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Biological invasions and host–parasite coevolution: different coevolutionary trajectories along separate parasite invasion fronts☆

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ABSTRACT

Host–parasite coevolution has rarely been observed in natural systems. Its study often relies on microparasitic infections introducing a potential bias in the estimation of the evolutionary change of host and parasite traits. Using biological invasions as a tool to study host–parasite coevolution in nature can overcome these biases. We demonstrate this with a cross-infection experiment in the invasive macroparasite *Mytilicola intestinalis* and its bivalve host, the blue mussel *Mytilus edulis*. The invasion history of the parasite is well known for the southeastern North Sea and is characterised by two separate invasion fronts that reached opposite ends of the Wadden Sea (i.e. Texel, The Netherlands and Sylt, Germany) in a similar time frame. The species' natural history thus makes this invasion an ideal natural experiment to study host–parasite coevolution in nature. We infected hosts from Texel, Sylt and Kiel (Baltic Sea, where the parasite is absent) with parasites from Texel and Sylt, to form sympatric, allopatric and naïve infestation combinations, respectively. We measured infection rate, host condition and parasite growth to show that sympatric host–parasite combinations diverged in terms of pre- and post-infection traits within <100 generations since their introduction. Texel parasites were more infective and more efficient at exploiting the host's resources. Hosts on Texel, on the other hand, evolved resistance to infection, whereas hosts on Sylt may have evolved tolerance. This illustrates that different coevolutionary trajectories can evolve along separate invasion fronts of the parasite, highlighting the use of biological invasions in studies of host–parasite coevolution in nature.

1. Introduction

Theoretical predictions of the dynamics underlying host–parasite coevolution (Van Valen, 1973; Hamilton et al., 1990; Sasaki, 2000) have been confirmed in study systems that employ experimental evolution (Schulte et al., 2010; Berenos et al., 2011; Gomez and Buckling, 2011; Joop and Vilcinskas, 2016). However, evidence from natural systems supporting these proof-of-principle observations is limited, although some natural systems (e.g., the water flea *Daphnia magna* and its castrating bacterial parasite *Pasteuria ramosa*) offer the opportunity to cross-infect different generations of hosts and parasites sampled directly from the environment, confirming that negative frequency-dependent selection can also act in natural populations (Decaestecker et al., 2007).

The above-mentioned studies use hosts with short generation times that are infected by microparasites. It is likely to observe coevolution in these systems, because often microparasites tend to be virulent, favouring host responses. Furthermore, short host generation times facilitate evolutionary responses. In contrast to host–microparasite interactions, host–macroparasite combinations have been investigated far less often and experimental studies are scarce (but see the study on *Potamopyrgus antipodarum* and its castrating trematode *Microphallus* sp.; Dybdahl and Lively, 1998; Koskella and Lively, 2007, 2009). More support for host–macroparasite interactions comes from local adaptation

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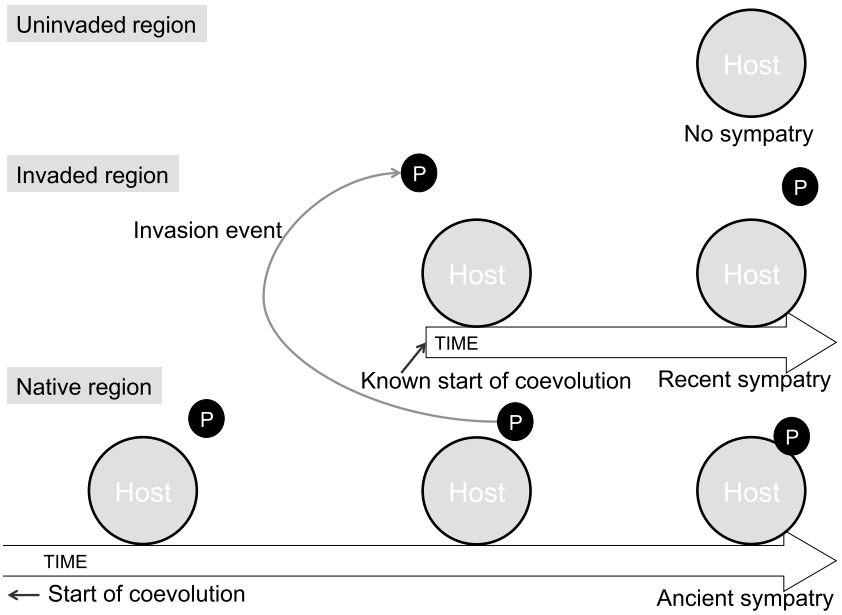

Fig. 1. Coevolution in biological parasite invasions. While in native regions host–parasite coevolution occurred over long and usually unknown time spans (ancient sympatry), invasions of parasites clearly define the onset of new coevolutionary interactions with new hosts (recent sympatry). In the uninvaded region there is no sympatry with naïve hosts. "P" symbolizes the parasite and the distance of the parasite to the host represents the coevolutionary relation between host and parasite. Reciprocal infection experiments with hosts and parasites of different levels of sympatry, covering a range from ancient sympatry over recent sympatry to no sympatry, can thus be used to estimate the rate of evolutionary change.

experiments (reviewed in Kaltz and Shykoff, 1998; Greischar and Koskella, 2007; Hoeksema and Forde, 2008) that represent proxies for coevolution, but often fail to capture the temporal dimension of coevolution.

It thus becomes clear that our empirical understanding of host–parasite coevolution is based on phylogenetic and experimental biases that fail to encompass the phylogenetic variety of host–parasite interactions, as well as the variety of their underlying dynamics that are present in nature.

Biological invasions can overcome several of these biases and therefore represent excellent opportunities to study the ecological and evolutionary effects of parasites and pathogens (Goedknegt et al., 2015), and thus, coevolutionary processes. Invasions are natural experiments with phylogenetically diverse combinations of hosts and parasites (Goedknegt et al., 2015). Additionally, the time frame of evolutionary changes is often known since it coincides with the time of invasion. It is therefore possible to estimate a rate of change by comparing native, invasive and naïve combinations of hosts and parasites after the invasion event (Fig. 1). In this way, biological invasions can add a time frame to local adaptation experiments. Finally, several invasion scenarios in which only the host or the parasite invade, or in which both host and parasite co-invade, lead to different predictions regarding the underlying evolutionary dynamics (reviewed in Goedknegt et al., 2015).

The invasive parasite *Mytilicola intestinalis* (Copepoda: Cyclopoida) offers a compelling natural history background to test several predictions of host–parasite coevolution in the wild. Originating from the Mediterranean Sea (Steuer, 1902, 1905) where it infests the Mediterranean mussel *Mytilius galloprovincialis*, the parasite invaded the North Sea and spread southwest and north in two fronts (see Fig. 2). The direct life cycle of this parasite limits coevolution to one principal host, the blue mussel *Mytilus edulis*, in its invaded range, and controlled infections (Hepper, 1953; Gee and Davey, 1986) can be applied to previously treated mussels (Blateau et al., 1992) to generate experimental combinations of host and parasite populations. Since *M. intestinalis* creates lesions in the epithelium of the intestinal walls of its host, especially at higher infection intensities (Couteaux-Bargeton, 1953; Watermann et al., 2008), and has been associated with mass mortalities (Korringa, 1950; Meyer and Mann, 1950; Blateau et al., 1992), selection for host resistance seems likely.

Pre- and post-infection traits of hosts and parasites can be separated within the mussel–*Mytilicola* system. The pre-infection traits are parasite infectivity, which is the ability to infect the host, and host resistance, i.e. the host's ability to prevent infections. Once infected, coevolution can occur for post-infection traits, i.e. host tolerance, which is the capability of the host to deal with infection, and parasite virulence, which is the harm inflicted on the host that should correlate with the ability to exploit the host. Both pre- and post-infection traits of hosts and parasites are tightly coupled and are therefore difficult to disentangle. While the proportion of successful infections resulting from exposure to a defined number of infective stages is a precise estimator of infectivity and resistance, tolerance and virulence can only be derived indirectly from host body condition in relation to parasite load. Nevertheless, pre- and post-infection traits can be separated in this system, offering the opportunity to investigate the evolutionary trajectories involving these traits.

Here, we describe these coevolutionary trajectories for the host *M. edulis* and the parasite *M. intestinalis* after its invasion in relation to naïve hosts lacking coevolutionary interactions. In particular, we answer how invasive parasites affect naïve hosts, and if similar host–parasite interaction patterns were found at the two different invasion fronts in the Wadden Sea.

2. Materials and methods

2.1. Field collection of mussels and treatment against previous infestations

Mussels in the size category of 3.5–5.0 cm shell length were collected from mixed mussel and oyster beds at the tidal flats of Vlakte

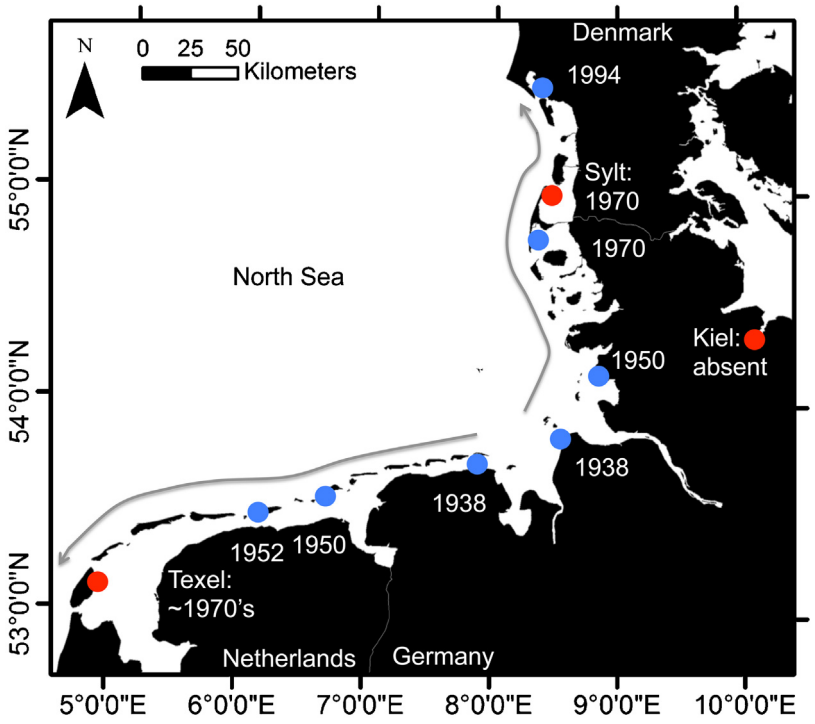

Fig. 2. Map of the invasion route of *Mytilicola intestinalis* in the Wadden Sea, a long stretch of intertidal areas and barrier islands in the southeastern North Sea. *M. intestinalis* was first found in 1938 near Wilhelmshaven and Cuxhaven (Caspers, 1939). The parasite spread north and southwest towards both ends of the Wadden Sea (Meyer and Mann, 1950) and reached Germany's most northern Wadden island, Sylt, by 1970 (Dethlefsen, 1972). Although *M. intestinalis* was still absent west of the Dutch Wadden island Ameland in the year 1968 (Korringa, 1968), it probably arrived on Texel during the 1970s.

van Kerken on the island of Texel, Netherlands (53°09′17.7″N, 04°53′36.1″E) and on the island of Sylt, Germany (55°02′17″N, 08°26′32″E) in June/July 2014 (Fig. 2). Naïve hosts were collected from a mussel bed in Kiel Harbour, Germany (Baltic Sea, 54°19′48.0″N, 10°09′00.0″E) and were adapted over a two-week period to the higher salinity of the North Sea. After transfer to the laboratory, mussels were kept in a climate-controlled room at 18 °C in an aquarium system with semi-continuous flow-through of fine-filtered seawater (30 μm) that was continuously re-filtered (50 μm) and UV-treated to prevent cross-infection throughout the experimental phase.

To eliminate previous infestations with *Mytilicola* spp. before the start of the experiment, all mussels were treated with Dichlorvos Pestanal (DDVP), following Blateau et al. (1992). Per bath, 100 mussels were put into 1 l of seawater with 30 mg DDVP for 4 h. After a recovery period of at least two weeks in aerated, filtered and UV radiation-treated seawater that was changed at least once per day, mussels were transferred again into a 1 l bath with 30 mg DDVP for 2 h. The experiment was initiated after a second recovery period of at least three weeks under the same protocol. At the start of the experiment, all mussels could retract their mantle fringes and close their shells completely, indicating that they had recovered from the DDVP treatment.

2.2. Experimental mussel infection

Egg sacks were obtained from individual gravid female *M. intestinalis* and were put into 24-well plates with ultra-filtrated seawater (0.45 μm). Eggs hatched within 1–12 days. Since the free-swimming larval stages are short-lived and only the first copepodid stage is infective to mussels (Hockley, 1951; Pesta, 1907), we infected individual mussels by pipetting 24 swimming copepodids into a 200 ml Kautex bottle containing a single mussel in ultra-filtrated seawater. Copepodids were taken from one mother or were a mix from two mothers. After 24 h the water was filtered through a 50 μm mesh sieve, which was checked under the stereomicroscope for remaining copepodids that were counted and removed. The bottle with the mussel was randomly placed into the aquarium system providing individual flow-through for each bottle. We infected mussels from Texel, Sylt and Kiel with parasites from Texel (each group, $n = 7$) and parasites from Sylt (each group, $n = 15$) and also included uninfected controls ($n = 15$).

Mussels were fed three times per week with 3.9×10^7 cells of *Isochrysis* 1800^{TM} (0.01 ml/mussel; CCMP 1324 T.ISO; Reed Mariculture, Campbell, CA, USA). During feeding, flow-through was interrupted for 3 h. Mortality was noted after each feeding event. Bottles were repositioned randomly twice per week.

After 80 days, mussels were dissected under a dissection microscope (6.3×). All parasites were removed from the gut and intestine. We measured host length to the nearest 0.01 mm with a digital calliper and flesh dry weight to the nearest 0.0001 g on a precision balance. Mussel flesh was separated from the shell and dried in an oven at 50 °C until constant weight. Parasites were sexed, counted and photographed under a dissection microscope for determining the length from head to tail (Leica QWIN imagining software; Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK).

2.3. Data handling and statistical analyses

All analyses were done in the R statistical environment version 0.99.893 (R Core Team, 2015). To optimise models, we used a stepwise selection procedure based on the Akaike information criterion (AIC). Results from the optimal models were not qualitatively different from those of the full models.

Host survival was tested with a binomial generalised linear model (GLM) estimating the effects of treatment (control or infected), host source and parasite source plus the interactions of treatment × host source and host source × parasite source.

Infection rate was used as a measure of infectivity of the parasite and resistance of the host. Infection rates were calculated by dividing the infection intensity by the exposure dose (number of parasites that infected the host, calculated as 24 minus the number of larvae that were still present in the water after 24 h, which were removed). We fitted optimal binomial GLMs testing successful against failed infections as a function of host and parasite sources plus the interaction term. We first calculated a model for all exposed mussels, and subsequently excluded Kiel hosts to separate recent sympatry combinations from naïve hosts.

Dry weight of the mussel flesh was used as a measure for the host condition, representing the relationship of host tolerance and parasite virulence. We defined parasite virulence as the reduction in host condition due to infection and host tolerance as the ability to deal with infection by parasites without suffering from condition loss, i.e. reduced slope of body condition as a function of infection intensity. Dry weight data were normally distributed after applying a log transformation (Shapiro–Wilk normality test, $p = 0.5573$). Because weight increases with body size, we corrected for host length by fitting it as the first term in the model. One mussel from Kiel infected with Sylt parasites was excluded from the dataset because the dry weight was more than 3 standard deviations away from the mean dry weight of that group (mean = 0.2237 g; sd = 0.2035; outlier = 0.9165 g), which was most likely due to a measurement error. Our sequential model fitting strategy for host condition was as follows. First, we tested for a treatment effect by optimising the full linear model using host dry weight (log-transformed) as a function of host length, host source, the treatment (control or infected) plus the interactions between all main effects. Second, to test for the effect of parasite source, we modelled log-transformed dry weight in a linear model as a function of host length, infection intensity, host source, parasite source and the interactions between infection intensity and host source, and host source and parasite source (terms in the full model). Finally, we fitted the same dry weight model with only Texel and Sylt hosts to separate recent sympatry combinations from naïve hosts.

We used mean parasite length and parasite development rate, expressed as the proportion of adults at time of dissection, as components of parasite fitness. Mean parasite length per host was taken as a measure for parasite growth and was normally distributed (Shapiro–Wilk normality test, $p = 0.508$). Mean parasite length was tested with a GLM as a function of infection intensity, sex ratio, host dry weight, parasite source and host source plus the two-way and three-way interaction terms between host dry weight, host source and parasite source (terms in the full model). We included host dry weight in the model to test if there is a dependency of the parasite growth rate on the availability of host resources. The slope of the parasite mean length by host dry weight relationship indicates exploitation efficiency. Parasite development rate was tested with a binomial GLM as a function of infection intensity, host source, parasite source and all interactions (full model). This test was also repeated for the dataset without naïve Kiel hosts.

3. Results

All mussels that were not exposed to parasites were free of infections at the end of the experiment, demonstrating that all found infections resulted from experimental exposure and not from any cross-infections between individual hosts or residual infections from the field. All parasites from the experimental infections were identified as *M. intestinalis* based on morphology (Steuer, 1905), showing that we successfully selected egg sacks from gravid *M. intestinalis* females only. Hosts in Kiel were indeed naïve, as no parasites were found in 190 mussels dissected before the experiment and as none were found in the baths after DDVP treatment. Sixteen mussels died throughout the experiment. Even though mortality was higher in the recent sympatry host sources (18.9% mortality in mussels from Texel and 16.2% in mussels from Sylt) than in the naïve host source (8.3% mortality in mussels from Kiel), we found no significant difference in host survival between host sources ($Deviance_{host\_source} = 1.97$, d.f. = 2, $p = 0.37$), between parasite sources ($Deviance_{parasite\_source} = 0.046$, d.f. = 1, $p = 0.83$), between treatments ($Deviance_{treatment} = 1.79$, d.f. = 1, $p = 0.18$) nor in the interaction between host and parasite sources ($Deviance_{host\_source \times parasite\_source} = 3.99$, d.f. = 2, $p = 0.14$).

3.1. Infectivity and resistance

Infection rates in relation to observed exposure dose ranged from 0 to 0.958 with a mean (±SE) of 0.526 ± 0.0265. In our experiment, parasites from Texel were on average more infective than parasites from Sylt (mean infection rate ± SE = 0.565 ± 0.0479 and 0.507 ± 0.0317, respectively, $Deviance_{parasite\_source} = 4.46$, d.f. = 1, $p = 0.035$; Fig. 3A) owing to the fact that the infection rate of Texel parasites was significantly higher in mussels from Sylt than from Texel and Kiel ($Deviance_{host\_source} = 7.77$, d.f. = 2, $p = 0.021$; $Deviance_{parasite\_source} = 4.46$, d.f. = 1, $p = 0.035$; $Deviance_{host\_source \times parasite\_source} = 15.40$, d.f. = 2, $p < 0.001$; total d.f. = 57; Fig. 3A). The infection rate of Sylt parasites did not show a significant difference between Texel and Sylt hosts (post-hoc $\chi^2_{d.f.=1,23} = 0.384$, $p = 0.54$) or between Sylt and Kiel hosts (post-hoc $\chi^2_{d.f.=1,25} = 2.973$, $p = 0.085$).

3.2. Virulence and tolerance

While hosts from different sources had significantly different dry weights, we could not find a significant difference in host condition between uninfected and infected hosts (treatment factor in Table 1A; Fig. 3B), indicating that infection did not lead to lower conditions in general. This effect could, however, mainly be attributed to the inclusion of naïve hosts into the analyses. Leaving naïve Kiel mussels out of the analyses, we observed that in sympatric combinations (Texel hosts with Texel parasites; Sylt hosts with Sylt parasites), condition was significantly lower than in the allopatric combinations (post-hoc tests, Texel: $\chi^2_{d.f.=1,11} = 0.474$, $p = 0.031$, Sylt: $\chi^2_{d.f.=1,22} = 6.872$, $p < 0.0001$) that did not differ significantly from the control animals (Table 1C and Fig. 3B). On Texel, sympatric parasites reduced the mean host dry weight by 28.21% and on Sylt, by 18.75%. Our model selection strategy only included length as a significant factor in the analyses with naïve hosts from Kiel. In the analyses only containing the reciprocal pairs of hosts and parasites (i.e. Texel and Sylt), host length was not significantly different. Infection intensity and the host × parasite interaction term explained a significantly larger proportion of the variation of host dry weight than in the model containing the naïve hosts. In naïve hosts, the parasite groups did not differ significantly (Table 1B and C). Although the slope of the sympatric combination on Texel was considerably steeper, we could not detect a significant three-way interaction term in the host dry weight models (Table 1B and C and Fig. 4).

Parasite development rate was negatively correlated with infection intensity (Fig. 5A). This effect was particularly strong for Sylt hosts infected with Texel parasites, reflecting the higher infection rate in the Sylt hosts by Texel parasites (Table 2A and Figs. 3 A

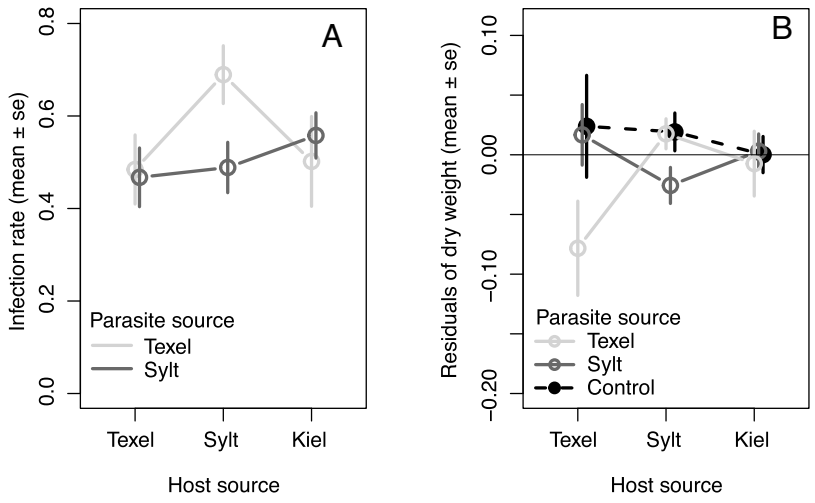

Fig. 3. Pre- and post-infection traits of host and parasite. (A) Mean parasite infection rate per host (±SE), representing host resistance vs. parasite infectivity. (B) Mean host dry weight (controlled for host length) as a measure of body condition (±SE), representing host tolerance vs. parasite virulence.

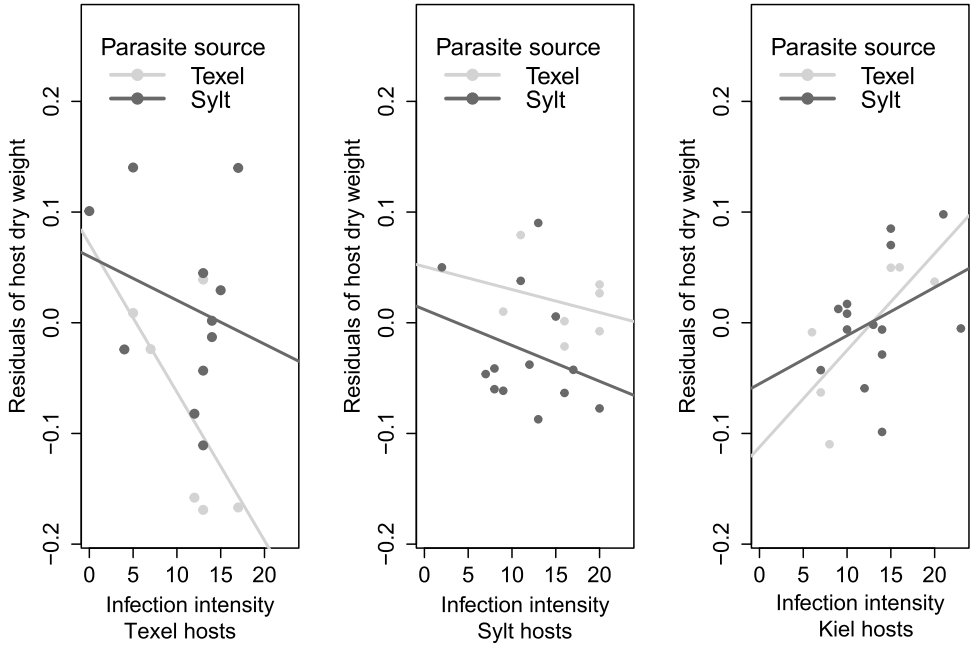

Fig. 4. Tolerance relationships between hosts and parasites, measured as host dry weight (corrected for host length) over infection intensity.

and 5 B). Host source was not a significant factor in the model when naïve hosts were excluded (Table 2B). Parasite length differed significantly between the sexes; females were on average 47.8% larger than male parasites (GLMM, d.f. = 2, F-value = 614.37, $p < 0.0001$). Mean parasite length per host varied depending on the sex ratio and on the interaction term between host dry weight, parasite source and host source in the model with all host sources (Table 2C), but varied depending on the sex ratio and the combination of host source × parasite source in the model without naïve hosts (Table 2D). Mean parasite length did not depend on the infection intensity (Table 2C and D). The slope of parasite mean length depending on host dry weight for the different host–parasite combinations represents the exploitation efficiency of the parasite (Fig. 5C). Parasites from both sources efficiently exploited naïve mussels from Kiel; however, in recent sympatry combinations, only Texel parasites were able to strongly exploit resources of Sylt hosts (Fig. 5C).

4. Discussion

Our results show that parasites from the Wadden Sea were able to exploit naïve hosts from Kiel but did not show any difference in pre-infection and post-infection traits. In recent sympatry combinations, however, coupled pre-infection traits (infectivity and resistance) and post-infection traits of parasites and hosts (virulence and tolerance) evolved differently along the separate invasion fronts of *M. intestinalis* in the Wadden Sea. Since it is hard to disentangle these coupled trait combinations, it is difficult to conclude whether hosts or parasites evolved at higher rates. Using a naïve host population can partly circumvent this problem, because a directed evolutionary response

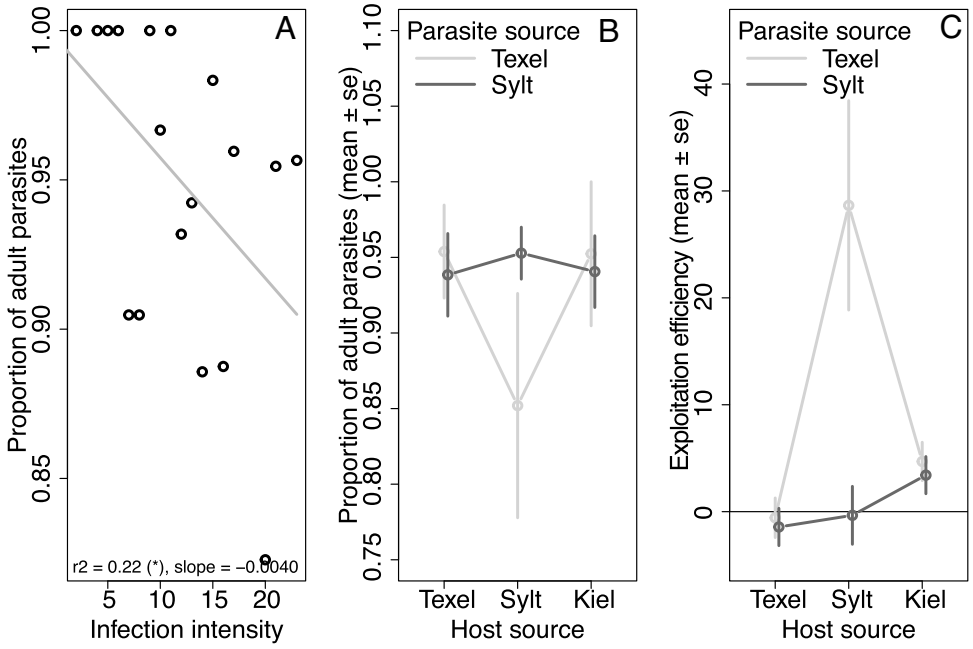

Fig. 5. Parasite development and exploitation efficiency. (A) Parasite development rate given as the proportion of adults as a function of infection intensity of all locations together. (B) Parasite development and (C) slope of the parasite mean length by host dry weight relationship, i.e. exploitation efficiency of the host's resources by the parasite. Positive values indicate a higher availability of resources that can be more efficiently exploited, while values around zero indicate that parasite mean length is independent of host dry weight and negative values would point out that more host resources are available for the host itself, for, e.g., immune response.

Table 1
Analysis of variance (ANOVA) of optimal models of the host condition (log-transformed host dry weight). Significant *p*-values are printed in bold.

	d.f.	sum of squares	mean square	*F*-value	*p*-value
(A) Infected vs. non-infected hosts					
Host length	1	1.3399	1.3399	9.7226	**0.0025**
Host source	2	13.2598	6.6299	48.1076	**<0.0001**
Treatment	1	0.1540	0.1540	1.1175	0.2934
Host length × treatment	2	0.5134	0.5134	3.7254	0.0568
Residuals	87	11.9898	0.1378		
(B) Infected hosts only					
Host length	1	0.8693	0.8693	7.1700	**0.0101**
Infection intensity	1	0.2036	0.2036	1.6792	0.2014
Host source	2	8.8141	4.4071	36.3476	**<0.0001**
Parasite source	1	0.0051	0.0051	0.0419	0.8388
Infection intensity × host source	2	0.7885	0.3943	3.2518	**0.0476**
Host source × parasite source	2	1.1566	0.5783	4.7696	**0.0130**
Residuals	47	5.6987	0.1212		
(C) Full reciprocal cross-infected combinations only (no naïve Kiel hosts)					
Host length	1	0.2095	0.2095	2.3673	0.1340
Infection intensity	1	0.8354	0.8354	9.4386	**0.0044**
Host source	1	6.8258	6.8258	77.1229	**<0.0001**
Parasite source	1	0.0662	0.0662	0.7482	0.3937
Host source × parasite source	1	1.2666	1.2666	14.3108	**<0.001**
Residuals	31	2.7437	0.0885		

of the host can be ruled out in the absence of the parasite.

4.1. Naïve vs. recent sympatry hosts

If mainly hosts evolved resistance or tolerance to the parasite infection we would expect that Kiel hosts, which do not share an evolutionary history with the parasite, would have higher infection rates and lower body conditions than coevolved hosts. However, Kiel hosts did not show a decrease in dry weight due to parasite infection, as was observed for sympatric infections in Texel and Sylt hosts, nor differences in susceptibility between parasite sources. As these parasites do not have any coevolutionary history with the Baltic hosts, they are not adapted to infect these hosts, and the host does not have a coevolved immune response that is specific to these parasites. Infection intensity and the host source × parasite source interaction term explained a larger proportion of the variation of host dry weight in the model without the naïve hosts, further supporting that coevolution could only be observed in the two localities where infections occur naturally, but not in the naïve population. Whereas the Sylt and Texel hosts seem to have a specific response to fight off sympatric infections, Kiel mussels did not show this response. Parasites from both sources were equally efficient in exploiting the naïve host, but their exploitation efficiency was reduced in sympatric combinations (Fig. 5C), further supporting the notion that coevolution is a local process.

The lack of specific patterns of all parasite traits in naïve hosts seems to suggest a lack of specific parasite adaptation. Mussel populations in the Baltic Sea are more distantly related to populations in the North Sea than populations within the North Sea are to each other, and the Baltic populations represent hybrids containing only 80% of the *M. edulis* genome (Stuckas et al., 2009). This strong differentiation might have masked any specific adaptation of the North Sea parasites. To test these assumptions, more populations from the hybrid zone gradient should be used in future experiments.

4.2. Sympatric vs. allopatric host–parasite combinations

Texel parasites and hosts seem to have evolved mainly along the infectivity and resistance trait space, because parasites from

Table 2
Analysis of deviance of the optimal models of parasite development (A and B) and parasite length (C and D). Significant *p*-values are printed in bold.

	d.f.	Deviance	Residual d.f.	Residual deviance	*p*-value
(A) Parasite development					
Null			54	104.410	
Infection intensity	1	7.1382	53	97.272	**0.0075**
Host source	2	7.1757	51	90.096	**0.0277**
Infection intensity × host source	2	15.6060	49	74.490	**<0.001**
(B) Parasite development without naïve Kiel mussels					
Null			34	72.191	
Infection intensity	1	22.18	33	50.011	**<0.0001**
(C) Parasite length					
Null			54	16.3575	
Sex ratio	1	6.2796	53	10.0780	**<0.0001**
Host dry weight	1	0.4704	52	9.6075	0.0841
Parasite source	1	0.0762	51	9.5313	0.4870
Host source	2	0.5358	49	8.9956	0.1829
Host dry weight × parasite source	1	0.0797	48	8.9159	0.4772
Host dry weight × host source	2	0.3625	46	8.5535	0.3168
Parasite source × host source	2	0.8874	44	7.6661	0.0600
Host dry weight × parasite source × host source	2	1.0436	42	6.6224	**0.0365**
(D) Parasite length without naïve Kiel mussels					
Null			34	11.5952	
Infection intensity	1	0.0316	33	11.5636	0.6691
Sex ratio	1	5.7242	32	5.8393	**<0.0001**
Host dry weight	1	0.0241	31	5.8153	0.7091
Parasite source	1	0.0612	30	5.7541	0.5521
Host source	1	0.0132	29	5.7409	0.7827
Host dry weight × parasite source	1	0.0013	28	5.7396	0.9301
Host dry weight × host source	1	0.1338	27	5.6058	0.3791
Parasite source × host source	1	0.9886	26	4.6172	**0.0168**
Host dry weight × parasite source × host source	1	0.2934	25	4.3238	0.1928

Texel had the highest infection rates when infecting Sylt hosts, whereas Sylt parasites did not show significant differences among host sources. Therefore, Texel parasites must have evolved a higher infection potential in comparison to Sylt parasites that, in turn, might have been selected for higher specific resistance in their sympatric hosts. Evolution of host resistance was also observed in a reciprocal infection experiment with Japanese and European eels and the invasive nematode *Anguillicola crassus*, where the sympatric combinations consistently had a lower infectivity than the allopatric combinations (Weclawski et al., 2013), indicating consistent resistance evolution of the host. However, *Mytilicola* infections had no consistent lower infectivity in sympatric combinations and this could be due to the invasion of the congener *M. orientalis*, which also infects mussels and is found on Texel but not in the northern part of Sylt (Elsner et al., 2010; pers. observation). Given that the host's resources are limited, co-infection by both parasites could lead to increased competition, which in turn may lead to selection for higher infectivity and more efficient exploitation by the parasite. However, *M. orientalis* only became common on Texel after the arrival of the invasive Pacific oyster on Texel (1983; Troost, 2010), making an evolutionary response to higher competition less likely.

Sympatric infections were more virulent than allopatric ones, or sympatric hosts less tolerant, and we only found indirect evidence for differential evolution of post-infection traits, i.e. tolerance and virulence (Table 1B and C and Fig. 4). In general, tolerance is more difficult to show experimentally (Råberg et al., 2009), since infectivity influences infection intensity, and thereby also our tolerance estimate. To properly investigate tolerance, a full range of infection intensities from all parasite sources is needed.

Indirectly, however, there are some indications for higher tolerance in Sylt hosts. Even though Sylt hosts had a higher infection rate by Texel parasites, and Texel parasites exploited Sylt hosts more than any other host–parasite combination, sympatric parasites caused an 18.75% reduction in dry weight in Sylt hosts. Texel hosts had equal infection rates for sympatric and allopatric parasites, and neither showed high exploitation efficiencies; nevertheless, the reduction of host dry weight in the sympatric infection combination was 28.21%, coupled with a steeper slope of the intensity vs. dry weight relationship (−0.013 on Texel vs. −0.0032 on Sylt). Therefore, indirectly, these results may indicate that Sylt hosts might be more tolerant to being parasitised by *M. intestinalis* than Texel hosts and that they evolved along the post-infection trait space. The specific patterns of pre- and post-infection traits in sympatric and allopatric combinations suggest that within the invasive population expansions towards Texel and Sylt, different evolutionary trajectories could be observed over approximately the same coevolutionary time frame (~45 years, corresponding to <100 generations). This means that coevolution does not necessarily have a fixed outcome, but can vary depending on the ecological conditions throughout the invasion of a parasite. In other systems, however, such as the rabbit–*Myxoma* system or the eel–nematode system, the same evolutionary outcome was repeatedly observed in several invasions (Kerr et al., 2015), or patterns were at least more uniform than for the *M. intestinalis* system (Weclawski et al., 2013). This could indicate that the ecological conditions in these systems were also more uniform and thus selected repeatedly for the same trait combinations, whereas selective forces of *M. intestinalis* invasions might differ more substantially.

The observed differences in evolutionary trajectories along the two invasion waves of the parasite could be connected to the ecological conditions encountered throughout the invasion process. Hydrodynamic regimes differ between the mixed oyster–mussel beds, as the Sylt site is located within a protected bay, while the site on Texel is more exposed. The hydrodynamic sheer could influence the number of *M. intestinalis* larvae (and parasites in general) that are retained within each host population. Prevalence data from both sites supports this idea (Goedknegt et al., unpublished data). Since transmission is often traded off against virulence (Frank, 1996; Alizon et al., 2009), selection on infectivity might be stronger for Texel parasites while the high number of infective stages on Sylt might select for tolerance in the host.

4.3. Disentangling host from parasite adaptation

In the present study there was only little evidence for local adaptation of the parasite to their sympatric host. The reduction of host body condition observed in sympatric host–parasite combinations could be interpreted as local adaptation of the parasite that evolved to exploit its local host. However, if the observed pattern was indeed due to increased host exploitation, this should be reflected in faster parasite development or a larger parasite mean length in sympatric combinations in comparison to allopatric combinations. We neither observed faster development in sympatric host–parasite combinations (Fig. 5B), nor a more efficient exploita-

tion of the host's resources in sympatric parasites (Fig. 5C), although competition among parasites occurred at higher infection intensities (Fig. 5A). Here, the negative correlation between parasite development rate and infection intensity (Fig. 5A) could indicate that resources within the hosts were limited, that there are host responses that are density-dependent, or that there is interference competition between parasites.

The reduction of host condition in sympatric combinations might reflect host adaptation and indicate that defence against parasite infection is costly. Increased specificity of immunological surveillance towards sympatric parasites might lead to better detection of the parasite and consequently divert more resources towards immunity or inflict more self-harm as a negative side effect of an increased immune response (Schmid-Hempel, 2009). To conclusively demonstrate the mechanistic basis of these effects in the interaction between *M. intestinalis* and its sympatric host combinations, future experiments should measure the immune response and potential self-harming immune substances (e.g., reactive oxygen species) directly or indirectly by investigating the underlying molecular mechanisms (e.g., transcriptional responses of both partners; Greenwood et al., 2016).

5. Conclusion

Non-native parasites are ideal candidates to reduce the existing biases in studies investigating host–parasite coevolution. Regarding a bias towards microparasites, our study now highlights the suitability of invasive macroparasites for investigating coevolution in nature. The possibilities to perform time-shift experiments on natural systems are restricted to few systems (Buckling and Rainey, 2002; Decaestecker et al., 2007; Schulte et al., 2010; Berenos et al., 2011), many of which rely on asexually reproducing hosts and parasites. However, as seen in our experiment, the effect of evolutionary time can be studied if invasive parasites are taken from different regions (space-for-time substitution) (Reusch, 2014), especially if these represent different fronts of biological invasions. The use of host–parasite pairs from the native range could then further extend the range of coevolutionary time from ancient to recent sympatry. In repeated invasion events or different invasion fronts one can further test whether host–parasite coevolution leads to predictable outcomes (Wendling and Wegner, 2015). Here, we can conclude that in the mussel–*Mytilicola* system independent invasions of parasites have led to different evolutionary trajectories on both the host and parasite side. Hosts on Texel seem to have evolved pre-infection traits, whereas hosts on Sylt may have evolved post-infection traits. This shows that the evolutionary outcome of host–parasite interactions may not always be predictable, even within the same host–parasite system. The outcome of coevolution therefore also depends on the ecological conditions in terms of the abiotic and biotic environment, which should be included into future studies of host–parasite coevolution to gain a better understanding of coevolution in nature.

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