forward: 5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (reverse: 5'-TCGGAAGGAACCAGCTACTA-3') and the 18S rDNA region of cestodes using PCR primers 1800 (forward: 5'-TAATGATCCTTCCGCAGGTT-3') and 18A1 (reverse: 5'-CCTACTCTGGTTGATCCTGCCAGT-3').

The 28S PCR reaction was conducted in a final reaction volume of 20 µl containing 10 µl ddH₂O, 2 µl 10x Buffer Y, 4 µl 5x Enhancer solution P (both PCR chemicals

from Peqlab), 2 μl dNTP (10 mM), 0.2 μl forward and reverse primers (20 pM/ μl), 0.1 μl Taq DNA Polymerase (5 U/ μl , Peqlab) and 1.5 μl template DNA (approximately 5-10 ng/ μl) using a thermocycler (Biometra) under the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of 30 sec at 94°C, 1 min at 55°C and 2 min at 72°C and a final extension at 72°C for 10 min.

The 18S PCR reaction was conducted in a final reaction volume of 40 μl containing 25.5 μl ddH₂O, 4 μl 10x Buffer, 4 μl 5x Enhancer solution P (both PCR chemicals from Peqlab), 4 μl dNTP (10mM), 0.2 μl forward and reverse primers (50 pM/ μl), 0.1 μl DNA Polymerase (5 U/ μl , Peqlab) and 2 μl template DNA (approximately 5-10 ng/ μl) using a thermocycler (Biometra) under the following conditions: initial denaturation at 94°C for 5 min, followed by 36 cycles of 45 sec at 94°C, 50 sec at 50°C and 200 sec at 72°C and a final extension at 72°C for 10 min. Sequencing was performed by the institute of clinical molecular biology (IKMB University hospital Kiel).

The 28S and 18S rDNA sequences were edited and assembled using Sequencher 4.8 software. To identify macroparasite species, we compared edited sequences against published sequences in NCBI GenBank using BLASTn algorithm. We chose 98% sequence similarity to assign species. Parasites with no representative sequence in GenBank were morphologically identified.

Phylogenetic reconstruction and analyses

The 16S rRNA sequences were edited and assembled using Sequencher 4.8 software. To identify close phylogenetic relatives of isolated Vibrionaceae, we compared edited sequences against published sequences in NCBI GenBank using BLASTn algorithm. We included 25 reference strains that were assessed from NCBI GenBank. Edited sequences and reference strains were aligned using ClustalX, version 1.83.1 (Larkin et al. 2007) and adjusted manually in JalView, version 2.8 (Waterhouse et al. 2009). A phylogenetic tree was constructed using the R package ape (Paradis et al. 2004), based on distance matrices using the Kimura'80 substitution model (Kimura 1980), which gave the best model fit. Statistical significance was assessed from 100 bootstrap replicates and tree topology was estimated using the Neighbor-joining algorithm,

taking *Shewanella* sp. as an outgroup. A radial cladogram was drawn using the interactive tree of life web service (<http://itol.embl.de/>), (Letunic and Bork 2011).

Data analyses

Prevalence (percentage of infected individuals in total sample) and abundance (mean number of parasites per infected hosts) of macroparasites from each fish species as well as for fish species separated by sampling season and year were calculated (Bush et al. 1997). Differences in infection parameters between sampling periods were analysed by generalized linear models (GLMs) using prevalence (family = binomial) and abundance (family = negative binomial) as dependent variables and fish species, sampling season and year as independent predictors as well as fish length and weight as a covariate to control the influence of host size. We used individual fish as statistical units and applied multiple post-hoc tests to identify pairwise differences of significant seasonal and annual effects on infection parameters. Family wise error rates were controlled by Bonferroni correction (Miller 1981), adjusting the statistical significance level at $P=0.008$ for seasonal effects (six multiple tests) and at $P=0.0125$ for annual effects (four multiple tests).

Furthermore, we determined diversity indices (Holmes and Price 1986) including total number of macroparasite species, parasitic burden, the Shannon-Wiener index (Hs) and species evenness (E) for each fish species as well as for fish species separated by sampling season and year. To describe the diversity of the Vibrionaceae community, we clustered all sequences into operational taxonomic units (OTU), based on 97% sequence similarity criteria, using uclust and calculated diversity indices analogous to macroparasite communities. OTUs were then transformed into a distance matrix based on unifracs distances (Lozupone and Knight 2005), incorporating phylogenetic and abundance information of Vibrionaceae OTUs. Community structure was analysed by permutational multivariate analysis of variances (PERMANOVA) using the Adonis function of the vegan package (Oksanen et al. 2013), partitioning variance between host species, sampling season and year.

To relate year wise differences of the diversity of Vibrionaceae and macroparasite communities to different temperature regimes, we calculated temperature and diversity differentials among the years (2012-2011) and analysed them by using

GLMs (family = Gaussian) with relative Hs as a measure of diversity and with relative water temperature, fish species and parasite group as independent predictors. Additionally, we calculated and analysed prevalence and abundance differentials of macroparasite groups and investigated year wise differences (2012–2011) of infection parameters to different temperature regimes analogous to diversity differentials.

All GLM model effects were tested by analysis of deviance and we assumed deviance change to be approximately χ^2 distributed. All statistical analyses were carried out using R statistical package (R Core Team 2013).

Results

Macroparasite community

In total, we detected 16 different macroparasite species from six phyla covering nematodes, acanthocephalans, monogeneans, digeneans, cestodes and parasitic copepods (table 2). Some parasite species only occurred in a single host species, e.g. *Lernaeenicus sprattae* in European sprat, *Acanthocephalus lucii* in Atlantic herring, while others were present in several hosts. The digenean *Brachyphallus crenatus*, the nematode *Hysterothylacium auctum* and the parasitic copepode *Caligus elongatus* were the dominant parasites, reaching highest prevalence in sand eel (65.2%), sprat (43.5%) and stickleback (28.6%) respectively.

The highest diversity was found in the parasite community of the three-spined stickleback consisting of 12 different macroparasite species, with all four cestode species occurring exclusively in this host (table 2, table 3). The diversity of the parasite communities from Atlantic herring, European sprat and lesser sand eel ranged from five to seven different parasite species and showed diversity indices in the range from 1.46 to 1.67 (table 3).

	<i>G. acul.</i>	<i>C. har.</i>	<i>S. spr.</i>	<i>A. tob.</i>	Similarity
Number of fish examined	84	96	62	66	
Mean total length (cm)	5.63 ± 1.1	9.69 ± 1.5	9.14 ± 1.4	11.88 ± 1.2	
Mean total weight (g)	1.76 ± 0.9	5.74 ± 2.3	5.06 ± 2.6	5.11 ± 2.2	
NEMATODA					
<i>Contraeaecum osculatum</i> (I, BC)	1.2 1 ± 0			1.5 1 ± 0	99%
<i>Contraeaecum rudolphi</i> (I, BC)	3.6 1.7 ± 1.2				99-100%
<i>Contraeaecum septentrionale</i> (I, BC)			1.6 2 ± 0		99%
<i>Hysterothylacium auctum</i> (I, BC)	13.1 2.4 ± 1.9	8.3 3.9 ± 6.6	43.5 5.1 ± 5.3	3.0 1 ± 0	99-100%
ACANTHOCEPHALA					
<i>Acanthocephalus lucii</i> (I)		3.1 1 ± 0			Morph.
MONOGENEA					
<i>Gyrodactylus sp.</i> (F)	11.9 1.5 ± 0.9				Morph.
<i>Mazocraes alosae</i> (G)	6.0 1.4 ± 0.9	14.6 1.4 ± 0.6	16.1 1.8 ± 1.9	4.5 1 ± 0	90-95% + Morph.
DIGENEA					
<i>Labratrema minimus</i> (I)			1.6 1 ± 0	1.5 1 ± 0	Morph.
<i>Brachyphallus crenatus</i> (St)	11.9 4.2 ± 4.4	22.9 2.7 ± 3.9	25.8 6.4 ± 10.3	65.2 5.4 ± 5.4	89-90%* + Morph.
CESTODA					
<i>Scolex pleuronectis</i> (I)	2.4 1 ± 0				98%
<i>Bothriocephalus scorpii</i> (I)	2.4 5 ± 1.4				98-99%
<i>Diphyllobothrium dentriticum</i> (I)	3.6 1.7 ± 1.2				99%
<i>Triaenophorus lucii</i> (I)	1.2 1 ± 0				Morph.
PARASITIC COPEPODA					
<i>Caligus elongatus</i> (F, S)	28.6 1.3 ± 1	5.2 1 ± 0	1.6 1 ± 0	1.5 1 ± 0	Morph.
<i>Lernaeenicus sprattae</i> (E)			19.4 1 ± 0		Morph.
<i>Thersitina gasterostei</i> (G)	3.6 1 ± 0				Morph.

Table 2 Summary of macroparasite communities. Mean prevalence (upper value) and abundance (lower value) ± standard deviation of macroparasites of three-spined stickleback (= *G. acul.*), Atlantic herring (= *C. har.*), European sprat (= *S. spr.*) and lesser sand eel (= *A. tob.*) and sequence similarity using NCBI BLASTn algorithm. Morph=Morphological identification. *Molecular identification: *Lecithocladium excisum* with 89-90% sequence similarity in GenBank, morphological identification: *Brachyphallus crenatus* (no representative sequence in GenBank). Letters in brackets give information about preferred target organ (microhabitat). B = Body Cavity, E = Eyes, F = Fins, G = Gills, I =Intestines/Intestinal wall, S= Skin, St = Stomac

	<i>G. acul.</i>	<i>C. har.</i>	<i>S. spr.</i>	<i>A. tob.</i>
Number of macroparasite species	12	5	7	6
Parasitic burden	23.2	10	18.3	10.4
Hs	2.31	1.46	1.67	1.47
E	0.93	0.90	0.86	0.90

Table 3 Diversity indices. Diversity of the macroparasite communities of three-spined stickleback (= *G. acul.*), Atlantic herring (= *C. har.*), European sprat (= *S. spr.*) and lesser sand eel (= *A. tob.*). Hs = Shannon-Wiener index, E=evenness

Beside significant effects of host species on parasite prevalence (figure 2, table 4) and abundance (figure 3, table 5), we found strong influences of sampling season on trematode and nematode infection parameters.

Trematodes followed the seasonal temperature course and occurred significantly more often in spring (55.3%) and summer (54.2%) than in autumn (29.8%) and winter (30.4%) (sp vs. au $\chi^2_1 = 2.73$, $P < 0.001$, sp vs. wi $\chi^2_1 = 2.23$, $P = 0.002$, su vs. au $\chi^2_1 = 2.42$, $P = 0.001$, su vs. wi $\chi^2_1 = 1.98$, $P = 0.004$). Highest abundance was detected in winter (4.8 ± 1.8), but significantly different abundances were only found between spring (3.8 ± 0.8) and autumn (2.5 ± 0.3) (sp vs. au $\chi^2_1 = 13.38$, $P < 0.001$) and between summer (4.6 ± 0.9) and autumn (su vs. au $\chi^2_1 = 14.82$, $P < 0.001$). Additionally, we found significant differences between sampling years on infection parameters of trematodes with increased prevalences in spring and autumn 2012 (figure 2, sp11 vs. sp12 $\chi^2_1 = 5.61$, $P < 0.001$, au11 vs. au12 $\chi^2_1 = 2.27$, $P < 0.001$) and increased abundances in spring 2012 (figure 3, sp11 vs. sp12 $\chi^2_1 = 30.38$, $P < 0.001$) compared to 2011.

In contrast to trematodes, nematodes were found significantly more often in winter (31.9%) compared to summer (12.5%) and autumn (6.4%) (wi vs. su $\chi^2_1 = 1.32$, $P = 0.005$, wi vs. au $\chi^2_1 = 2.58$, $P < 0.001$), whereas abundance was significantly higher in spring (5.4 ± 1.4) and winter (3.8 ± 1.0) compared to autumn (2.0 ± 0.7) (sp vs. au $\chi^2_1 = 12.20$, $P < 0.001$, wi vs. au $\chi^2_1 = 20.81$, $P < 0.001$). Nematode prevalence was also significantly affected by fish weight. Lighter fish, after controlling for species affiliation, were significantly more often infected with nematodes (table 4).

Cestodes and parasitic copepods were comparatively rare (table 2) and we could not detect any seasonal or annual pattern, probably owing to low prevalences.

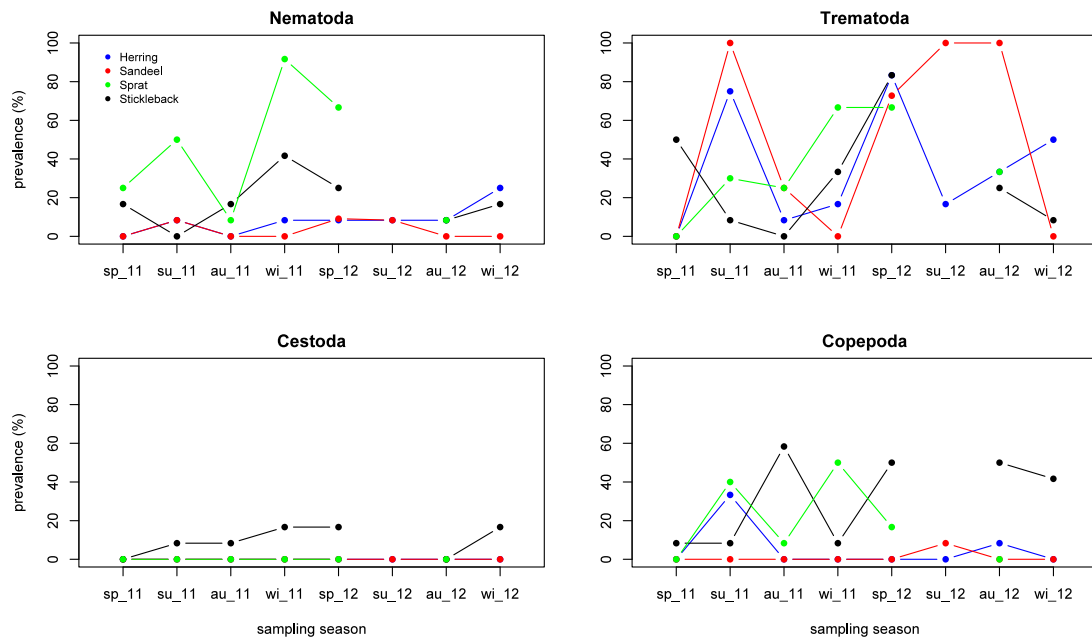


Figure 2 Prevalence of macroparasite phyla separated by fish species and sampling period. Gaps represent seasons with missing fish species.

Response	Nematoda prev					Trematoda prev				
	Df	Dev	ResDf	ResDev	P(>Chi)	Df	Dev	ResDf	ResDev	P(>Chi)
Null			307	282.9				307	419.5	
Fish species (F)	3	39.6	304	243.2	<0.001	3	25.5	304	394.0	<0.001
Year (Y)	1	0.03	303	243.2	0.861	1	15.9	303	378.1	<0.001
Season (S)	3	26.5	300	216.7	<0.001	3	20.1	300	358.0	<0.001
Fish length	1	1.8	299	214.9	0.180	1	3.1	299	354.9	0.079
Fish weight	1	7.5	298	207.4	0.006	1	1.6	298	353.4	0.211
F*Y	3	2.7	295	204.7	0.443	3	2.1	295	351.3	0.555
F*S	9	14.6	286	190.1	0.102	9	55.8	286	295.5	<0.001
Response	Cestoda prev					Copepoda prev				
	Df	Dev	ResDf	ResDev	P(>Chi)	Df	Dev	ResDf	ResDev	P(>Chi)
Null			307	74.2				307	259.7	
Fish species (F)	3	21.4	304	52.8	<0.001	3	40.9	304	218.8	<0.001
Year (Y)	1	0.2	303	52.7	0.669	1	0.2	303	218.6	0.637
Season (S)	3	2.2	300	50.4	0.527	3	3.2	300	215.4	0.363
Fish length	1	0.3	299	50.1	0.568	1	3.0	299	212.4	0.082
Fish weight	1	0.4	298	49.7	0.509	1	1.2	298	211.2	0.272
F*Y	3	0	295	49.7	1.000	3	11.9	295	199.3	0.008
F*S	9	0	286	49.7	1.000	9	23.6	286	175.8	0.005

Table 4 Summary of seasonal and annual effects on macroparasite prevalence. Generalized linear models containing prevalence (prev) of macroparasite phyla as dependent variables and fish species, year, season, fish length and fish weight as independent predictors. Significant effects are shown in bold. Family = binomial

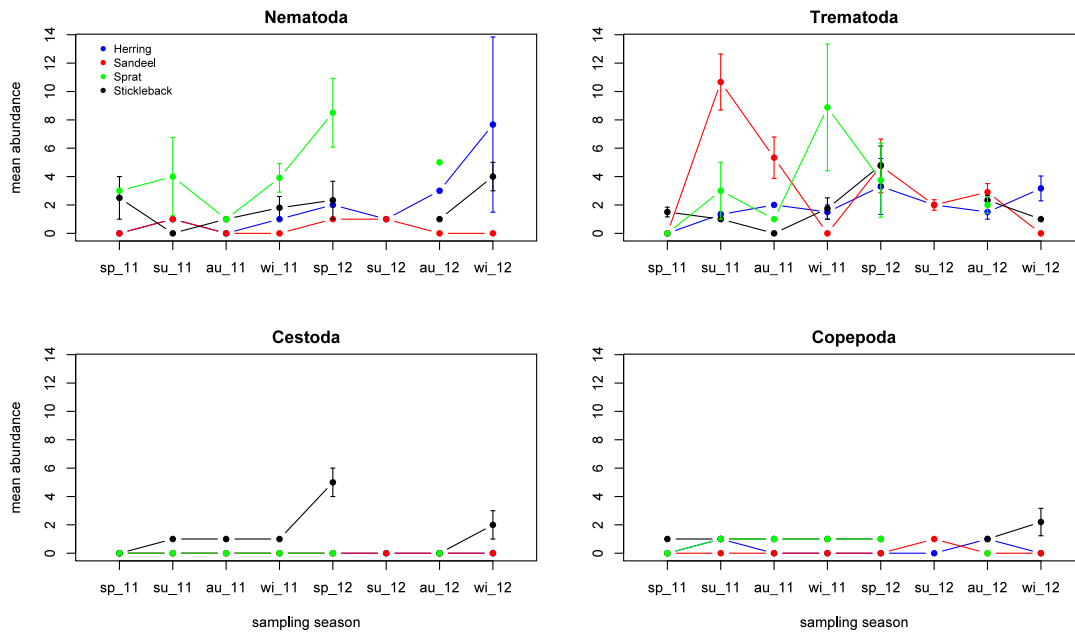


Figure 3 Abundance of macroparasite phyla separated by fish species and sampling period.

Gaps represent seasons with missing fish species.

Response	Nematoda abund					Trematoda abund				
	Df	Dev	ResDf	ResDev	P(>Chi)	Df	Dev	ResDf	ResDev	P(>Chi)
Null			307	278.8				307	424.8	
Fish species (F)	3	78.5	304	200.3	<0.001	3	48.2	304	376.6	<0.001
Year (Y)	1	1.9	303	190.9	0.168	1	7.2	303	369.4	0.007
Season (S)	3	33.0	300	157.8	<0.001	3	37.2	300	332.3	<0.001
Fish length	1	0.7	299	157.1	0.400	1	0.03	299	332.2	0.857
Fish weight	1	0.6	298	156.5	0.450	1	2.6	298	329.6	0.107
F*Y	3	5.4	295	151.1	0.142	3	23.6	295	306.0	<0.001
F*S	9	17.9	286	133.2	0.037	9	52.9	286	253.1	<0.001
Response	Cestoda abund					Copepoda abund				
	Df	Dev	ResDf	ResDev	P(>Chi)	Df	Dev	ResDf	ResDev	P(>Chi)
Null			307	63.3				307	200.9	
Fish species (F)	3	33.2	304	30.1	0.002	3	43.9	304	157.0	<0.001
Year (Y)	1	3.5	303	26.6	0.061	1	1.6	303	155.4	0.204
Season (S)	3	1.9	300	24.7	0.583	3	5.7	300	149.7	0.126
Fish length	1	0.05	299	24.6	0.831	1	1.0	299	148.7	0.307
Fish weight	1	0	298	24.6	0.987	1	0.3	298	148.3	0.558
F*Y	3	0	295	24.6	1.000	3	11.6	295	136.7	0.009
F*S	9	0	286	24.6	1.000	9	17.9	286	118.8	0.036

Table 5 Summary of seasonal and annual effects on macroparasite abundance. Generalized linear models containing abundance (abund) of macroparasite taxa as dependent variables and fish species, year, season, fish length and fish weight as independent predictors. Significant effects are shown in bold. Family = negative binomial

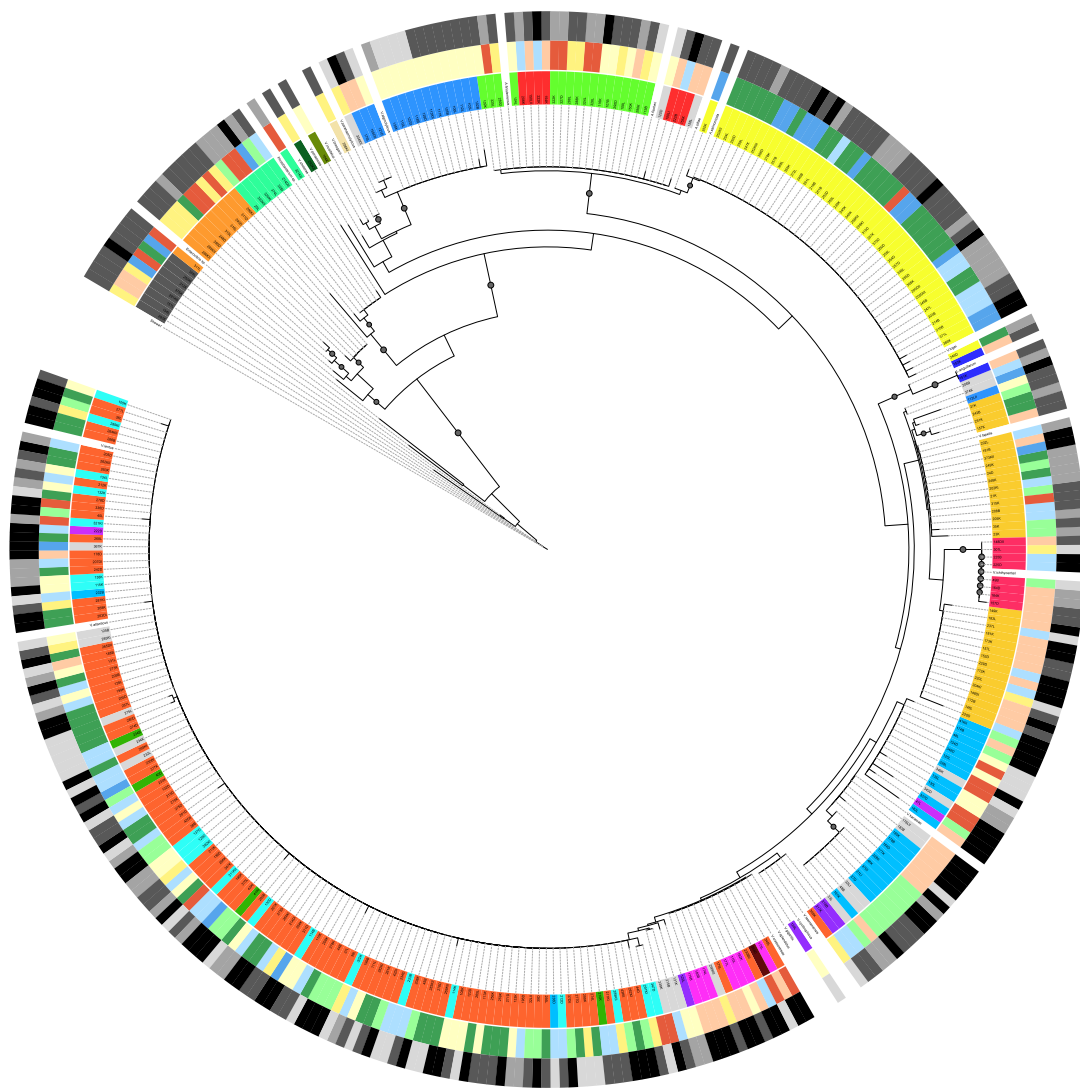
Vibrionaceae community

We found 20 OTUs affiliated to 20 different species from the family Vibrionaceae in the organs of all four marine fish species (figure 4) consisting of 14 species of the genus *Vibrio* (V.), three species of *Aliivibrio* (A.), one species each of *Listonella* (L.), *Enterovibrio*, and *Photobacterium*. Most strains were assigned to *V. splendidus* (28.6%), followed by *A. logei* (13.3%) and *V. tapetis* with 9.6%, which were found in all four or at least three of the investigated fish species. The bacterial species diversity differed significantly between fish species (table 6). The highest diversity was found in the Atlantic herring, consisting of 18 out of 20 different Vibrionaceae species and showing a diversity index (Hs) of 1.54, whereas the lowest diversity could be found in lesser sand eel with only 10 species and an Hs value of 1.23. The Vibrionaceae communities of both fish species were comprised of rare species, which were only once detected. These were *V. breoganii*, *V. inusitatus* and *V. penaeicida* in Atlantic herring, and *V. crassostreae* in the lesser sand eel.

In addition to differences between host species, we also found seasonal variation in bacterial species diversity with the highest diversity of Vibrionaceae species in summer (Hs = 1.53), followed by autumn (Hs = 1.49), spring (Hs = 1.38) and winter (Hs = 1.16). The rare Vibrionaceae species from Atlantic herring were only detected in summer, whereas *V. crassostreae* was only found in autumn. Furthermore, the Vibrionaceae communities differed significantly between sampling years (table 6).

Response	Variance of Vibrionaceae communities					
	Df	sumSq	meanSq	F	R2	Pr(>F)
Fish species (F)	3	0.007	0.002	4.39	0.28	0.011
Year (Y)	1	0.003	0.003	5.24	0.11	0.009
Season (S)	3	0.002	0.001	1.28	0.08	0.293
F*Y	3	0.003	0.001	2.33	0.15	0.057
F*S	9	0.005	0.001	1.14	0.22	0.382
Residuals	8	0.004	0.000		0.17	
Total	27	0.024			1.00	

Table 6 Summary of seasonal and annual effects on the variance of Vibrionaceae communities. Permutational multivariate analysis of variances of distance matrices for Vibrionaceae (unifrac distance), including independent predictors fish species, sampling year and season. Significant effects are shown in bold.



Color ranges:

Grey	Shewanella sp.
Orange	Enterovibrio sp.
Green	V. inusitatus
White	Reference strain
Yellow	V. breoganii
Blue	V. alginolyticus
Light grey	V. sp.
Dark green	V. penaeicida
Purple	V. cyclitrophicus
Red	V. splendidus
Pink	V. gigantis
Brown	V. crassostreae
Cyan	V. atlanticus
Light green	V. tasmaniensis
Light purple	V. lentus
Light blue	V. kanaloae
Yellow	V. tapetis
Pink	V. ichthyenteri
Blue	L. anguillarum
Red	A. fischeri
Light green	A. finisterrensis
Yellow	A. logei
Light green	Photobacterium sp.

Legend:

Dataset season

Light blue	winter_11
Light green	spring_11
Yellow	summer_12
Light yellow	summer_11
Orange	autumn_12
Light orange	autumn_11
Green	spring_12
Blue	winter_12

Dataset fish species

Light grey	sprat
Dark grey	herring
Black	stickleback
White	sand eel

Figure 4 Summary of Vibrionaceae communities. Phylogenetic relationship based on the Neighbor-joining method (Kimura'80 model) using partial 16s rRNA sequences (n=332) of Vibrionaceae isolates from four marine fish species. Shewanella was used as an outgroup. Inner colour circle represents different Vibrionaceae strains, whereas middle circle and outer circle show different sampling periods and different fish species. White slots represent reference strains assessed from NCBI GenBank.

Comparison of communities

We found strong year-wise differences of water temperature and species diversity with significantly increased relative diversities of infectious species at higher temperature differentials (figure 5, table 7). The relative diversity differed also significantly between fish species (table 7). The highest temperature and diversity difference could be seen in spring with an increased water temperature of 6.5°C corresponding to an increased macroparasite diversity of 1.29 in Atlantic herring in spring 2012 (figure 5).

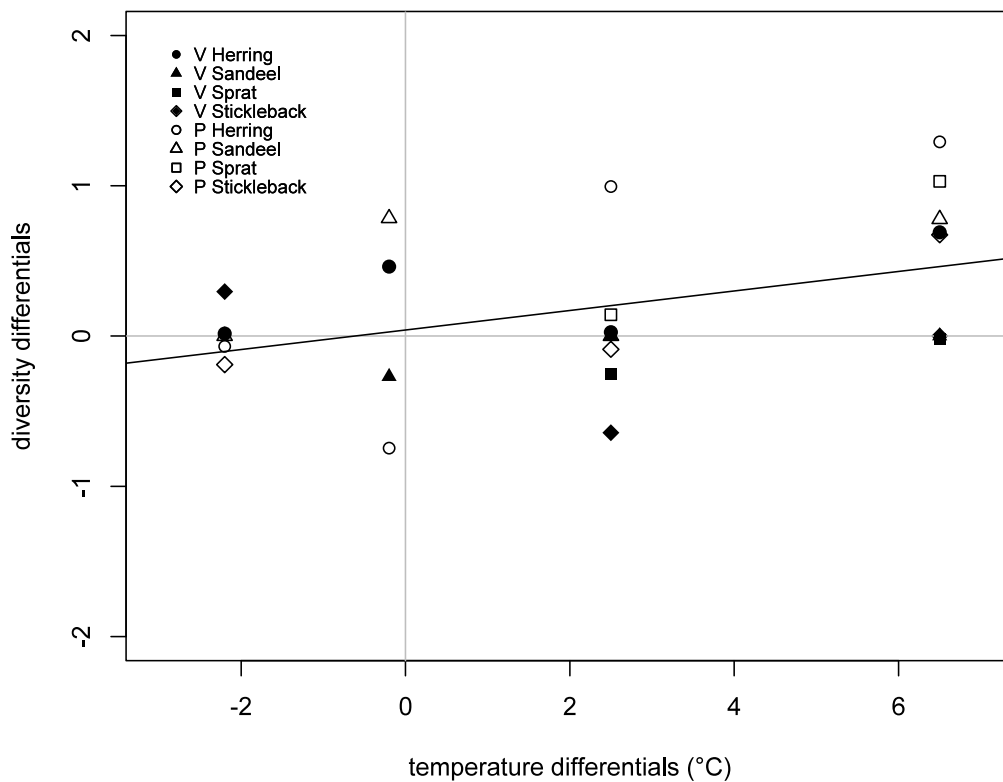


Figure 5 Diversity and temperature differentials of Vibrionaceae and macroparasites separated by fish species. V = Vibrionaceae, P=macroparasites

Response	Relative diversity				
	Df	Dev	ResDf	ResDev	P(>Chi)
Null			31	12.26	
Relative temperature (T)	1	3.52	30	8.74	<0.001
Fish species (F)	3	2.32	27	6.42	0.003
Parasite group (P)	1	0.30	26	6.12	0.175
T*F	3	1.07	23	5.05	0.087
T*P	1	1.47	22	3.59	0.003

Table 7 Summary of relative temperature effects on relative diversities. Generalized linear models containing relative diversity as dependent variable and relative temperature, fish species and parasite group (macroparasite and Vibrionaceae pooled) as independent predictors. Significant effects are shown in bold. Family = Gaussian

Next to increased relative diversities, we found significantly increased relative prevalences of some parasite groups at higher temperature differentials (figure 6, table 8). The relative prevalence of nematodes ($\chi^2_1 = 460.2$, $P = 0.005$) and trematodes ($\chi^2_1 = 10052.0$, $P = 0.026$) followed the temperature differential between the years with highest prevalence differences in spring (figure 6).

Even if we could not find overall relative temperature effects on parasite abundance differentials (table 8), post-hoc tests detected also increased relative abundances of trematodes ($\chi^2_1 = 21.3$, $P = 0.039$) at relative higher temperatures (figure 7).

Response	Relative prevalence					Relative abundance				
	Df	Dev	ResDf	ResDev	P(>Chi)	Df	Dev	ResDf	ResDev	P(>Chi)
Null			33	32249				33	259.8	
Relative temp (T)	1	7273	32	24975	0.002	1	10.0	32	249.8	0.204
Fish species (F)	3	1870	29	23105	0.474	3	33.4	29	216.4	0.144
Parasite group (P)	3	2936	26	20168	0.268	3	32.6	26	184.8	0.164
T*F	3	1804	23	18364	0.490	3	32.4	23	152.5	0.155
T*P	3	3453	20	14910	0.201	3	29.0	20	123.5	0.195

Table 8 Summary of relative temperature effects on relative infection parameters. Generalized linear models containing relative prevalence and abundance as dependent variables and relative temperature, fish species and parasite groups (nematoda, tematoda, cestoda, copepoda pooled) as independent predictors. Significant effects are shown in bold. Family = Gaussian

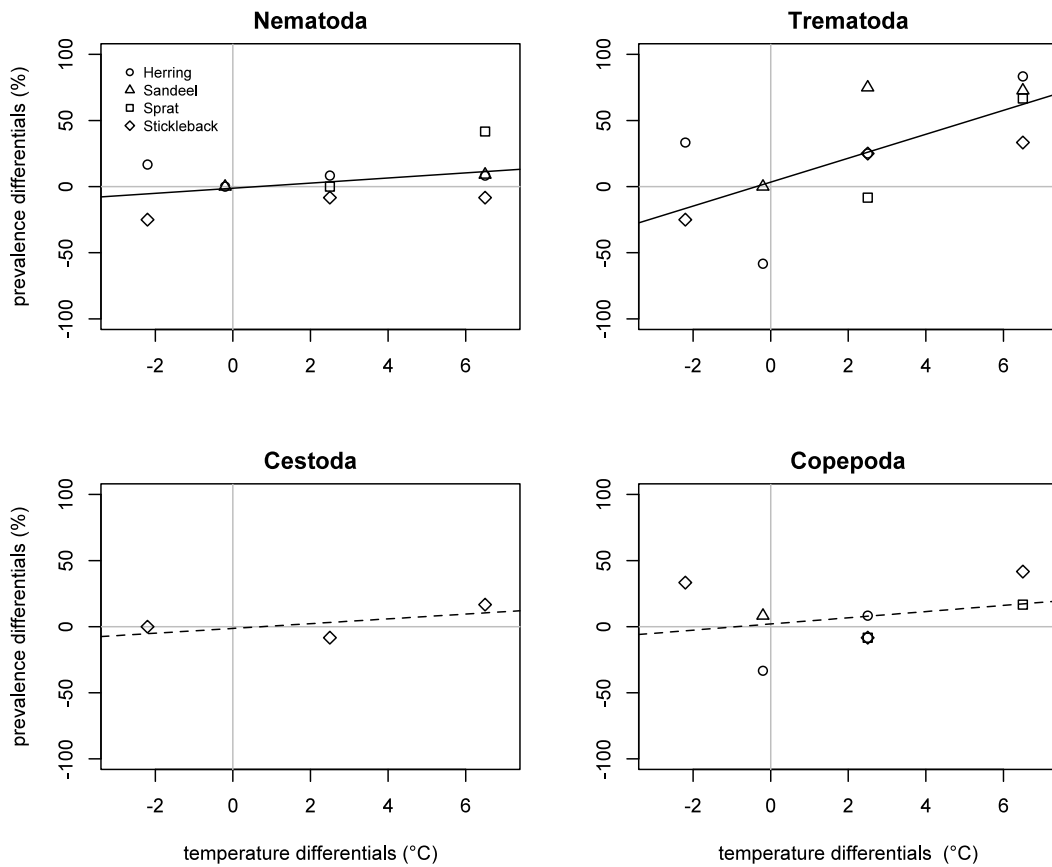


Figure 6 Prevalence and temperature differentials of macroparasites separated by fish species and parasite groups. Solid lines represent significant relative temperature effect on relative parasite prevalence, whereas dashed lines represent no significant relative temperature effect on relative parasite prevalence. Statistical significance level at $P = 0.05$.

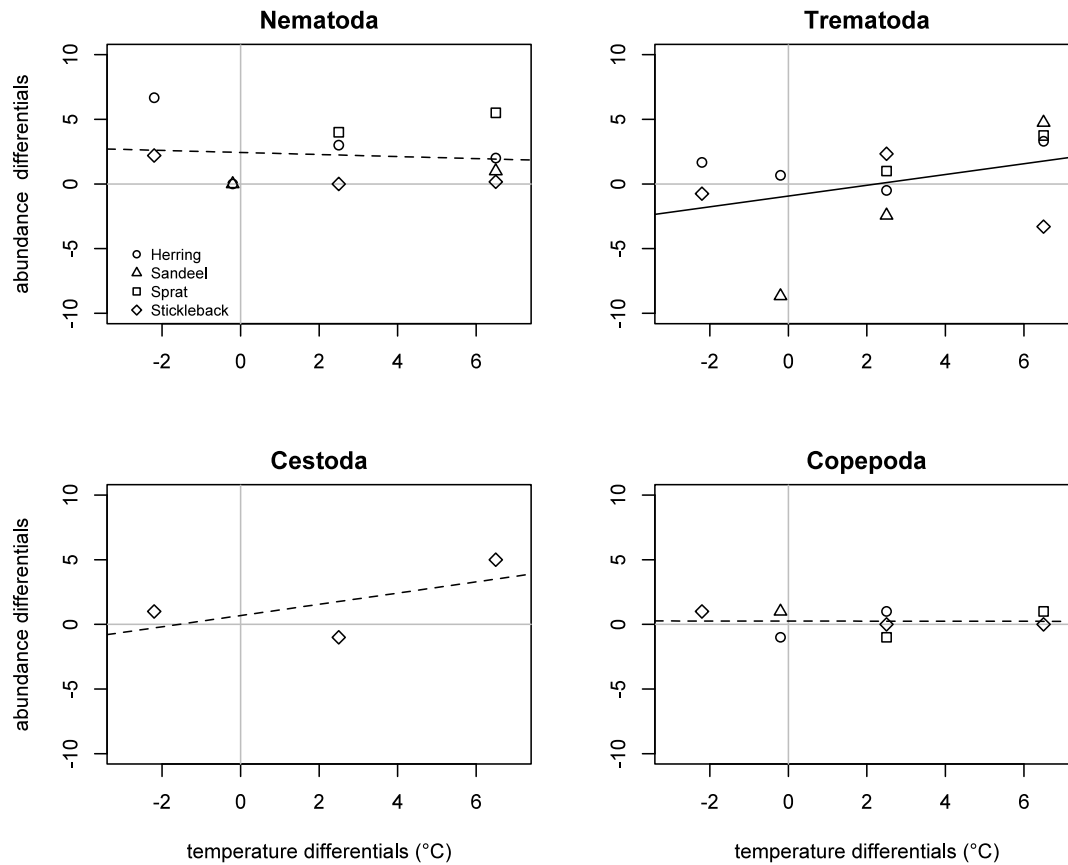


Figure 7 Abundance and temperature differentials of macroparasites separated by fish species and parasite groups. Solid lines represent significant relative temperature effect on relative parasite prevalence, whereas dashed lines represent no significant relative temperature effect on relative parasite prevalence. Statistical significance level at $P = 0.05$.

Discussion

Environmental conditions in the Wadden Sea are changing, mainly driven by rising water temperatures (Oost et al. 2005), which are likely to alter host-parasite interactions.

In this study, we detected inter-annual variation in micro- and macroparasite communities that were correlating to short periods of relative increase in water temperature between years (figure 1). Next to increased diversities of macroparasites as well as Vibrionaceae, we also observed a forward shift and extension in peak prevalence and abundance of trematodes following warmer spring seasons. For nematodes, highest prevalence differences could be also found in spring, while other parasite groups showed no such correlation between temperatures and infection parameters.

In general, parasitism seems to be common in small fish of the Wadden Sea (Groenewold et al. 1996; Zander 2005), reflecting also the outcomes of our study. We detected 16 different macroparasite species from six different phyla. The highest diversity of macroparasites could be found in the three-spined stickleback, which consists of mixes of marine and anadromous populations. Owing to its greater mobility in fresh- and seawater habitats, the parasite richness and the infection risk is higher for anadromous species than for pure marine fish species.

The diversity of the parasite communities from Atlantic herring, European sprat and lesser sand eel ranged from five to seven different parasite species and showed diversity indices in the range from 1.46 to 1.67, which is considerably higher than reported H_s values from the German Wadden Sea of 0.95 for European sprat and 0.20 for lesser sand eel (Groenewold et al. 1996). In the past, many parasitological studies were exclusively based on morphological identification, but particularly nematodes or larval stages of parasites are difficult to identify at the species-level (Bhadury and Austen 2010; Locke et al. 2010). Molecular techniques, such as DNA barcoding, can be powerful tools to distinguish between morphologically similar parasites at any stage in their live cycle (Moszczyńska et al. 2009). Next to erroneous identification in early parasitological studies, higher diversities may be attributable to changed environmental conditions in the Wadden Sea area (Reise and van Beusekom 2008).

Nevertheless, we revealed significant temperature effects on trematode and nematode infection parameters. Trematodes showed significantly increased abundances and

prevalences in spring and summer, coinciding with previous results (Mouritsen 2002; Poulin 2006; Thieltges and Rick 2006). More interestingly, we detected increased prevalences in spring and autumn 2012 as well as increased abundances in spring 2012 in comparison with sampling in 2011. With a temperature increase of 6.5°C in spring and 2.5°C in autumn, both seasons were much warmer in 2012 (figure 1). Particularly, warmer spring seasons may not only increase infectivity of trematodes, but also shift and extend the length of transmission periods (Sankurathri and Holmes 1976; Mouritsen 2002; Thieltges and Rick 2006). On the other hand nematodes showed significantly decreased infection parameters in warmer seasons (summer, autumn), reflecting an altered phenology of the parasite life cycle. Dependent on their thermal tolerance some life cycles stages might be more susceptible to temperature changes than others (Harley et al. 2006; Lafferty 2009; Karvonen et al. 2010).

Additionally, we revealed a significant interaction of reduced fish weight and increased parasite prevalence. We found, that lighter fish were significantly more often infected with nematodes than heavy ones, reflecting previous results of a common carp fingerling (Boerlage et al. 2012). Either infection weakened immune system and disturbed the development or already immune-compromised hosts with lighter weight got more often infected.

The Vibrionaceae communities of the four fish species were also highly diverse with 20 different species. The highest diversity of Vibrionaceae was found in Atlantic herring, suggesting that filter feeding is an effective way to accumulate Vibrionaceae strains in the body.

In total, the Vibrionaceae communities were mostly dominated by the species *Vibrio splendidus*. Some strains of *V. splendidus* can cause vibriosis in fish species, resulting in acute mortalities, particularly in summer months (Gatesoupe et al. 1999; Diggle et al. 2000; Jensen et al. 2003). The occurrence of potentially pathogenic strains from the family Vibrionaceae is highly correlated with temperature, leading to increased virulence and the occurrence of emerging agents in warmer waters (Thompson et al. 2004; Vezzulli et al. 2010).

Bacterial studies in the Wadden Sea have mainly concentrated on oysters and mussels (Lhafi and Kuhne 2007; Schets et al. 2010; Thieltges et al. 2013), and to our knowledge only one study has yet been conducted on the microparasite community of

flatfish (Wegner et al. 2012). Beside *Vibrio splendidus*, we detected other strains of potential pathogens of fish (*Listonella anguillarum*), crustaceans (*V. penaeicida*) and bivalves (*Vibrio tapetis*), which were associated with mass mortality events in some cases (Allam et al. 2002; Aguirre-Guzman et al. 2003; Planas et al. 2005; Pernet et al. 2012). Increases in temperature are likely to intensify these events (Cerrano and Bavestrello 2009).

However, relative diversities of both macroparasites and Vibrionaceae followed the relative temperature differential between the years, showing highest diversity differentials at larger temperature differences. Along with the shifts in phenology of parasite life cycles and disease occurrence, we conclude that temperature may be an important driver of parasite population dynamics, although they are buffered from direct environment by the host physiology (Paperna and Overstreet 1981). Even small changes in seasonal temperatures might lead to extended epidemiology of parasites as well as opportunistic pathogens. And even if only a subset of pathogenic bacteria and parasites react to environmental temperatures, shifts in life cycle and infection parameters can potentially have severe consequences for sensitive ecosystems.

5 Chapter II

Impact of thermal stress on evolutionary trajectories of pathogen resistance in three-spined stickleback (*Gasterosteus aculeatus*)

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Abstract

Pathogens are a major regulatory force for host populations, especially under stressful conditions. Elevated temperatures may enhance the development of pathogens, increase the number of transmission stages, and can negatively influence host susceptibility depending on host thermal tolerance. As a net result, this can lead to higher prevalence of epidemics during summer months. These conditions also apply to marine ecosystems where the possible ecological impacts, population-specific potential for evolutionary responses to changing environments and increasing disease prevalence are, however, less known. Therefore, we investigated the influence of thermal stress on the evolutionary trajectories of disease resistance in three marine populations of three-spined sticklebacks *Gasterosteus aculeatus* by combining the effects of elevated temperature and infection with a bacterial pathogen affiliated to the *Vibrio tubiashii* group using a common garden experiment.

We found strong impacts of thermal stress on life history traits and on survival after infection after only short periods of thermal acclimation. Environmental stress reduced genetic differentiation (Q_{ST}) between populations by releasing cryptic within-population variation. While life history traits displayed positive genetic correlations across environments and genotype by environment interactions (GxE), environmental stress led to negative genetic correlations across environments in pathogen resistance. This reversal of genetic effects, governing resistance, is probably attributable to changing environment-dependent virulence mechanisms of the pathogen interacting

differently with host genotypes, i.e. $G_{\text{Pathogen} \times G_{\text{Host}}} \times E$ or $(G_{\text{Pathogen} \times E}) \times (G_{\text{Host} \times E})$ interactions, rather than to pure host genetic effects, i.e. $G_{\text{Host} \times E}$ interactions.

To cope with climatic changes and the associated increase in pathogen virulence, host species require wide thermal tolerances and pathogen-resistant genotypes. The higher resistance we found for some families at elevated temperatures showed that there is evolutionary potential in both thermal environments. The negative genetic correlation in pathogen resistance between thermal environments, on the other hand, indicates that adaptation to current conditions can be a weak predictor for performance in changing environments. The observed feedback on the selective gradients exerted on life history traits may exacerbate this effect as it can also modify the response to selection for other vital components of fitness.

Introduction

Pathogens are strong selective forces that can influence biodiversity by regulating host population dynamics (Hudson et al. 2002; Altizer et al. 2003). Environmental conditions can amplify these effects (Cattadori et al. 2005), and many infectious diseases are temperature-dependent (Harvell et al. 2002). An increase in ambient temperature will affect the pathogen by, for example, enhancing its metabolism resulting in faster development and a higher number of transmission stages per generation. Especially for many bacterial disease agents, population growth rates increase exponentially with increasing temperature (Shiah and Ducklow 1994; Karvonen et al. 2010). Since population growth rate closely resembles fitness for prokaryotes, improved pathogen fitness can also lead to a higher prevalence of diseases (Pounds et al. 2006).

Rising temperatures are also likely to stress the host organism, thereby increasing host susceptibility to diseases (Kim et al. 2000; Lafferty 2009). This also applies to marine organisms (Harvell et al. 1999), which are often highly vulnerable to climatic changes (Arnell 1999). Thermal stress causes an alteration of the immune response, which generally results in a reduction of pathogen resistance (Alborali 2006). Despite these multifaceted effects, the specific interactions of increased thermal stress due to

climatic warming with infectious diseases are sparsely explored, particularly in marine ecosystems.

The ecological and evolutionary consequences of thermal stress-disease interactions will strongly depend on the thermal tolerance and rate of adaptation of populations to climate warming (Harley et al. 2006). A high potential for acclimation within and between generations and the capacity for adaptation by selection of pathogen-resistant genotypes will ensure population viability and biodiversity (Visser 2008; Eliason et al. 2011). Species with rapid turnover of generations are likely to adapt fast enough to cope with the changing environment (Perry et al. 2005). For vertebrates, the potential for adaptation by microevolution is considered to be alarmingly low (Gienapp et al. 2008). Standing genetic variation supplies the raw material for adaptation, and different genotypes will show different reaction norms leading to genotype by environment (G x E) interactions. GxE interactions as well as phenotypic plasticity can potentially compensate for declines in mean fitness of populations with low evolutionary potential. Furthermore, it also has been shown that changing environmental conditions can release otherwise cryptic genetic variation (McGuigan et al. 2011), which can consequently alter the evolutionary potential of a population.

Here, we tested whether environmental change can also alter the genetic components of resistance to pathogen challenges, and whether such changes can feed back on selection gradients of other components of fitness. To do so, we set up a common garden breeding experiment using three populations of marine three-spined sticklebacks *Gasterosteus aculeatus* and challenged the offspring with an infection by a bacterial strain affiliated to the *Vibrio tubiashii* group in two different temperature environments (ambient and stressful).

In general, bacteria of the genus *Vibrio* include many facultative symbionts and pathogenic strains (Austin and Austin 2007). They are strongly temperature-dependent with elevated population sizes during summer (Thompson et al. 2004; Vezzulli et al. 2010), and moreover, they are able to adapt rapidly to environmental changes due to high genome plasticity including frequent mutation, recombination and lateral gene transfers, (Oberbeckmann et al. 2012) making them ideal candidates for our purpose to study host-parasite interactions in changing environments. *Vibrio tubiashii*, in particular, has been described as a pathogen of bivalves (Hada et al.

1984; Elston et al. 2008), but is also known to harm several marine fish species (Egidius 1987) (Austin et al. 2005), where Vibriosis can lead to superficial skin lesions, muscle necrosis and haemorrhages which are likely to cause mortality (Woo and Bruno 2011).

The three-spined stickleback represents an ideal model system to study the evolutionary ecology of host-parasite interactions (Barber et al. 2001; Kurtz et al. 2004; Wegner et al. 2008) especially in connection with environmental change. These fish occur in shallow freshwater and marine habitats with a temperature range from 4 to 20°C (Jordan and Garside 1972), but show preferences for intermediate temperatures (15-18°C (Macnab and Barber 2012)). Our experimental temperature environments were set to simulate the average summer temperature of coastal North Sea areas (i.e. 17°C) and elevated temperatures with reference to recent climate change predictions for the North Sea (21°C) (Sheppard 2004), which was shown to result in diminished growth rates after long-term exposure in one of the populations used here (Ramler et al., submitted). With this study, we estimated the effects of short-term thermal stress on pathogen resistance and its interactions with other life-history traits. Short-term fluctuations and extreme events like heat waves that are predicted to increase in the nearer future (Schär et al. 2004) could have very different effects on populations with different adaptive thermal optima. By additionally determining the within- and between-population genetic components of resistance and life-history traits, we can also project if environmental stress will modify the results of population-specific evolutionary trajectories by releasing or absorbing genetic variance for traits under selection. The connection between environmentally modified pathogen-induced selection to its consequences for other components of fitness will help to deepen our understanding of how populations can or cannot cope with increasingly stressful and pathogen rich environments.

Methods

Sampling and fish breeding

During April and June 2011, adult marine sticklebacks were caught by dip netting in an oyster farm in Yerseke (Oosterschelde, The Netherlands, 51°49' N, 4°05' E) and in tidal channels on the islands of Texel (The Netherlands, 53°14' N, 4°82' E) and Sylt (Germany, 55°05' N, 8°41' E). Between 50-70 individuals were sampled from each location and transferred in groups of 5-7 fish to 20 l aquaria (38 x 25 x 20 cm) equipped with permanent seawater flow-through at a constant temperature of 17°C. Between July and August, laboratory-bred F1 families were produced for each population (20 families per population) using a modified North Carolina II breeding design (five replicates of crosses between two dams x two sires i.e. four half-sib families). Since not all crosses were successful, we produced additional families (with different parents) for the Sylt population (n=10) to increase sample size and achieve better estimates of within population variation. Eggs were kept in aerated 1 l glass beakers until hatching. Hatchlings were then transferred to 10 l flow through tanks (19 x 25 x 20,5 cm) with a water temperature of 17°C and a 16 h: 8 h light: dark cycle. In total, 14 families from Oosterschelde, 11 families from Texel and 24 families from Sylt hatched.

One month after hatching, the density of each family was reduced to 20 fish per 10 l aquaria. Fish were fed daily ad libitum with live *Artemia* sp. until 12 weeks of age and afterwards with frozen chironomid larvae until the end of the experiment. During the early rearing phase, we lost several families due to electrical failure of aeration pumps. These affected only newly hatched families from all populations and did not lead to a systematic bias in mortality ($\chi^2_2 = 0.584$, $P = 0.747$). In the end, 12 families remained from the Oosterschelde population, nine families from Texel and 16 families from Sylt that had sufficient family sizes (min. 20 fish per family) for the infection experiment.

Infection experiment

Eight months after hatching, families were separated into smaller groups of 10 fish per 2,6 l aquarium (12 x 18,5 x 11,8 cm). In line with recent climate change predictions for the North Sea (Sheppard 2004), half of each family (n=10) was experimentally exposed to elevated temperatures by increasing the water temperature up to 21°C. The remaining half (n=10) was kept at their rearing temperature of 17°C as a control unit. After a four week thermal acclimation period, fish from both temperature treatments were injected either with 10 µl of a solution containing a bacterial strain closely related to a pathogen strain of *Vibrio tubiashii* (10^7 cells per ml, 17°C n=5, 21°C n=5) or with the same amount of PBS medium as control (17°C n=5, 21°C n=5). Infected and control fish were then held in ambient or elevated water temperature without flow through and susceptibility was determined by monitoring induced mortality every 12 hours for 10 days. Dead fish as well as fish remaining at the end of the experiment were weighed (wet mass, to 0.01 g) and measured (standard length, to 0.1 cm). All experiments were conducted in accordance with German animal welfare laws (Permission No. V312-72241.123-16).

Microsatellite genotyping

To measure drift-based neutral divergence between populations, each parental fish (n=70) was genotyped at 15 microsatellite loci. DNA was extracted from the caudal fin using the DNAeasy Blood and Tissue Kit (Qiagen, Hilden). The 20 µl multiplex PCR reactions consisted of 4 µl of 5x PCR Flexi buffer, 2 µl 10mM dNTP, 0.6 µl 25 mM MgCl₂, 0.1 µl 500 U GoTaq DNA Polymerase (all PCR chemicals from Promega, Mannheim), 5 pmol of each fluorescently labelled forward and unlabelled reverse primer and 2 µl DNA template. Thermal cycling for PCR group 1 (containing markers 5196 HEX, 4170 6_FAM, 1125 6_FAM, 1097 NED and 7033 NED, (Largiadere et al. 1999)) started with an initial denaturation step at 94°C for 3 min followed by 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min. PCR group 2 (containing markers STN 18 HEX, STN 32 6_FAM, STN 75 HEX and STN 84 NED) started with an initial denaturation step at 94°C for 3 min followed by 30 cycles

of 94°C for 45 sec, 56°C for 45 sec and 72°C for 1 min. PCR group 3 (containing markers STN 2 6_FAM, STN 170 HEX and STN 174 HEX) and as well as PCR group 4 (STN 36 6_FAM, STN 114 6_FAM and STN 167 HEX, (Peichel et al. 2001)) started with an initial denaturation step at 94°C for 2 min followed by 30 cycles of 94°C for 45 sec, 56°C for 45 sec, 72° for 45 sec and a final extension step at 72°C for 20 min.

One µl of each PCR reaction was denatured in HiDi-formamide (Life Technologies, Darmstadt) at 94°C for 2 min. Fragments were separated using an ABI prism 3100 XL (Life Technologies, Darmstadt) capillary sequencer. Genotype scoring was performed using GeneMarker software (SoftGenetics, version 1.91), and neutral genetic differentiation (measured as F_{ST}) was calculated after Weir and Cockerham (Weir and Cockerham 1984) using the software GENETIX (Belkhir K 1996-2004). Significance of the observed estimates was calculated by bootstrapping using 1000 random permutations of the data. Confidence intervals of F_{ST} values for the Q_{ST} - F_{ST} comparison were determined following Whitlock (Whitlock 2008) by using the range of single-locus estimates of F_{ST} for all 15 loci.

Statistical analyses

We used generalized linear models (GLM) to determine the effects of temperature, infection, population affiliation and family (nested within population) on length, weight and survival. For these initial analyses, we used family means as our unit of replication to ensure independence of data and to avoid pseudo-replication of fish stemming from the same tank and family. Afterwards, we used Welch's two sample t-test as a post-hoc test to identify pairwise differences of significant treatment effects on length and weight among populations.

Pathogen-induced mortality was analysed using GLMs containing temperature, population and either length or weight as independent predictors and survival as the dependent variable using proportions of survivors (approximately Gaussian distributed) from infected fish only. All models contained age of fish and density per aquarium as covariates to control for any potentially confounding effects. All model effects were tested by analysis of deviance and we assumed deviance change to be approximately χ^2 distributed.

We used generalized linear mixed models (GLMM) to calculate population and genetic variance components using data from individual fish. Divergence in quantitative traits (Q_{ST}) was calculated as

$$Q_{ST} = \frac{\sigma_B^2}{\sigma_B^2 + 2\sigma_W^2} \quad (\text{Spitze 1993}),$$

where σ_B^2 and σ_W^2 are the between- and within-population components of genetic variation. We fitted animal models as multivariate generalized linear mixed models with length and weight in both temperatures as Gaussian response variables and survival as a binomial variable using the MCMCglmm package (Hadfield 2010) of the R statistical environment (R Development Core Team 2008). Models for length and weight contained density and age as fixed effects and variance components were determined from the random terms population and animal. The animal variance component was taken as the measure of within-population variance while the population variance component was taken as a measure of between population variation. Additionally, we used the driftsel package (Karhunen et al. 2013) to test for the probability of positive selection (S-test). We used the suggested default priors and ran chains for neutral divergence as well as quantitative trait divergence with 50000 iterations (burn-in 30000, thinning interval 50). For traits with positive genetic correlations between environments (length and weight), we used the resulting G-matrix to test for selection, while for traits with negative genetic correlations, we estimated the probability of selection separately in each environment.

We used a character state approach (Falconer 1952), treating life history and resistance traits in the different thermal environments as separate traits, to calculate genetic correlations of traits across environments. Model fits were assessed by their respective DIC scores (Spiegelhalter et al. 2002) including random effects. We used weak but informative priors of half the observed phenotypic variance and set the covariance between temperature-specific trait measurements (length, weight, survival) to zero to account for measurements stemming from separate individuals. Markov chains were run for 500'000 iterations and we kept every 100th value after removing 300'000 iterations of burn-in to generate posterior distributions of random and fixed

parameters. Genetic correlations across environments (r_G) were calculated as the covariance between traits divided by the square root of the product of both traits, and were visualized by plotting the 95% confidence interval of the G-matrices resulting from variance and covariance estimates obtained for the scaled and standardized environment specific trait measurements. Significance of genetic correlations across environments was assessed by estimating the proportion of estimates from the posterior distributions that overlapped with zero. A genetic correlation across environments that is significantly smaller than 1 is a strong indicator that genetic components are differentially utilized in different environments, thus representing genotype by environment (GxE) interactions (Windig 1997). Since the upper limit for the calculation of r_G using MCMCglmm is also 1, we estimated the confidence interval around the upper boundary by calculating genetic correlations across environments of a trait with itself and took the lowest posterior estimate as the threshold against which significance was assessed (Shama et al. 2011). All statistical analyses were carried out using R statistical package (R Development Core Team 2008).

Results

Effects of thermal stress on life history traits and pathogen resistance

Thermal stress was visible in life history traits of sticklebacks after the acclimation and experimental period (figure 1). Fish from Oosterschelde and Texel were smaller and lighter at 21°C than at 17°C, but temperature had the most significant effect on weight (table 1), a trait likely reacting faster to environmental change. Furthermore, we found significant population effects on length and weight (table 1). The population from Sylt was significantly larger ($t_{4.5} = 420$, $P < 0.001$ for Sylt vs. Oosterschelde, $t_{6.4} = 409.6$, $P < 0.001$ for Sylt vs. Texel) and heavier ($t_{1.4} = 443.2$, $P = 0.017$ for Sylt vs. Oosterschelde, $t_{4.8} = 468.8$, $P < 0.001$ for Sylt vs. Texel) compared to the other populations. Infection as well as interactions between temperature and infection had no significant effects on life history traits (table 1).

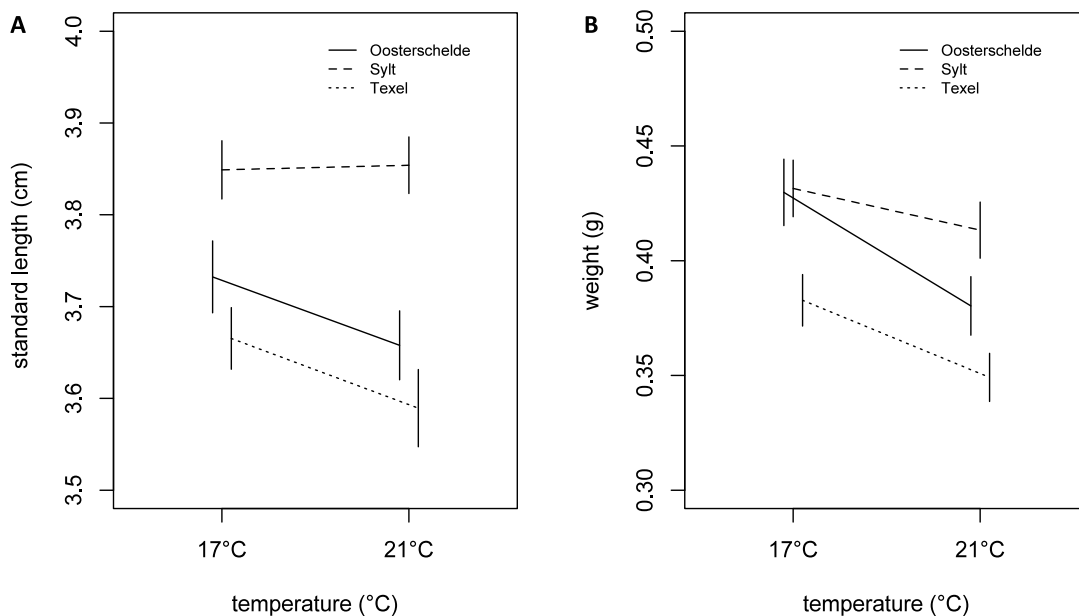


Figure 1 Effect of thermal stress on life-history traits of different stickleback populations. Lines represent means (with standard error) of length (A) and weight (B) of three different populations connecting different temperature treatments. Data from all fish were used.

Response	Length			Weight			Survival								
	Df	Dev	ResDf	ResDev	P(>Chi)	Df	Dev	ResDf	ResDev	P(>Chi)	Df	Dev	ResDf	ResDev	P(>Chi)
Null			139	12.41				139	2.01				139	14.30	
Temperature (T)	1	0.12	138	12.29	0.034	1	0.05	138	1.95	<0.001	1	0.64	138	13.66	<0.001
Infection (I)	1	0.02	137	12.27	0.429	1	0.01	137	1.94	0.087	1	5.11	137	8.55	<0.001
Population (P)	2	1.89	135	10.38	<0.001	2	0.15	135	1.79	<0.001	2	0.02	135	8.53	0.254
Density	1	3.35	134	7.03	<0.001	1	0.68	134	1.11	<0.001	1	0.00	134	8.53	0.543
Age	1	0.08	133	6.95	0.089	1	0.04	133	1.07	<0.001	1	0.51	133	8.02	<0.001
T*I	1	0.04	132	6.92	0.247	1	0.01	132	1.07	0.175	1	0.13	132	7.89	<0.001
T*P	2	0.08	130	6.84	0.227	2	0.01	130	1.06	0.385	2	0.03	130	7.85	0.089
I*P	2	0.04	128	6.80	0.440	2	0.00	128	1.06	0.723	2	0.16	128	7.69	<0.001
T*I*P	2	0.13	126	6.66	0.081	2	0.01	126	1.05	0.215	2	0.05	126	7.65	0.042
T*Family P	65	5.03	61	1.63	<0.001	65	0.86	61	0.19	<0.001	65	7.21	61	0.43	<0.001

Table 1 Summary of the effects of thermal stress on life history traits and pathogen resistance. Generalized linear models containing dependent variables length, weight and survival and independent predictors temperature, infection, population, family nested within population, density and age (family means of all fish were used). Significant effects are shown in bold.

Infection strongly reduced survival (figure 2). There was a significant difference between the survival of fish injected with PBS medium and fish injected with the *Vibrio tubiashii* isolate (table 1). Control fish showed low levels of mortality towards the end of the experiment. Furthermore, temperature and the interaction between temperature and infection had strong effects on survival (table 1). Infected fish held at the elevated water temperature (21°C) died faster (30.1% mortality within the first 12 hours of the experiment) and in higher numbers (53.4 % total mortality after 10 days post injection) than infected fish at 17°C (8.7% mortality in the first 12 hours, 33.5% total mortality after 10 days post injection). Survival rates differed among populations when infection and temperature effects were accounted for (infection x population and population x temperature; see table 1).

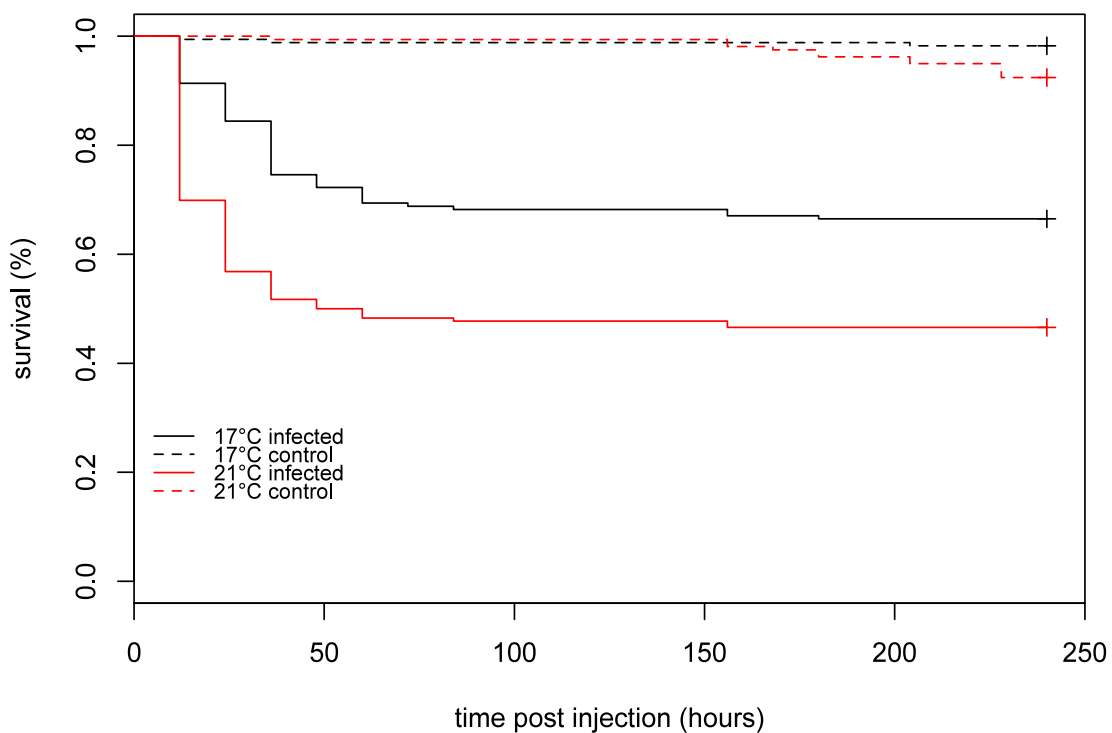


Figure 2 Survival plot of sticklebacks from different temperature treatments after infection with *Vibrio tubiashii* or PBS medium. Lines show means for all populations pooled.

Effects of thermal stress on quantitative genetic variation

We calculated the drift-based neutral divergence between populations for all populations combined (global $F_{ST} = 0.00825$, C.I. -0.00055 - 0.03901) as well as for each population pair (pairwise F_{ST} , Sylt vs. Texel $F_{ST} = 0.00339$, $P = 0.19$, Sylt vs. Oosterschelde $F_{ST} = 0.00926$, $P = 0.01$, Oosterschelde vs. Texel $F_{ST} = 0.02187$, $P = 0.003$). We observed small, but significant neutral differentiation between the Wadden Sea populations (Sylt and Texel) and the Oosterschelde. Our F_{ST} estimates were somewhat smaller than previously reported F_{ST} values for marine stickleback populations (range of 0.08 to 0.19 (Reusch et al. 2001; Leinonen et al. 2006; Raeymaekers et al. 2007)), but reflected results obtained for other marine fish from the Wadden Sea (Hoarau et al. 2002).

For all investigated traits, quantitative genetic differentiation (Q_{ST}) exceeded the overall neutral expectation (F_{ST} , figure 3) at 17°C , suggesting directional selection is driving populations to different phenotypic optima at ambient temperatures (Merila and Crnokrak 2001). Divergent selection was also detected with high probabilities for combined estimates of length and weight (driftsel S-test $P = 1$ in both cases). Q_{ST} values were on average lower at elevated temperature (21°C , red lines in figure 3) than at ambient temperature (17°C , blue lines in figure 3) for all traits owing to a trend of reduced genetic differentiation between populations ($V_{\text{Population}}$) and a release of within-population genetic variance of the selected traits (V_{Animal} , table 2). This result was most obvious for survival, where Q_{ST} values at elevated temperatures did not exceed neutral expectations (S-test: $P = 0.595$ at 21°C), and only at ambient temperature did divergent selection become visible (S-test: $P = 0.933$ at 17°C).

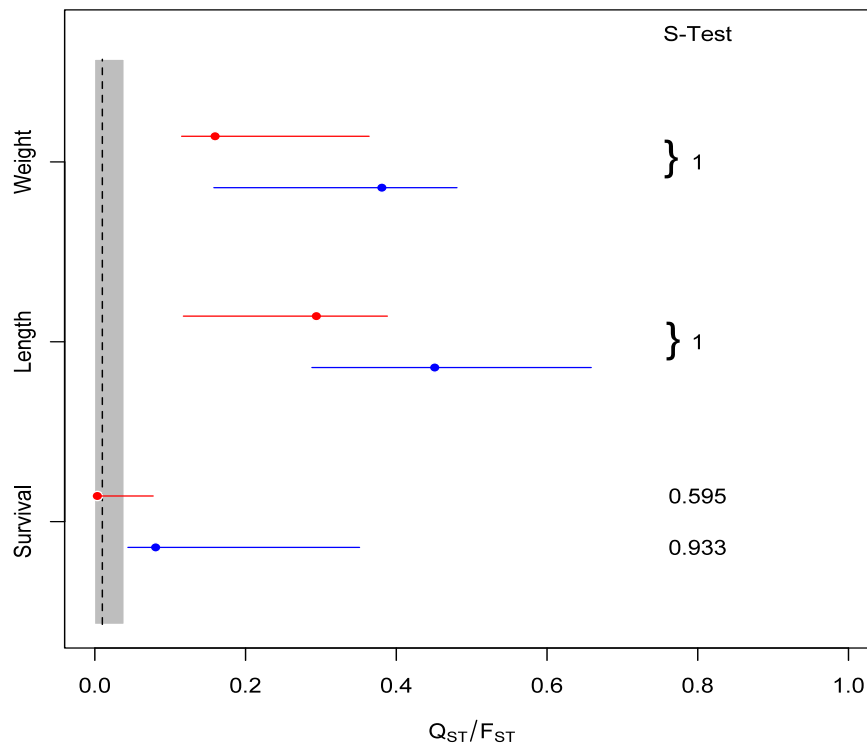


Figure 3 Quantitative trait and neutral marker differentiation of stickleback populations at different temperature treatments.

The grey box represents confidence intervals of global F_{ST} estimates. Blue lines represent 95% confidence intervals of Q_{ST} values for all phenotypic traits at 17°C, while red lines represent 21°C. Dots indicate position of highest densities of posterior distributions. All data were used for weight and length; only data from infected fish were used for survival. Probabilities of divergent selection (S-test) are given behind each trait. For length and weight, probability was estimated jointly for both environments due to positive genetic correlations, while probabilities of selection were separately estimated for survival due to negative genetic correlations.

		17°C		21°C		
Weight	17°C	V_A	0.006 (0.003 - 0.011)	r_G	0.669 (0.302 - 0.823)	
		V_{pop}	0.003 (0.002 - 0.028)	$P_{rG=0}$	< 0.001	
		V_U	0.011 (0.006 - 0.013)	$P_{rG=1}$	< 0.001	
		h^2	0.165 (0.069 - 0.252)			
	21°C	V_A		0.011 (0.006 - 0.014)		
		V_{pop}		0.004 (0.002 - 0.024)		
		V_U		0.005 (0.003 - 0.008)		
		h^2		0.441 (0.318 - 0.619)		
Length	17°C	V_A	0.026 (0.014 - 0.062)	r_G	0.626 (0.281 - 0.832)	
		V_{pop}	0.029 (0.016 - 0.227)	$P_{rG=0}$	< 0.001	
		V_U	0.086 (0.072 - 0.118)	$P_{rG=1}$	< 0.001	
		h^2	0.378 (0.137 - 0.556)			
	21°C	V_A		0.073 (0.035 - 0.118)		
		V_{pop}		0.046 (0.013 - 0.204)		
		V_U		0.065 (0.047 - 0.101)		
		h^2		0.236 (0.161 - 0.449)		
Survival	17°C	V_A	0.988 (0.250 - 2.738)	r_G	-0.807 (-0.992 - -0.193)	
		V_{pop}	0.831 (0.228 - 1.399)	$P_{rG=0}$	< 0.001	
		V_U	1*	$P_{rG=1}$	< 0.001	
		h^2	ne*			
	21°C	V_A		2.604 (0.705 - 135.630)		
		V_{pop}		0.609 (0.244 - 2.071)		
		V_U		1*		
		h^2		ne*		

Table 2 Estimated variance components of different traits and temperature treatments.

Variance components for V_{Animal} , $V_{Population}$, V_{Unit} (representing residual variation including environmental effects) and resulting heritability (h^2) as well as genetic correlations across environments (r_G) with 95% confidence interval. *fixed residual variation because categorical traits were used and heritabilities were not estimable.

We found relatively small variation between families but increased variation within some families for life-history traits. In comparison, pathogen resistance showed larger variation and increased resistance in a higher number of families at elevated temperature in all populations (figure 4a), suggesting that population mean fitness may be partly resilient to environmental change. Also, the increase in within-population genetic variation (table 2) shows that there is evolutionary potential in both environments. Within populations, we found significant interactions between temperature and family (nested within population) for all traits (table 1), indicating GxE interactions. This was further supported by the results of the character-state genetic correlations across environments. Figure 4b shows the respective G-matrices

for length, weight and survival. All traits showed genetic correlations and genotype by environment ($G_{\text{Host}} \times E$) interactions (slopes significantly different from 1). For life history traits, genetic correlations were similar and positive, whereas survival showed a negative genetic correlation between temperature environments.

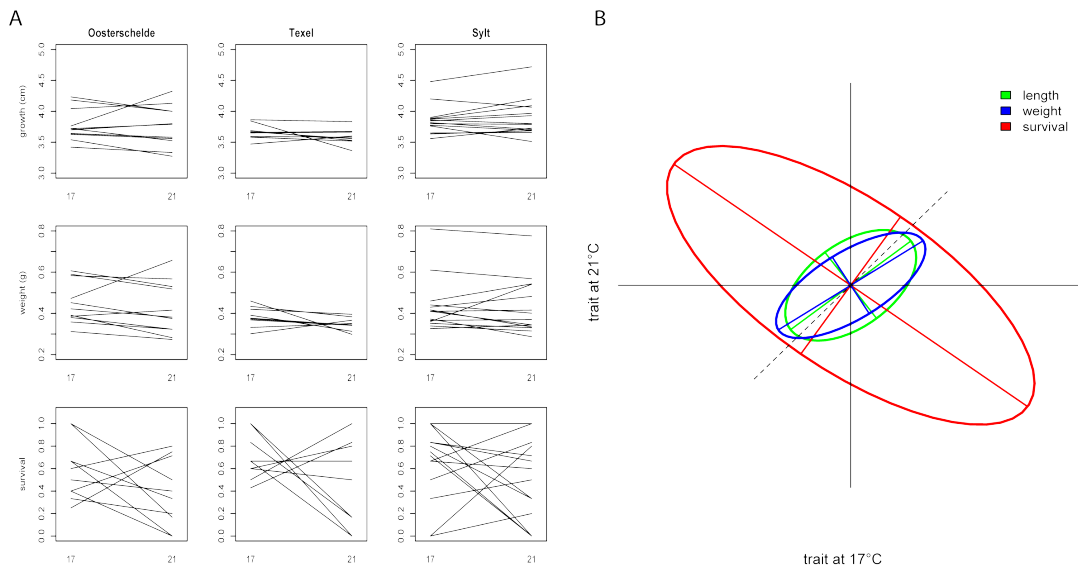


Figure 4 Family-specific reaction norms and genetic correlations across environments.

A Family-specific reaction norms of length, weight and survival from three different stickleback populations after infection with *Vibrio tubiashii*. Lines represent different families connecting means of different temperature treatments. **B** Character state genetic correlations represented by G-matrices between scaled and standardized trait values at different temperatures. Colour code for different traits, with ellipses marking 95% confidence intervals. The dashed line shows a slope of 1 indicative of no genotype x environment interactions (GxE). All data were used for weight and length; only data from infected fish were used for survival.

Pathogen-induced mortality also exerted correlated selection on life history traits. At ambient temperature, surviving fish were significantly longer ($\chi^2_1 = 4.23$, $P < 0.001$, figure 5a) and heavier ($\chi^2_1 = 4.90$, $P < 0.001$, figure 5b) than dying ones. At elevated temperature, the selection differential for body length increased in two out of three cases (Texel and Oosterschelde), whereas selection differentials for weight decreased uniformly in all populations.

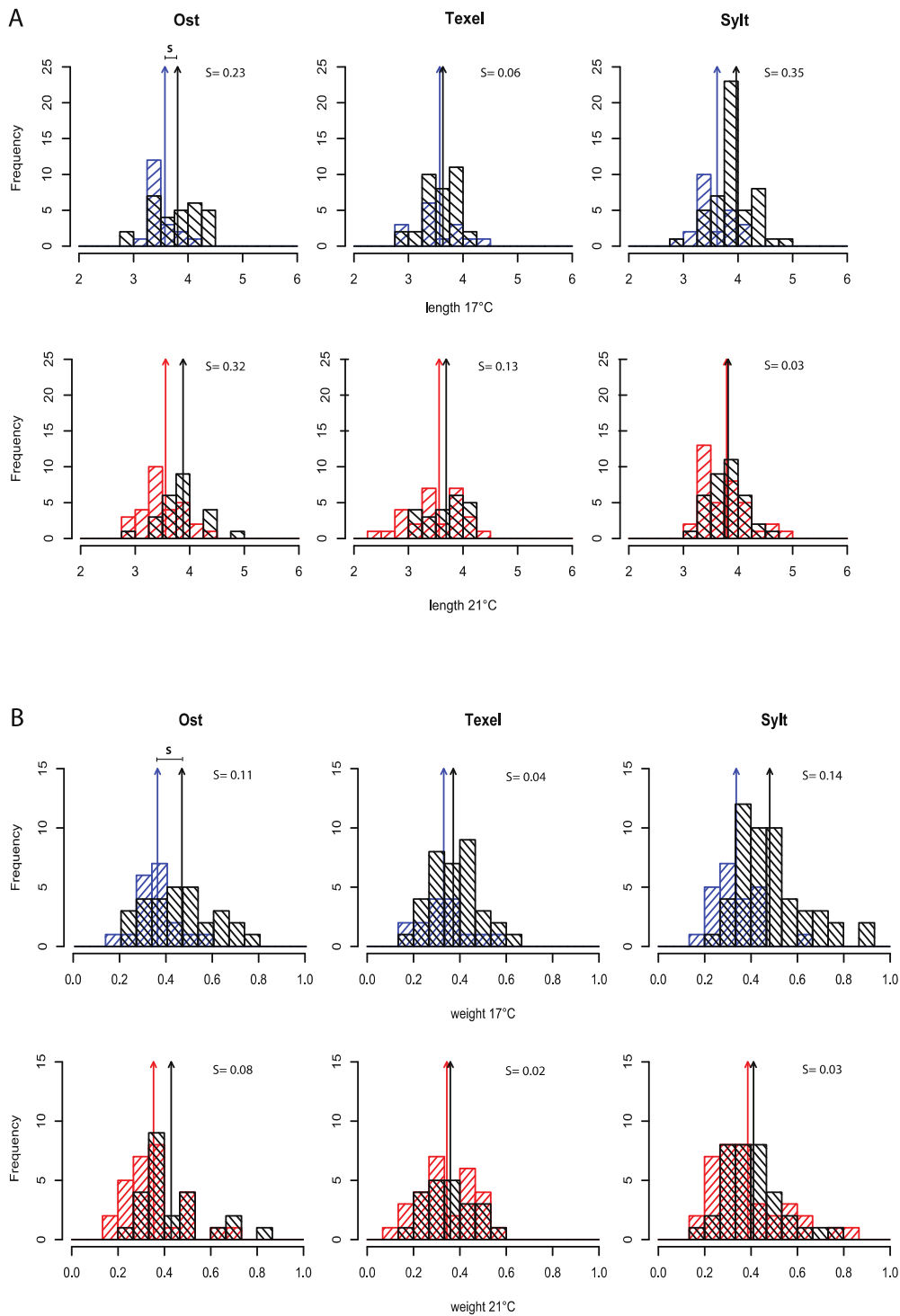


Figure 5 Selection gradients of life-history traits under pathogen induced selection.

Length (A) and weight (B) of dead fish (red bars=died at 17°C, blue bars=died at 21°C) and surviving fish (black bars) from different populations. Arrows show the means of the respective distributions marking the selection differential S (mean length survived - mean length dead) resulting from pathogen-induced mortality. Only data from infected fish were used for selective gradients. Ost=Oosterschelde

Discussion

In this study, we tried to connect the effects of elevated temperature on life history traits, pathogen resistance and population-specific potential for evolutionary responses in marine sticklebacks. After only a short acclimation period of elevated temperature, we found strong impacts of thermal stress on life history traits and survival after infection. Furthermore, thermal stress reduced genetic differentiation (Q_{ST}) between populations by releasing cryptic within-population variation. While life history traits showed positive genetic correlations between temperatures and genotype by environment interactions (GxE), thermal stress led to negative genetic correlations across environments in pathogen resistance. These trait-specific patterns indicate that evolutionary potential for life history traits with positive genetic correlations across environments can be reliably predicted from levels of standing genetic variation. For survival, on the other hand, negative genetic correlations across environments indicate that current genetic variation in resistance may be a weak predictor for future resistance evolution, highlighting the crucial importance of diseases in adaptation to new environmental conditions potentially favouring pathogens and parasites.

In general, thermal stress is likely to affect fish biochemistry, physiology and behaviour (Beitinger et al. 2000), and can negatively influence fish development and survival (Dominguez et al. 2004). Interactions between elevated temperatures and decreased size and weight have been demonstrated in several studies (Allen and Wootton 1982; Guderley and Leroy 2001; Lefebure et al. 2011; Donelson et al. 2012; Macnab and Barber 2012). It is believed that higher temperatures lead to lower food conversion efficiency in temperate sticklebacks, as energy is preferably used to maintain high metabolic rate (Guderley and Leroy 2001). Surprisingly, fish from Sylt were less sensitive to thermal stress throughout the experiment. This indicates the presence of different phenotypic optima as well as different thermal tolerances among the studied populations. Temperature preferences of sticklebacks for ideal growth were previously reported in the range between 15-18°C (Garside et al. 1977), at 19°C (Allen and Wootton 1982) as well as at 21,7°C (Lefebure et al. 2011), showing that thermal optima can differ substantially between populations. Temperate species are able to adapt and shift their thermal window through changes in mitochondrial densities as

well as via other molecular and systemic adjustments (Portner 2001). The wide range of thermal optima reported in the literature in conjunction with our results on quantitative genetic differentiation (figure 3) seem, however, to suggest that stickleback populations harbour substantial amounts of standing genetic variation and can adapt quickly to local environmental conditions.

The reduced sensitivity of the Sylt population can either be explained by increased tolerance due to phenotypic plasticity, or alternatively, by more favourable experimental conditions matching local conditions when compared to the other populations. We can assume that the allopatric populations from Oosterschelde and Texel were not only exposed to temperature stress but also had to cope with different water chemistry (common garden experiment used seawater from Sylt). This might have led to less favourable overall conditions amplifying negative effects of temperature stress, thus resulting in stronger declines of life-history traits at elevated temperature. Although we showed that short-term acclimation to increased temperature negatively influenced fish development, previous studies have shown that long-term acclimations to higher temperatures (within the natural range) can potentially lead to enhanced development and increased growth rates (Portner 2001; Green and Fisher 2004; Sponaugle et al. 2006). Sylt stickleback populations, on the other hand, continue to show decreased body size when exposed to higher temperatures throughout their lifetime (Ramler et al., submitted), indicating that our thermal conditions exceeded their potential for acclimation.

Our experiment also revealed that elevated temperatures have profound impacts on pathogen resistance of marine stickleback populations. Infected fish held in water at 21°C died faster and showed higher overall mortality than infected fish at 17°C. Suboptimal temperatures may influence innate immunity directly (Nikoskelainen et al. 2004) or indirectly as a result of stress-linked overproduction of immunosuppressive cortisol (Weyts et al. 1999). It has also been shown that elevated temperature negatively affects the resistance of fish to diseases by affecting antibody production and leucocyte activity (Alborali 2006; Landis et al. 2012). Either way, stressed organisms are likely to be more susceptible to infectious diseases than non-stressed ones (Lafferty and Holt 2003). Increased susceptibility due to stress can additionally be amplified by increased virulence of pathogens at higher temperatures. *Vibrios* are generally favoured at warmer temperatures (Thompson et al. 2004; Vezzulli et al. 2010), and the

temperature optimum for *Vibrio tubiashii* is reported to be at 25°C (Temperton et al. 2011). We can thus assume that the elevated temperature of 21°C supported faster development and a higher concentration of bacteria in the fish resulting in higher pathogenicity. Additionally, surviving fish were significantly longer and heavier, with weight reacting faster to environmental change than length. Especially for weight, we consistently observed decreased selection differentials at elevated temperatures. The altered response to selection imposed by infection might indicate that responses of other fitness components also can change unpredictably in changing environments.

Such change in response to selection on single traits will ultimately also feed back on past and future evolutionary responses of the host. By using a Q_{ST} - F_{ST} approach, we revealed that environmental stress reduced quantitative genetic differentiation (Q_{ST}) between populations. While the genetic components underlying phenotypic variation tended to be less divergent for traits assayed at higher temperature (21°), thermal stress increased genetic variance (V_{Animal} and heritability) for most of the selected traits (table 2). The effects of environmental change on quantitative genetic differentiation have only rarely been addressed. Hoffmann and Merilä (Hoffmann and Merila 1999) summarized several outcomes of how unfavourable conditions can affect the genetic variation of a trait. First, stressful conditions can increase genetic variation in traits by increasing rates of recombination and mutation (Hoffmann and Parsons 1991), by removing low fitness alleles due to selection (Kawecki et al. 1997), or because phenotypic differences among genotypes are only expressed as resources become limiting (Hartl et al. 1985). Second, genetic variance and heritability are decreased by increased environmental variation (Blum 1988) or due to limited genetic potential of organisms under poor nutrition (Gebhardthenrich and Vannoordwijk 1991). And third, stressful conditions have unpredictable effects on the genetic variation of a trait (Ebert et al. 1993; Gavrilets and Scheiner 1993; McGuigan et al. 2011). Our comparison of indices of population differentiation in quantitative traits (Q_{ST} of length, weight and survival) and neutral genetic divergence (F_{ST} of microsatellite markers) showed that divergent directional selection was the driving force for reaching different optima in length, weight and survival at 17°C in stickleback populations ($Q_{ST} > F_{ST}$).

Even if the comparison between Q_{ST} - and F_{ST} - values can reveal the presence and type of selection acting on traits (Tubiash et al. 1965; Knopp et al. 2007; Roberge et al. 2007; Leinonen et al. 2008), an essential requirement for evolutionary change is the amount of genetic variability expressed for the trait under selection (Charmantier and Garant 2005). We observed positive genetic correlations between character states of traits assayed at different temperatures. Slopes of the genetic correlations across environments as well as family-specific reaction norms were significantly different than 1, indicating significant genotype by environment interactions ($G_{Host \times E}$) in life history traits, while pathogen resistance showed a negative genetic correlation across environments. This reversal of genetic effects is probably due to changing environment-dependent virulence mechanisms of the pathogen interacting differently with host genotypes, i.e. $G_{Pathogen \times G_{Host \times E}}$ or $(G_{Pathogen \times E}) \times (G_{Host \times E})$ interactions, rather than to pure host genetic effects, i.e. $G_{Host \times E}$ interactions. On the other hand, some families within each of the populations showed improved performance at elevated temperature, which may lead to higher fitness in changing environments and adaptation of the population as a whole.

The maximal rate of adaptation is of crucial importance for estimating the ecological and evolutionary consequences of climatic warming. Populations need to shift the distribution of phenotypes to maintain optimal fitness in the changed environment (Visser 2008). Fast adaptation to rising temperature either requires short generation times or a substantial amount of standing genetic variation within populations (Poertner and Farrell 2008). Even though we found evolutionary potential in both environments, the negative genetic correlation in pathogen resistance between thermal environments demonstrates that adaptation to current conditions can, in certain cases, be a weak predictor for performance in changing environments. The negative genetic correlation across environments in survival might also have changed the selection gradients of associated life history traits, which might indicate that due to pathogen-induced selection, responses of other fitness components can be variable under unfavourable conditions.

Our study showed that thermal stress negatively impacts life-history traits and pathogen resistance of marine three-spined sticklebacks and reduces genetic differentiation between populations. Moreover, environmental stress led to negative genetic correlations across environments for pathogen resistance. Although we found

evolutionary potential in both thermal environments, it is hard to predict the capacity for adaptation by selection of pathogen resistant genotypes from ambient conditions. While positive genetic correlations of life history traits between environments indicate similar responses to selection, the altered selective gradient imposed by infection shows that responses of vital fitness components can change unpredictably in changing environments.

6 Chapter III

Within- and transgenerational effects of ocean acidification on life history of marine three-spined stickleback (*Gasterosteus aculeatus*)

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Abstract

Many studies have demonstrated that elevated CO₂ concentrations in ocean waters negatively impact metabolism and development of marine fish. Particularly early developmental stages are more susceptible to ocean acidification due to insufficient regulations of their acid-base balance. Transgenerational acclimation can be an important mechanism to mediate impacts of increased CO₂ on marine species, yet very little is known about the potential of parental effects in teleosts. Here, transgenerational effects on life history in juvenile three-spined sticklebacks *Gasterosteus aculeatus* were investigated by acclimating parents and offspring to ambient (~400 µatm) and elevated (~1000 µatm) CO₂ concentrations and measuring parental fecundity as well as offspring survival, growth and otolith characteristics. Exposure to elevated CO₂ concentrations led to an increase in clutch size in adults as well as increased juvenile survival and growth rates between 60 and 90 days post-hatch. Increased growth rates were also reflected in enlarged otolith areas. Moreover, transgenerational effects were also observed as otolith area was significantly increased when both parents were acclimated to the high CO₂ environment. This may suggest that elevated CO₂ concentrations do not need to have negative effects on life history traits of three-spined sticklebacks and that parental acclimation can modify these

effects. Nevertheless, it should be considered that acclimation is energetically costly, which might have severe impacts on other traits such as longevity or immunity.

Introduction

Approximately a third of the constantly increasing atmospheric carbon dioxide (CO_2) is dissolved in marine waters causing a decline in ocean pH and leading to an acidification of the ocean (Caldeira and Wickett 2005; Peters et al. 2012). Additionally, elevated oceanic CO_2 concentrations reduce the availability of carbonate ions required by many marine organisms to form calcium carbonate (CaCO_3) for skeletal structures (Feely et al. 2004; Doney et al. 2009; Dupont et al. 2010). Although ocean acidification primarily affects calcifying organisms such as corals, mollusks or echinoderms, recent studies also demonstrated severe impacts on teleosts (Frommel et al. 2012a; Munday et al. 2012; Wittmann and Pörtner 2013). Adult fish are considered to be tolerant to changes in ambient CO_2 due to active adjustments of their acid-base balance through bicarbonate accumulation and ion exchange across gills (Claiborne et al. 2002; Evans et al. 2005; Melzner et al. 2009). This regulation is insufficient in early developmental stages such as fish eggs and larvae making them more susceptible to ocean acidification (Brauner 2008; Ishimatsu et al. 2008). Along this line, it has been found that elevated CO_2 concentrations negatively impact behaviour (Munday et al. 2009a; Munday et al. 2009b; Ferrari et al. 2011), development (Frommel et al. 2012a; Miller et al. 2012), survival (Baumann et al. 2012) and otolith characteristics (Checkley et al. 2009; Munday et al. 2009b; Munday et al. 2011; Maneja et al. 2013) of marine fish larvae.

However, most of the studies investigate only short-term effects of ocean acidification by acclimating a single life-history stage to different CO_2 concentrations. But transgenerational acclimation can be an important mechanism to cope with changing environmental conditions (Donelson et al. 2011; Donelson et al. 2012; Salinas and Munch 2012). Parents can influence the fitness of their offspring by both genetic and non-genetic factors (Kirkpatrick and Lande 1989). Non-genetic parental inheritance is any effect on offspring phenotype based on

molecular (e.g. epigenetic inheritance) or nutritional (e.g. maternal provisioning) processes but is not caused by changes in the underlying DNA sequence (Bonduriansky and Day 2009).

These mechanisms have the potential to alter basic evolutionary predictions on one side, but might be energetically costly on the other side leading to different consequences for the fitness of parents and their offspring (Angilletta et al. 2003). To date, only few studies have considered parental effects in research on ocean acidification. While Parker et al. (Parker et al. 2012) revealed positive parental effects of elevated CO₂ on offspring growth, development and survival in the Sydney rock oyster (*Saccostrea glomerata*), Dupont et al. (Dupont et al. 2012) found negative parental effects on juvenile settlement success and survival in green sea urchins (*Strongylocentrotus droebachiensis*). With the exception of one study on tropical fish (Miller et al. 2012) transgenerational effects of ocean acidification in non-calcifying species are largely unexplored and to our knowledge no study has yet been conducted on how this mechanism will affect highly stress tolerant species.

Therefore we used a fish species with a high tolerance to fluctuations in water chemistry and temperature, the three-spined stickleback *Gasterosteus aculeatus* (Östlund-Nilsson et al. 2006). In this species, high CO₂ levels can induce behavioural defects in juveniles (Jutfelt et al. 2013), but the effects of ocean acidification on life history and components of fitness within and between stickleback generations are largely unexplored. A recent study (Shama and Wegner submitted) on marine sticklebacks revealed positive transgenerational effects of elevated temperature on juvenile growth. To deepen our understanding of how the parental environment can mediate transgenerational effects of elevated CO₂ in the offspring, we acclimated marine three-spined sticklebacks to ambient and elevated CO₂ concentrations and investigated the impacts of ocean acidification on the reproductive output of the parents and especially on early life history traits of their offspring. While we focused on survival and growth as central components of fitness, we also examined shape and size characteristics of otoliths (earbones). In contrast to fish skeleton, otoliths are bony structures that are primarily composed of aragonite, which will be reduced in acidified seawater (Sabine et al. 2004). These ear bones are used by fish for balance, orientation and sound detection and thus any change in otolith structure can lead to serious implications for individual

performances such as swimming behaviour (Popper et al. 2005) or survival (Gagliano et al. 2008).

Since we investigated the combined effects of elevated CO₂ on fecundity in adults with transgenerational effects of survival, growth rates as well as otolith characteristics in the offspring, these results will help our understanding of transgenerational acclimation and its role in coping with changing environmental conditions.

Methods

Experimental design

Seawater treatment

Seawater was acidified by the standard method of dissolving additional CO₂ into seawater (Leclercq et al. 2002). Temperature and pH were monitored every second day (during the breeding period twice per day) with a pH meter (WTW, 330i) and a calibrated pH electrode (SenTix, 81 plus). Every two weeks a subsample of seawater from every treatment was measured for temperature, salinity, pH (calibrated on total scale with Dickson buffer (Tris buffer solution in synthetic seawater, batch 8, bottle 69, Dr. Andrew Dickson CO₂ QcLab)) and total alkalinity to investigate the carbonate chemistry of the water (table 1) using CO₂calc software (Robbins 2010), with dissociation constants K₁ and K₂ according to Mehrbach et al. (Mehrbach et al. 1973), refitted by Dickson and Millero (Dickson and Millero 1987).

Treatment	T [°C]	S [‰]	pH _T	A _T [μmol kg ⁻¹]	pCO ₂ [μatm]
P control	17,0 ± 0,3	29,7 ± 1,1	8,01 ± 0,05	2291 ± 45	482 ± 15
P high CO ₂	16,9 ± 0,5	29,7 ± 1,1	7,72 ± 0,03	2300 ± 60	1013 ± 122
F1 control	17,4 ± 0,8	29,8 ± 0,4	8,03 ± 0,02	2261 ± 19	435 ± 27
F1 high CO ₂	17,5 ± 0,5	29,8 ± 0,4	7,65 ± 0,07	2267 ± 20	1167 ± 176

Table 1 Water chemistry for parental (P) and offspring (F1) treatments. T, Temperature, S, Salinity, pH (total scale), A_T, total alkalinity (all measured) and calculated pCO₂, partial pressure of CO₂. Values are means ± SD.

Parental acclimation

In April 2012 adult marine sticklebacks were caught on a day cruise with the RV “Mya” in the Sylt-Rømø-Bight, North Sea, Germany (55°03' N, 8°44' E). A total of 60 fish were transferred to twelve 20 l aquaria (38 x 25 x 20 cm) with permanent seawater flow through (15°C, pH ambient conditions) in groups of five. After a two week period fish were randomly assigned to two acclimation groups: One group was kept in seawater with ambient CO₂ concentration of 482 ± 15 µatm (control), while the other group was kept in seawater with elevated CO₂ concentration of 1013 ± 122 µatm (high), based on future CO₂-induced ocean acidification scenarios (IPCC 2007). This acclimation period lasted for three months and throughout this time adult fish were kept at 16 h:8 h light:dark cycle and fed daily with chironomid larvae *ad libitum*.

Breeding and offspring treatment

After parental acclimation laboratory-bred F1 families ($n_{\text{total}}=19$) were produced within each parental treatment (i.e. sire_{control} x dam_{control} and sire_{high} x dam_{high}) as well as between parental treatments (sire_{control} x dam_{high} and sire_{high} x dam_{control}). Dams were measured (standard length, to 0.1 cm) and weighed before and after stripping of egg batches to calculate weight of eggs (to 0.01 g). Egg clutches were photographed under a stereomicroscope for digital analyses of egg number and egg size (0.001 cm) using an image analysis program (Leica QWin, Image Processing & Analysis Software V3.5.0).

Egg clutches were then separated into two equal parts and transferred to sealed 1 l glass beakers, containing seawater with ambient CO₂ concentration of 435 ± 27 µatm (control) and elevated CO₂ concentration of 1167 ± 176 µatm (high). Water was exchanged twice per day (90% of total volume) with pre-acidified seawater until hatching. Hatchlings were then transferred to 2,6 l flow through aquarium (12 x 18,5 x 11,8 cm) with a density of 10 fish per tank, referring to respective CO₂ concentrations (control or high), and kept for additional three months. Throughout the experiment, juvenile fish were kept at 16 h:8 h light:dark cycle and fed daily

with live *Artemia* nauplii *ad libitum*. All experiments were conducted in accordance with German animal welfare laws (Permission No. V312-7224.123-16).

Data collection

Survival and growth rates

At 30, 60 and 90 days post-hatch, all offspring from each clutch were counted and photographed under a stereomicroscope for digital analysis of size (standard length, to 0.001 cm) using an image analysis program (Leica QWin, Image Processing & Analysis Software V3.5.0). At the end of the experiment (100 days post-hatch) fish were frozen individually in tubes at -80°C for further investigations.

Measurement of otolith characteristics

To extract otoliths, the top of the cranium was sliced off with a small scissor and both sagittae were dissected from beneath the posterior end of the brain using fine forceps under a stereomicroscope. They were cleaned with distilled water, dried and collected in 96 well plates before pictures were taken under a stereomicroscope with highest resolution for digital analysis of size (to 0.001 mm) and area (to 0.0001 mm^2) with an image analysis program (ImageJ (Rasband 1997-2012)). Critical illumination and high video contrast were used to improve the image. Otoliths were then weighed (to 0.001 g) using a MC5 microbalance (Sartorius, Göttingen).

Data analyses

After we found no significant difference of dam size between CO_2 treatments using Welch's two sample *t*-test, we continued with generalized linear models (GLM) to determine the effects of maternal CO_2 treatment and dam size as independent predictors on fecundity using egg number (Poisson function), egg weight and egg diameter (Gaussian function). Survival and growth rates at 30 days, 60 days and 90 days post-hatch were analysed using GLMs (Gaussian function), containing the offspring CO_2 , maternal CO_2 , paternal CO_2 as independent

predictors using family means to guarantee statistical independence. Since density had a significant effect on growth, we corrected all growth rates by using the residuals from a linear model containing standard length (cm).

To determine the effects of CO₂ on fish otoliths, we used GLMs (Gaussian function) containing dependent variables otolith size, otolith area and otolith weight and independent predictors offspring CO₂, maternal CO₂, paternal CO₂, offspring size, density and age using family means. Because density and age had no significant effect on otolith characteristics we excluded both predictors from the reported models.

All GLM effects were tested by analysis of deviance and we assumed deviance change to be approximately χ^2 distributed. All statistical analyses were carried out using R statistical package (R Development Core Team 2008).

Results

Fecundity

Dam size between CO₂ treatments was not significantly different ($t_{12} = 0.486$, $P = 0.636$), but we found strong impacts of dam size ($\chi^2_1 = 41.095$, $P < 0.001$) and maternal CO₂ treatment on egg number ($\chi^2_1 = 25.552$, $P < 0.001$, figure 1). Dams acclimated to elevated CO₂ (high) produced significantly more eggs (in average $n=123.1 \pm 5.8$) in comparison with dams acclimated to ambient CO₂ (control, in average $n=98.7 \pm 4.8$). Although we found a slight trend for decreased average egg weight at elevated CO₂, there was no significant correlation between egg number and egg size ($\chi^2_1 = 67.074$, $P = 0.793$) as well as between egg number and average egg weight ($\chi^2_1 = 525.79$, $P = 0.457$), suggesting that there was no trade-off between fecundity and egg quality.

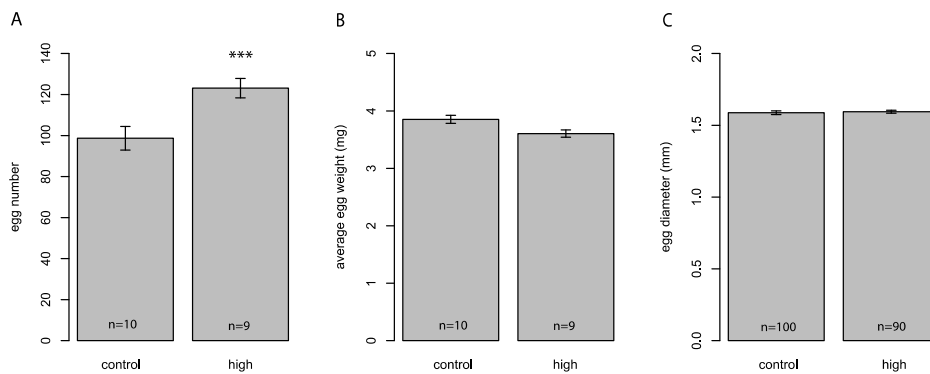


Figure 1 Effect of maternal CO₂ on fecundity. Bars represent means (with standard error) of egg number (a), average egg weight (b) and egg diameter (c) separated by CO₂ treatment of dams. Numbers in bars are the number of replicates per treatment. *** denotes significant differences $P < 0.001$.

Survival

After 30 days post-hatch, offspring survival was mostly influenced by paternal and parental treatment (table 2). Survival between offspring CO₂ treatment did not differ significantly at this time (table 2, figure 2). After 60 and 90 days post-hatch, potential transgenerational effects were overruled by offspring CO₂ treatment. At the end of the experiment, fish from control treatment showed significantly reduced densities per tank (survival) than fish from high CO₂ treatment (table 2, figure 2).

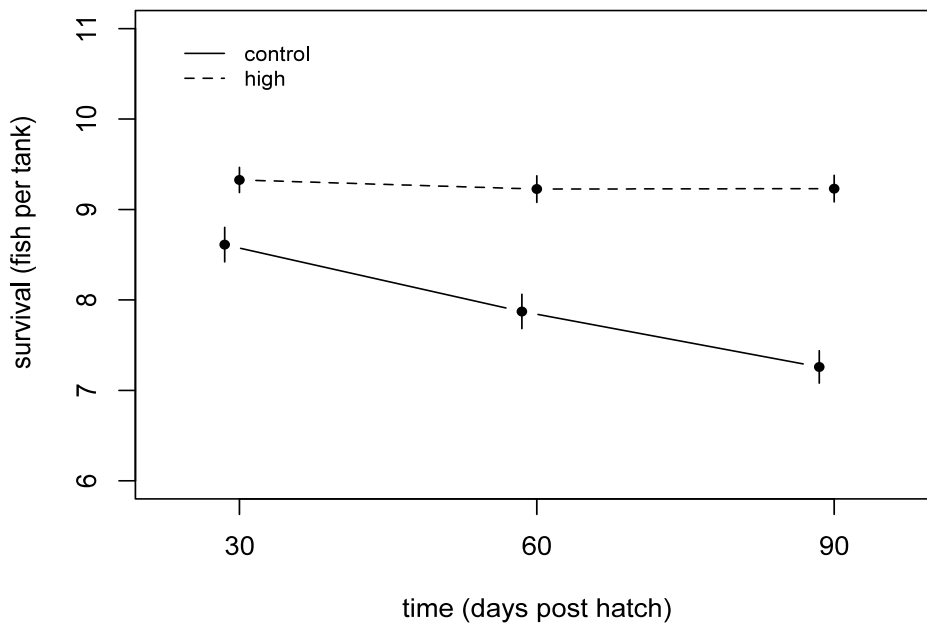


Figure 2 Effect of offspring CO₂ on fish survival. Lines represent mean tank survival (with standard error) grouped by days post-hatch (x-axis) and offspring CO₂ treatment (solid and dashed lines).

Response	Survival 30 days post-hatch			Survival 60 days post-hatch			Survival 90 days post-hatch								
	Df	Dev	ResDf	ResDev	P(>Chi)	Df	Dev	ResDf	ResDev	P(>Chi)	Df	Dev	ResDf	ResDev	P(>Chi)
Null			30	148				29	146				30	154	
CO ₂ F1 (F1)	1	10.4	29	137	0.086	1	20.0	28	126	0.027	1	25.4	29	129	0.014
CO ₂ dam (D)	1	3.2	28	134	0.344	1	0.01	27	126	0.952	1	2.4	28	126	0.445
CO ₂ sire (S)	1	17.0	27	117	0.029	1	15.6	26	111	0.051	1	9.9	27	116	0.125
F1*D	1	1.1	26	116	0.575	1	0.6	25	110	0.708	1	6.2	26	110	0.223
F1*S	1	6.9	25	109	0.162	1	10.4	24	100	0.111	1	5.2	25	105	0.268
D*S	1	14.4	24	95	0.044	1	4.9	23	95	0.276	1	4.4	24	100	0.309
F1*D*S	1	13.0	23	82	0.055	1	4.6	22	90	0.289	1	3.9	23	97	0.338

Table 2 Summary of the effects of parental and offspring CO₂ treatment on fish survival. Generalized linear models containing dependent variables of density at 30 days, 60 days and 90 days post-hatch and independent predictors CO₂ offspring (CO₂ F1), CO₂ dam (D) and CO₂ sire (S). Significant effects are shown in bold.

Response	Growth 30 days post-hatch			Growth 60 days post-hatch			Growth 90 days post-hatch								
	Df	Dev	ResDf	ResDev	P(>Chi)	Df	Dev	ResDf	ResDev	P(>Chi)	Df	Dev	ResDf	ResDev	P(>Chi)
Null			22	8.1				21	23.3				21	65.3	
CO ₂ F1 (F1)	1	0.22	21	7.9	0.395	1	9.21	20	14.1	<0.001	1	28.6	20	36.7	<0.001
CO ₂ dam (D)	1	0.16	20	7.8	0.472	1	1.31	19	12.8	0.194	1	0.79	19	36.0	0.534
CO ₂ sire (S)	1	2.44	19	5.3	0.005	1	0.02	18	12.8	0.886	1	2.53	18	33.4	0.266
F1*D	1	0.03	18	5.3	0.740	1	0.12	17	12.7	0.693	1	0.53	17	32.9	0.611
F1*S	1	0.41	17	4.9	0.245	1	0.24	16	12.4	0.577	1	0.23	16	32.7	0.733
D*S	1	0.33	16	4.5	0.297	1	1.57	15	10.9	0.155	1	3.97	15	28.7	0.164
F1*D*S	1	0.00	15	4.5	0.999	1	0.03	14	10.8	0.840	1	0.06	14	28.6	0.870

Table 3 Summary of the effects of parental and offspring CO₂ treatment on fish growth. Generalized linear models containing dependent variables family mean growth at 30 days, 60 days and 90 days post-hatch and independent predictors CO₂ offspring (CO₂ F1), CO₂ dam (D) and CO₂ sire (S). Growth was estimated as the residuals of standard length corrected for effects of density. Significant effects are shown in bold.

Growth

Significant transgenerational effects of paternal CO₂ environment on offspring growth were found at 30 days post-hatch (figure 3a, table 3). Offspring from sires acclimated to high CO₂ showed reduced growth, while growth rates were not significantly impacted by maternal treatment or the interaction of both (table 3).

Offspring growth at 60 days and 90 days post-hatch was strongly influenced by offspring CO₂ treatment (table 3) showing reflecting patterns found for offspring survival. Despite of no significant decrease in densities for fish stemming from high CO₂ treatment, growth was significantly increased for this experimental group. We could not detect overall parental effects on offspring growth for 60 days and 90 days, but offspring with both parents acclimated to high CO₂ (HH) tended to be larger compared to offspring from other crosses (figure 3b, c).

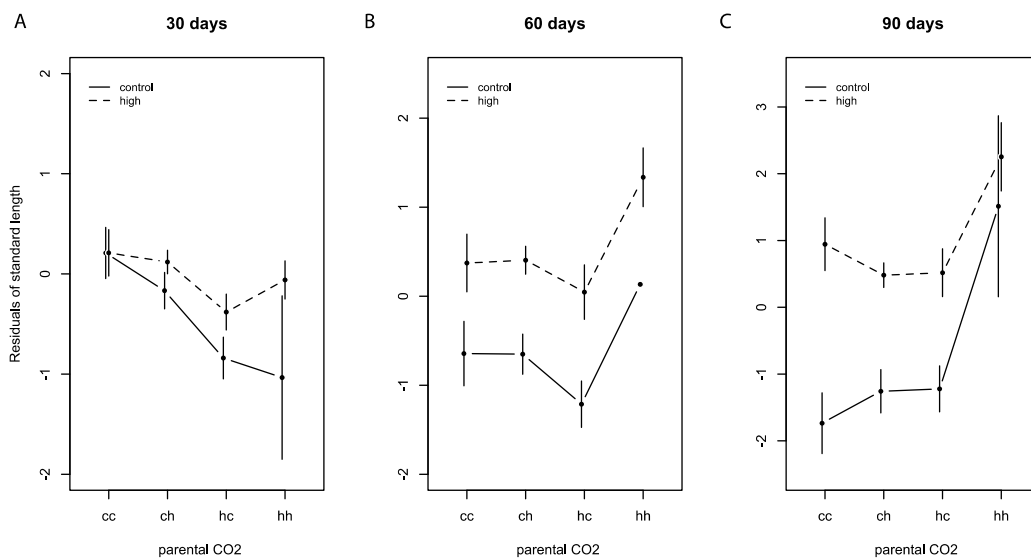


Figure 3 Transgenerational effects of CO₂ on fish growth. Lines show means (with standard error) of growth controlled for density at 30 days (a), 60 days (b) and 90 days (c) post-hatch grouped by parental CO₂ treatment (x-axis) and offspring CO₂ treatment (solid and dashed lines). Residuals of standard length were used to adjust density effect. CC sire control/dam control, CH sire control/dam high CO₂, HC sire high/dam control, HH sire high/dam high.

Otoliths

Otolith characteristics (length, area and weight) were strongly influenced by fish growth (table 4), but parental and offspring CO₂ treatment also showed significant effects on otolith characteristics.

Although we found paternal transgenerational effects on survival and growth only in early life stages, we observed strong influences of parental treatment on otolith size as well as from paternal and offspring CO₂ treatment on otolith area (figure 4, table 4) until the end of the experiment (100 days post-hatch). Offspring from fathers acclimated to high CO₂ showed larger otoliths (figure 4a), whereas otolith area was significantly increased, when both parents were acclimated to the high CO₂ environment (figure 4b).

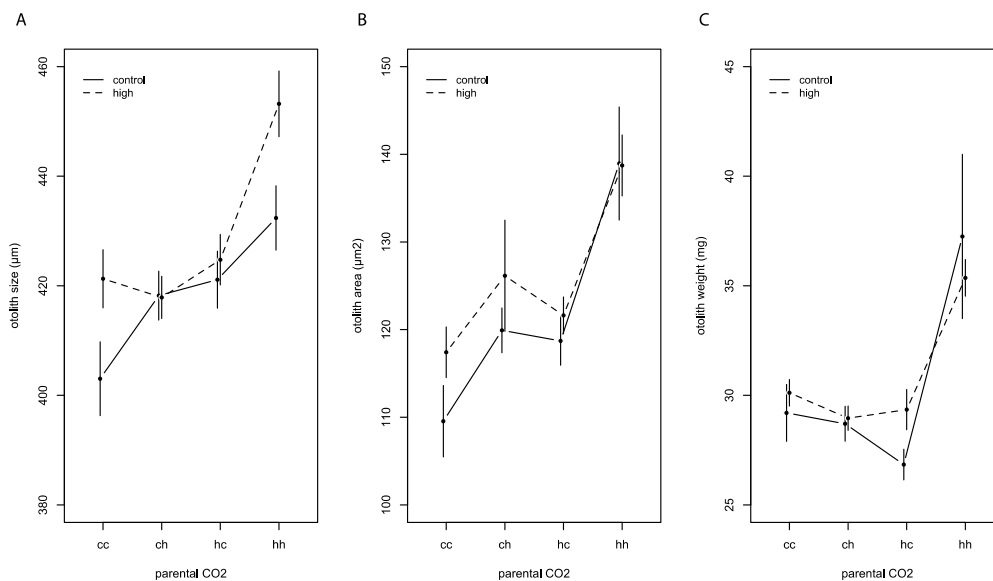


Figure 4 Transgenerational effects of CO₂ on fish otolith characteristics. Lines show means (with standard error) of otolith size (a), otolith area (b) and otolith weight (c) separated by parental CO₂ treatment (x-axis) and offspring CO₂ treatment (solid and dashed lines). CC sire control/dam control, CH sire control/dam high CO₂, HC sire high/dam control, HH sire high/dam high.

Response	Otolith size			Otolith area			Otolith weight		
	Df	Dev	P(>Chi)	Df	Dev	P(>Chi)	Df	Dev	P(>Chi)
Null	22	9933		22	2450		22	422	
CO ₂ F1 (F1)	1	987	0.099	1	362	0.009	1	23	0.172
CO ₂ dam (D)	1	224	0.432	1	518	0.002	1	15	0.269
CO ₂ sire (S)	1	1646	0.033	1	500	0.002	1	30	0.122
size F1	1	1501	0.042	1	274	0.023	1	139	<0.001
F1*D	1	18	0.823	1	1	0.883	1	7	0.468
F1*S	1	19	0.818	1	12	0.631	1	2	0.660
D*S	1	24	0.798	1	34	0.425	1	27	0.142
F1*D*S	1	429	0.277	1	0.5	0.923	1	2	0.699

Table 4 Summary of the effects of parental and offspring CO₂ treatment on fish otoliths. Generalized linear models containing dependent variables otolith size, otolith area and otolith weight and independent predictors CO₂ offspring, CO₂ dam, CO₂ sire and offspring size at the end of the experiment (family means were used). Significant effects are shown in bold.

Discussion

In general, adult fish can maintain their internal pH within an optimal range and are therefore relatively tolerant of changes in ambient CO₂ (Ishimatsu et al. 2005; Ishimatsu et al. 2008; Melzner et al. 2009), suggesting that effects of OA can be compensated. Compensation can be metabolically costly and could reduce the amount of energy available for respiration, reproduction and survival (Portner et al. 2004; Kroeker et al. 2010). In contrast we found strong positive within-generation impacts of maternal CO₂ treatment on egg number. Dams acclimated to elevated CO₂ produced significantly more eggs (19.8%) compared to dams acclimated to ambient CO₂. Previous studies tended to find negative or no effects of elevated CO₂ on reproduction in fish (Inaba et al. 2003; Frommel et al. 2010; Frommel et al. 2012b; Sundin et al. 2013). However, one recent study reported increased breeding activity in cinnamon anemonefish (*Amphiprion melanopus*) due to increased CO₂ (Miller et al. 2013), suggesting that higher CO₂ levels can have stimulating effect on behaviours associated with reproduction as well as reproduction itself. It is possible that our results were based on hormonal stimulation, as changes in hormone levels can be generated by environmental stressors and lead to increased reproductive performance due to accelerated physiological processes (Costantini et al. 2010; Schreck 2010). Here, the elevated CO₂ concentration in seawater seems to be rather beneficial than harmful for the reproduction in three-spined sticklebacks. Larger clutch sizes can lead to increased female fitness, if current reproduction is not traded off against future reproduction. The non-significant correlation of egg number and egg weight or size seems to suggest that the increase in clutch size did not occur on the cost of reduced egg quality. Moreover, offspring from elevated CO₂ treatment showed higher survival rates during the experiment leading to the assumption that sticklebacks can cope well with changing CO₂ concentrations. The three-spined stickleback, a highly stress tolerant fish, is found in a wide range of habitats showing strong physiological plasticity and a large potential for acclimation to environmental stressors (Pottinger et al. 2002; Östlund-Nilsson et al. 2006; Barrett et al. 2011). So far, most studies on three-spined sticklebacks deal with thermal or osmotic stress (Jordan and Garside 1972; Moran et al. 2010; Hopkins et al. 2011; Lefebure et al. 2011), but

only one study has revealed negative effects of ocean acidification on stickleback behaviour (Jutfelt et al. 2013). Although the ancestral form of *G. aculeatus* was purely marine, the population used here is a mix of anadromous and marine fish. This anadromous heritage, containing fresh water life stages that regularly experience lower pH values, might be an explanation of why sticklebacks cope better with acidified seawater than other species lacking such habitat shifts.

Several studies showed negative impacts of ocean acidification on growth in fish (Franke and Clemmesen 2011; Baumann et al. 2012; Miller et al. 2012). For marine sticklebacks we now show that offspring growth can also be positively affected by elevated CO₂ between 60 and 90 days post-hatch (figure 2b, c, table 3). These results coincide with recent ocean acidification studies on larval clown fish *Amphiprion percula* (Munday et al. 2009b) and Atlantic cod *Gadus morhua* (Frommel et al. 2012a). Since the fish in this study were fed *ad libitum*, enhanced growth rates in high CO₂ treatment could be based either on increased energy intake due to stimulate appetite and feeding activity in juveniles (Munday et al. 2009b) or juveniles allocate more of the available energy to growth under elevated CO₂, which in turn might come at the cost of severe organ damages (Frommel et al. 2012a). However, in general increased growth and accelerated development is known to be advantageous for fish survival and reproduction. Larger fish have usually higher feeding success and decreased mortality through predation (Searcy and Sponaugle 2000; Bergenius et al. 2002; Meekan et al. 2003) and larger fish also show higher reproductive potential due to earlier sexual maturity and improved fecundity e.g. larger females produce larger egg clutches (Portner et al. 2004; Portner et al. 2005; Denman et al. 2011).

Additional to positive effects of high CO₂ on growth, we found increased otolith area in offspring kept in elevated CO₂ environment (figure 4b, table 4), confirming results of other recent studies (Checkley et al. 2009; Munday et al. 2011; Bignami et al. 2013; Maneja et al. 2013). Otolith characteristics depend on fish growth (Jones and Hynes 1950), but can be also influenced by other physiological responses of fish to high CO₂ (Claiborne and Heisler 1984). Adjustment of extracellular pH, for example, leads to an increase in carbonate ions, which can in turn cause an increase in the aragonite saturation state of the otolith endolymph (Checkley et al. 2009). The consequences of altered otolith characteristics are under debate, but a

substantial change to otolith size, shape or symmetry can be harmful to organisms (Gagliano et al. 2008). An increase in otolith size can, for example, could influence the swimming behaviour (Popper et al. 2005), particularly in fast and agile swimmers such as three-spined sticklebacks.

Next to direct effect of ocean acidification our breeding design mainly aimed at investigating the transgenerational effects of parental acclimation on offspring life history. Transgenerational acclimation is a potentially powerful mechanism to mediate the effects of rapid environmental change (Donelson et al. 2012; Salinas and Munch 2012; Munday et al. 2013). In our study, growth rates of stickleback larvae were only influenced by parental environment in early life stages (30 days post-hatch). Later in life (60 and 90 days post-hatch) growth was mainly determined by offspring CO₂, where environmental effects overruled transgenerational effects. In contrast to body size, parental effects persisted for otolith characteristics (size and area) indicating that different mechanisms or downstream consequences act in transgenerational acclimation.

In marine fish, transgenerational plasticity has mainly been investigated in conjunction with changing temperatures and several studies showed that parental acclimation could at least partly mediate detrimental effects of suboptimal temperatures (Donelson et al. 2012; Salinas and Munch 2012; Shama and Wegner submitted). So far, only a single study tried to elucidate the role of transgenerational acclimation in terms of ocean acidification (Miller et al. 2012), where negative effects of elevated CO₂ (~1000 µatm) on the growth rate of juvenile anemonefish (*Amphiprion melanopus*) were absent, when parents also experienced high CO₂.

In total, elevated CO₂ concentrations, which are predicted to occur in ocean waters in the year 2100 (IPCC 2007), did on average not influence the investigated traits of sticklebacks negatively. However, the direct translation of increased growth or altered otolith characteristics into fitness is not straightforward and may take several generations to be expressed (Munday et al. 2012). Additionally, within- as well as transgenerational acclimation might generate additional costs or involve trade-off with other traits (Angilletta et al. 2003). It is believed that ocean acidification could result in diminished longevity and lifetime fecundity (Portner and Peck 2010) or

may be have severe impacts on immunity, leading to altered host-parasite interactions (Dupont et al. 2012; Dupont and Thorndyke 2012). At least, after short-term acclimation we could not reveal negative impacts of elevated CO₂ on survival. Nevertheless, the potential for adaptation and transgenerational acclimation are crucial considerations to determine a species' susceptibility to environmental change (Skelly et al. 2007; Munday et al. 2013), although the effects can be reversed dependent on investigated trait or environmental stressor.

7 Conclusions and perspectives

The ongoing increase of the greenhouse gas carbon dioxide in the atmosphere causes a rise in global mean air and water temperatures (IPCC 2012). When atmospheric CO₂ dissolves in seawater, oceanic pH declines, leading to an acidification of the ocean (Caldeira and Wickett 2005; Peters et al. 2012). When the oceans becoming warmer and more acidic, many marine species will be exposed to unsuitable environments, likely impacting their performance and survival (Harley et al. 2006; Bijma et al. 2013).

To reveal potential effects of rising water temperature and ocean acidification on marine fish populations, I used a prime model organism, the three-spined stickleback *Gasterosteus aculeatus*, for my studies.

After exposure to a simulated four-week summer heat wave (21°C), as an extreme event of global change, juvenile sticklebacks showed decreased length and weight, in comparison to fish held at 17°C (chapter II). In general, rising temperatures are likely to stress organisms, dependent on their thermal tolerance (Harley et al. 2006). Thermal stress can alter biochemistry and physiology, which in turn may negatively influence fish development (Beitinger et al. 2000; Dominguez et al. 2004).

Moreover, thermal stress can alter immune functions of organisms, thereby increasing susceptibility to infectious diseases (Harvell et al. 2002; Lafferty et al. 2004; Alborali 2006). Thermally stressed sticklebacks showed strongly reduced survival after infection with a bacterial pathogen (chapter II). A recently published study revealed long-lasting immune disorders in sticklebacks after exposure to an experimental heat wave (Dittmar et al. 2014), which might explain reduced pathogen resistance.

On the other hand, the development of many parasites and pathogenic bacteria is positively correlated with temperature, increasing the risk of infection and diseases during summer (Harvell et al. 2002). Short periods of elevated water temperatures resulted in increased macroparasite and bacterial diversities in three-spined stickleback, Atlantic herring, European sprat and lesser sand eel (chapter I). In

addition, some parasite groups showed a forward shift and extension in infection peaks, following warmer spring seasons (chapter I). Increased virulence of pathogens at higher temperatures in combination with immune-compromised hosts can result in acute fish mortalities, particularly in summer months (Gatesoupe et al. 1999; Diggles et al. 2000; Jensen et al. 2003).

Next to rising ocean temperatures, marine organisms have to cope with ocean acidification. Adult fish species appear to be relatively tolerant to increasing CO₂ concentrations, due to active adjustments of their acid-base balance (Claiborne et al. 2002; Evans et al. 2005; Melzner et al. 2009). Compensation can be metabolically costly, reducing the amount of energy available for reproduction, development and survival (Portner et al. 2004; Kroeker et al. 2010). After a three-month acclimation period of marine three-spined sticklebacks to elevated CO₂ concentrations (~ 1000ppm), dams showed increased breeding activity, compared to dams acclimated to ambient CO₂ (chapter III), contrasting results obtained for other marine fish species (Inaba et al. 2003; Frommel et al. 2010; Frommel et al. 2012b; Sundin et al. 2013). This stimulating CO₂ effect was also reflected in increased survival and growth rates of offspring reared in elevated CO₂ treatment (chapter III). To a certain extent, environmental stressors can accelerated physiological processes and metabolism, leading to advantageous conditions (Costantini et al. 2010; Schreck 2010). Even if one study has revealed negative effects of ocean acidification on stickleback behaviour (Jutfelt et al. 2013), increases in ambient CO₂ concentrations in seawater seems to be rather beneficial than harmful for investigated stickleback traits (chapter III). The population, used in this study, is a mix of marine and anadromous sticklebacks. Anadromous species regularly experience lower pH values, probably explaining, why sticklebacks cope better with acidified seawater than other species lacking such habitat shifts.

In general, sticklebacks are highly stress tolerant species, coping well with fluctuations in water chemistry and temperature (Pottinger et al. 2002). When comparing the ecological effects of ocean warming and acidification on stickleback performance and survival (figure 1), this fish species copes better with acidified seawater than with thermal stress, due to active regulations of their acid-base balance and the anadromous heritage. The metabolism and physiology of

heterothermic organisms, such as fish, are fundamentally influenced by environmental temperatures (Alborali 2006).

As a first response to ocean warming, fish may migrate to suitable habitats (Bijma et al. 2013; Jones et al. 2013). But shifts in fish distribution and migration behaviour may also increase the risk of emerging parasites and diseases (Perry et al. 2005). During the two-year investigation of the parasite communities of marine fish species in the northern Wadden Sea, the newcomer *Labratrema minimus* could be detected (chapter I, table 2). This digenean is well known in Mediterranean, French Atlantic and British waters (Faliex and Morand 1994; Malek 2001) and was likely introduced in the Wadden Sea region during the last decades by changed range expansion of the final host, the sea bass *Dicentrarchus labrax*. Introduced species can influence native ecosystems immensely (Cohen and Carlton 1998), particular when native species are susceptible to introduced parasites (Lafferty and Kuris 1999).

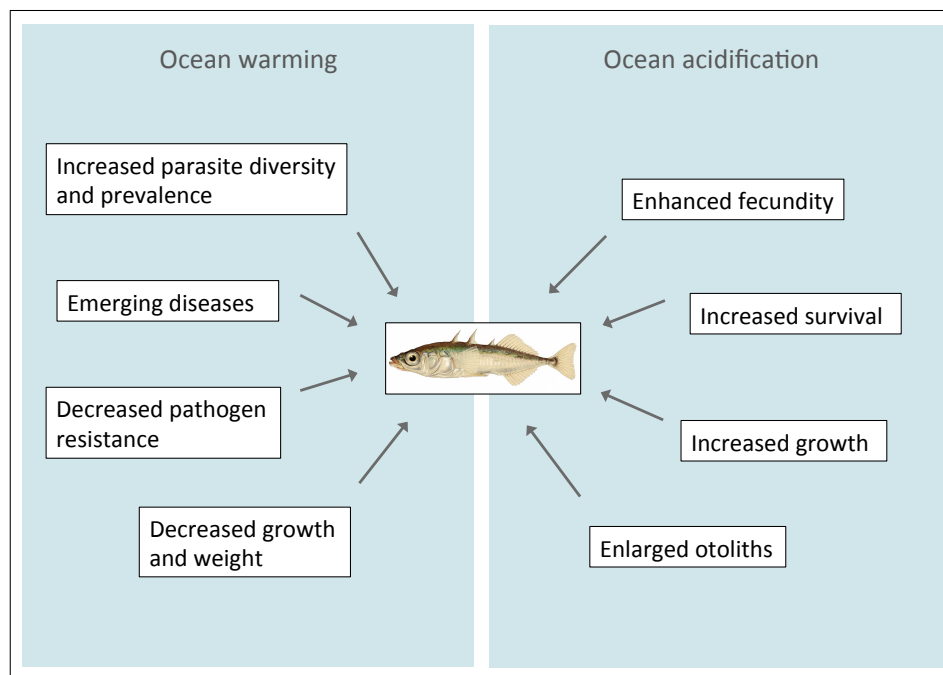


Figure 1 Overview of direct and indirect ecological effects of ocean warming and acidification on marine three-spined stickleback. Results were obtained from projects that are presented in the chapters I-III.

7 CONCLUSIONS AND PERSPECTIVES

However, when migration is impossible due to limited dispersal capacities or unavailable suitable habitats, fish have to adapt to changing environmental conditions (Bijma et al. 2013; Jones et al. 2013). Adaptation can work via evolutionary response or via phenotypic plasticity (Fuller et al. 2010). The basis for any evolutionary change is a substantial amount of standing genetic variation (May 1994). Unfavourable conditions can affect the genetic variation of a trait (Hoffmann and Merila 1999). Whether global change will increase or decrease genetic diversity for traits under selection will be one decisive factor for population viability (Pauls et al. 2013). By setting up a common garden breeding experiment using three populations of marine three-spined sticklebacks, it was possible to study the combined effects of thermal stress and bacterial infection on evolutionary trajectories (chapter II). Elevated temperature reduced genetic differentiation between populations, by releasing cryptic within-population genetic variation of selected traits. Additionally, some stickleback families (F1) in all populations showed improved performance under changed environmental conditions (GxE), reflecting evolutionary potential in both thermal environments. The negative genetic correlation in pathogen resistance between thermal environments, on the other hand, indicates that current genetic variation in resistance may be a weak predictor for future resistance evolution.

Next to genetic evolution, plasticity as well as transgenerational plasticity is a powerful mechanism to cope with global change, especially for long-lived species (Donelson et al. 2012; Munday et al. 2013). Parental acclimation has been shown to partly mediate detrimental effects of suboptimal temperature and ocean acidification in tropical fish species (Donelson et al. 2012; Miller et al. 2012; Salinas and Munch 2012). Transgenerational effects of ocean acidification in temperate fish species could recently be detected (chapter III). Stickleback offspring showed slightly increased growth rates and significantly enlarged otolith areas, when both parents were acclimated to high CO₂, suggesting that parental acclimation can modify ocean acidification effects.

To summarize, three-spined sticklebacks cope better with ocean acidification than with rising water temperatures. Transgenerational plasticity enables sticklebacks to respond quickly to environmental changes and provides time for genetic evolution,

as long-term adaptive mechanism, to catch up. The high tolerance to fluctuations in water chemistry and temperature as well as the substantial amount of standing genetic variation suggest that stickleback populations can adapt fast enough to changing environmental conditions.

However, in nature, environmental stressors do not act in isolation (O'Donnell et al. 2009). Temperature and CO₂ interact with each other and with other stressors, such as hypoxia, pollution or eutrophication (Portner 2010; Boyd 2011). Multiple stressor effects can be much greater than any individual influence (Hoffman et al. 2003; Wittmann and Pörtner 2013), highlighting the importance of multifactor experiments for future studies (Boyd 2011). Moreover, few studies have considered parasites and bacterial pathogens in the context of ocean acidification (Koprivnikar et al. 2010; Joint et al. 2011). How host-parasite-interactions will be affected, due to elevated CO₂ concentrations in ocean waters, are largely unexplored. Remaining questions need to be answered to get a comprehensive understanding about the consequences of future global change in marine environments.

8 Authors' contributions

Chapter I

Seasonal variation in parasite infection patterns of marine fish species from the northern Wadden Sea in relation of interannual temperature fluctuation

Authors: Franziska M Schade, Michael J Raupach, K Mathias Wegner

(in preparation)

Contributions: FSM wrote the manuscript, conducted the fish monitoring surveys, dissected the fish, performed the molecular analyses, was involved in designing the project and performing statistical analyses. MJR was involved in barcoding and writing the manuscript. KMW was involved in designing the project, performing statistical analyses and writing the manuscript.

Chapter II

Impact of thermal stress on evolutionary trajectories of pathogen resistance in three-spined stickleback (*Gasterosteus aculeatus*)

Authors: Franziska M Schade, Lisa NS Shama, K Mathias Wegner

(submitted to BMC Evolutionary Biology)

Contributions: FSM wrote the manuscript, conducted the fish breeding, performed the experiment, was involved in designing the project, genotyping and performing statistical analyses. LNSS was involved in designing the project, running pilot studies and writing the manuscript. KMW was involved in designing the project, genotyping, performing statistical analyses and writing the manuscript.

Chapter III

Within- and transgenerational effects of ocean acidification on life history of marine three-spined stickleback (*Gasterosteus aculeatus*)

Authors: Franziska M Schade, Catriona Clemmesen, K Mathias Wegner

(submitted to Marine Biology)

Contributions: FSM wrote the manuscript, conducted the fish breeding, performed the experiment, dissected the otoliths, performed statistical analyses and was involved in designing the project. CC and KMW were involved in designing the project and writing the manuscript.

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11 Curriculum Vitae

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05/2009 - 04/2010	Diploma thesis at University of Rostock, Germany (Supervisor: Prof. Dr. H. Schubert) and Institute of Fisheries Ecology, Cuxhaven, Germany (Supervisor: Dr. T. Lang) Thesis: Blood analysis of European flounder <i>Platichthys flesus</i> : Spatial patterns and possible environmental effects
10/2004 - 04/2010	Study of Biological Science at University of Rostock, Germany Main subjects: Marine Biology, Zoology, Animal Physiology, Medical Parasitology

Work Experience

- 05/2010 - 07/2010 Research Associate, University of Rostock, Dep. Aquatic Ecology (Project: A comparison of phototrophic bacteria in dimictic lakes)
- 08/2009 – 09/2009 Research Assistant on Cruise with RV “Walther Herwig III (Project: Biological effects of contaminants and fish diseases in the North Sea and Baltic Sea)
- 08/2008 - 10/2008 Research Assistant, University of Rostock, Dep. Animal Physiology (Project: Spatio-temporal control of Wnt/beta-catenin signaling during fate commitment of human neural progenitor cells)
- 07/2008 - 04/2009 Research Assistant, University of Rostock, Dep. Aquatic Ecology (Project: A comparison of phototrophic bacteria in dimictic lakes)

Poster Presentations

Schade FM, Shama LNS, Wegner KM (2012): The impact of climate change on pathogen resistance in three-spined sticklebacks. Workshop ‘Evolutionary potential in marine populations’, Sylt, Germany.

Schade FM, Lang T (2011): Blood analysis of European flounder - A suitable indicator of environmental pollution? ASLO Aquatic Sciences Meeting, San Juan, Puerto Rico.

12 Declaration

Hiermit versichere ich, dass die vorliegende Arbeit - abgesehen von der Beratung durch meinen Betreuer Dr. K. Mathias Wegner - nach Inhalt und Form meine eigene Arbeit ist.

Ich habe keine anderen als die angegebenen Hilfsmittel und Quellen verwendet und die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft erstellt.

Die Arbeit wurde bisher weder ganz noch zum Teil an anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegt.

Teile dieser Arbeit wurden als Manuskripte bei wissenschaftlichen Fachzeitschriften eingereicht: Kapitel II in BMC Evolutionary Biology mit Lisa N. S. Shama und K. Mathias Wegner als Koautoren, Kapitel III in Marine Biology mit Catriona Clemmesen und K. Mathias Wegner als Koautoren.

Kiel, den 31. Januar 2014

Franziska Maria Schade