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Temporal and spatial infection patterns of the rhizocephalan parasite *Parasacculina leptodiae* (Guérin-Ganivet, 1911) in the crab *Leptodius exaratus* along the shores of Kuwait

Zainab Al-Wazzan[1,2] · **Andreas M. Waser**[3] · **Henrik Glenner**[4,5] · **Luis Gimenez**[2,6] · **David W. Thieltges**[7]

Abstract

Rhizocephalan cirripedes are a very unique group of parasites infecting decapod crustaceans, but apart from a few well-studied species, little is known on their ecology and impact on hosts. Here we report on the results of a 14-month study of infestations of the rhizocephalan *Parasacculina leptodiae* in the rocky shore crab *Leptodius exaratus* along the shores of Kuwait in the Persian Gulf (Arabian Gulf). Monthly samples along an intertidal gradient revealed a slightly higher prevalence of *P. leptodiae* in female (18%) compared to male crabs (11%) and marked differences in prevalence among the sampling sites. Crabs from more sheltered locations in Kuwait Bay showed lower prevalence of *P. leptodiae* compared to crabs from more exposed sites. Seasonal patterns were largely absent, but prevalence in female crabs showed some monthly variation depending on the site. Rhizocephalan prevalence was generally highest in both crab sexes at the lower shores. This possibly resulted from lower exposure of crabs to infective stages in the higher intertidal and movements of infected crabs to lower parts of the shore. Prevalence of ovigerous females significantly declined with increasing local parasite prevalence. This suggests that the well-known castrating effects of rhizocephalans on individual hosts can also affect local crab reproduction at the population level which has not been shown before. Our results indicate that the rhizocephalan *P. leptodiae* is a common parasite of the rocky shore crab *L. exaratus* along the shores of Kuwait, with potential effects on the crab's population dynamics which warrants further study.

Keywords Brachyura · Distribution · Host · Parasitic barnacle · Persian Gulf (Arabian Gulf)

Introduction

Among the more unusual groups of parasites in marine systems are the rhizocephalans. They belong to the Cirripedia, but their adult morphology does not resemble their acorn and gooseneck barnacle relatives, which are omnipresent on marine hard surfaces worldwide. Rhizocephalans infect decapods and other crustaceans and are recognizable by a conspicuous sack-like structure, the externa, underneath the pleon of their hosts (Fig. 1). The non-visible internal part of a rhizocephalan, the interna, is an extensive net-like structure branching throughout the host's body (Noever et al. 2016). Only the free-living larval stages, which are released

✉ Zainab Al-Wazzan
zoology13@gmail.com

1 Environment Public Authority, Kuwait, Kuwait

2 School of Ocean Sciences, Bangor University, Menai Bridge, Isle of Anglesey, UK

3 Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Wadden Sea Station Sylt, Hafenstraße 43, 25992 List, Sylt, Germany

4 Department of Biological Science, University of Bergen, Bergen, Norway

5 Centre of Macroecology, Evolution and Climate (CMEC), Globe Institute, University of Copenhagen, Copenhagen, Denmark

6 Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Marine Station of Helgoland, 27498 Helgoland, Germany

7 Department of Coastal Systems, NIOZ Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB Den Burg, the Netherlands

Fig. 1 A rocky shore crab *Leptodius exaratus* infected by the rhizo-cephalan parasite *Parasacculina leptodiae*, showing an externa (containing the parasite ovary) below the abdomen of the host

from the externa, resemble their barnacle relatives. In one group of rhizocephalans, the "akentrogonid-type", the cypris larva is the only free-swimming larval type and acts both as a spreading, searching, settling, and host injection stage. In another group, "the kentrogonid-type", which includes *P. leptodiae*, female larvae develop via a series of naupliar stages into a final settlement stage, the cypris. The female cypris larva settles on the integument of a suitable host and metamorphoses into a kentrogon, an infective stage that via a syringe-like devise, the stylet, penetrates the integument of the host and injects the parasitic material into its haemolymph. The injected parasitic material develops a root-like trophic organ, the interna, in the host's blood system. Later, a reproductive organ, the externa, emerges through the host's cuticle on the ventral side of the abdomen. Here, exposed to the ambient water, the virgin externa is fertilized by 1–2 male cypris larvae and can now start the reproductive cycle (Høeg et al. 2005). In kentrogonid-type rhizocephalans belonging to the genus *Parasacculina*, infection usually leads to single externa, although it is not known whether there may be more individually different internas that just do not develop an externa. Prevalence of infections in crustacean hosts can vary widely even over relatively small spatial scales (Werner 2001; Pernet et al. 2010), and abiotic drivers such as water depth and salinity have been discussed to underlie such spatial heterogeneities in infection levels (Reisser and Forward 1991; Walker and Lester 1998; Waser et al. 2016). Infection prevalence can also vary temporarily, and seasonality in water temperature or other factors may be the underlying cause (Alvarez et al. 2002; Sloan et al. 2010; Mouritsen et al. 2018).

Hosts, which are infected by rhizocephalans, usually undergo various morphological, physiological, and behavioural changes induced by the parasite (for review, see Høeg (1995); Høeg and Lützen (1995); Waiho et al. (2020,

2021)). Among the induced changes that have a particularly strong effect on host populations is functional host castration (Fazhan et al. 2020)). This effect does not only remove a part of the reproductive adult host population, but it may also affect mating success of uninfected conspecifics as male crabs have been observed to mate with sterilized infected conspecifics (Shields and Wood 1993). While general distribution patterns and effects on hosts are relatively well studied for a few rhizocephalan species, very little is known about the ecology of the majority of the approximately 250 rhizocephalan species (see reviews by Høeg (1995); Høeg and Lützen (1995); Høeg et al. (2005)).

In this study, we investigated infections of the rhizocephalan *Parasacculina leptodiae* in the rocky shore crab *Leptodius exaratus* along the shores of Kuwait in the Persian Gulf (also known as Arabian Gulf (Fig. 1). This rhizocephalan species has first been described on *L. exaratus* from the Gulf of Aden (Guérin-Ganivet 1911), and since then, the species has been recorded at other locations in the Pacific Ocean (Boschma 1948, 1955; Moazzam and Moazzam 2004). In the Persian Gulf, it has been recorded on *L. exaratus* in Kuwait (Al-Wazzan 2012) and at a few localities along the Iranian coast, although the species was not identified by the authors (Afkhami et al. 2016). Apart from these limited local observation records, not much is known about the ecology of the species. In this paper, a 14-month sampling along the shores of Kuwait (6 sites with 8 sampling stations along a tidal gradient at each site) was conducted (1) to determine spatial and temporal patterns of infection of *P. leptodiae* and (2) to identify its potential effects of infections on host populations. The results of the study will contribute with new knowledge about the biology of this unique group of parasites in marine systems.

Materials and methods

Field sampling

Monthly surveys were carried out at six sites dominated by rocky substrate along the shores of Kuwait between November 2013 and December 2014 (Fig. 2). Three of these sites were relatively sheltered shores located inside Kuwait Bay, and the other three were more exposed shores located along the open coast to the south of the Bay. At each site, three line transects were established with eight sampling stations in each transect covering the intertidal zone (stations 1 and 2, high intertidal; stations 3 to 5, mid intertidal; stations 6 to 8, low intertidal), resulting in a total of 24 sampling plots per location (see Al-Wazzan et al. (2020) for more details). At each sampling station, a 1 m² quadrat was used to collect crabs, and they were subsequently sexed and their carapace width (CW) measured to the nearest 0.1 mm using a dial
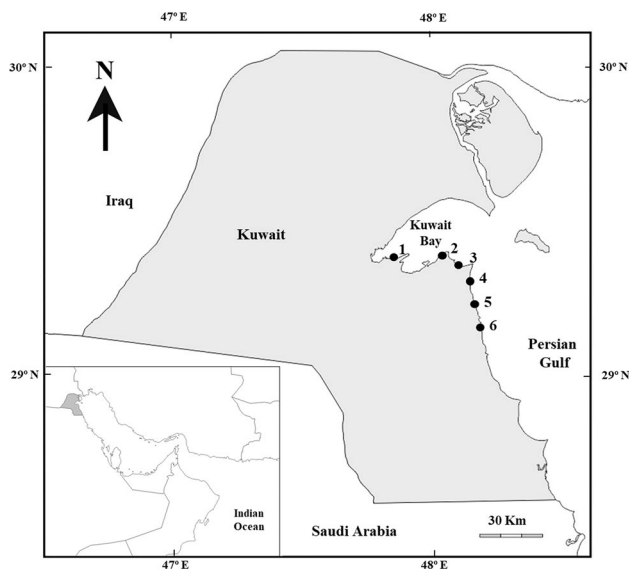
Fig. 2 Sampling sites (1–6) along the shore of Kuwait in the Persian Gulf

Vernier calliper. All crabs were checked for the presence of rhizocephalan parasites (clearly visible externa on the ventral abdomen, see Fig. 1), and all female crabs were also checked for the presence of eggs.

Species ID

A random sample of 15 infected crabs was collected and fixed in 70% ethanol for parasite identification. The morphology of the external part (the externa) of the parasites from Kuwait in the present study corresponds to the original description of *Sacculina leptodiae* by Guérin-Ganivet in 1911 from the Gulf of Aden and the extended description in Boschma (1955). In both cases, the rhizocephalans were found on the host *Leptodius exaratus* (WoRMS 2021). Based on this, the rhizocephalans of the present study are identified as *Parasacculina leptodiae* (Guérin-Ganivet, 1911). To further confirm the identification of the morphological species, a molecular identification was performed (details in the subsequent Molecular techniques sequence), which unequivocally showed that the DNA sequences from the present Kuwait study differ from the reference sequences of *P. leptodiae* deposited in GenBank.

Molecular techniques

DNA extraction and gene amplification

Genomic DNA was extracted from ~ 1 mm^3 of tissue from the mantle of individual externa of *Parasacculina leptodiae* and the distal (dactylus) segment of walking leg number 4

(left) using the QiagenDNeasy Blood & Tissue Kit following the QiagenDNeasy Protocol for Animal Tissues 07/2006.

Mitochondrial COI amplification was performed using standard DNA Barcoding protocols with Folmer primers (LCO1490 and HCO2198). The nuclear 18 s gene were amplified and sequenced using the primers published in (Korn et al. 2020). The concatenated sequences produced 702 nucleotides of the mitochondrial CO1 gene and 2136 of the ribosomal nuclear 18 s gene.

All PCR reactions were carried out using a Bio-Rad C1000 Thermal Cycler in 25 ml volumes containing 1 ml of DNA extract, 2.5 ml 10_PCR buffer, 1.2 ml of dNTP mixture (2.5 mM each), 1 ml of each 10 mM primer, and 0.75U of Takara polymerase. Conditions for all amplifications were as follows: initial denaturation at 94 °C for 5 min then 35 cycles of 30 s denaturation at 94 °C, 1 min primer annealing at 52 °C, and 1 min extension at 72 °C, with a final 7 min 72 °C extension. All PCR products were visualized on 1% agarose gels and stored at 4 °C prior to purification and sequencing. PCR products were cleaned by the addition of 0.1 ml (1U) exonuclease I, 1 ml (1U) of shrimp alkaline phosphatase, and 0.9 ml of ddH2O to 8 ul of PCR product. This was followed by incubation at 37 °C for 30 min and deactivation of the enzymes at 85 °C for 15 min. Sequence reactions were performed using the BigDye v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Norwalk, CT, USA) with the same primers used for initial PCR amplification. Both strands of all PCR products were sequenced using an ABI 3730 capillary sequencer.

Evolutionary analysis

P distances

Estimates of evolutionary divergence between CO1 sequences (SI 1): The number of base differences per site from between sequences is shown. This analysis involved 15 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 702 positions in the final dataset. P-distance analyses were conducted in MEGA X (Kumar et al. 2018).

Phylogenetic analysis using maximum likelihood

The evolutionary history was inferred by using the maximum likelihood method and Tamura-Nei model (Tamura and Nei 1993). The concatenated sequences of 702 nucleotides of the mitochondrial CO1 gene and 2136 of the ribosomal nuclear 18 s gene were used for the phylogenetic analysis. The tree with the highest log likelihood (− 18,022.09) is shown (SM 2). The percentage of trees in which the associated taxa

clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 23 nucleotide sequences. There were a total of 2838 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018; Stecher et al. 2020).

Field data analysis

As a first step, the total number of crabs caught during all sampling events and at all sites was used to derive size frequency distributions of infected and uninfected crabs (both for males and females separately) as well as that of ovigerous females. The minimum size of crabs showing a parasite externa and females bearing eggs was then used as a size cut-off for further analyses, enabling us to focus on crab sizes that are (a) potential hosts for rhizocephalan parasites and (b) equal to sizes of mature female crabs. Following this, the proportions of infected crabs (larger than the cut-off size) were calculated for each sex separately based on either the total number of males or females. Differences in prevalence between sexes were tested with likelihood-ratio tests (G-tests). A potential relationship between local prevalence of infection and crab density was investigated with Pearson correlations and using crab density data from Al-Wazzan et al. (2020). As there was no significant relationship, we excluded crab density from further analyses.

As prevalence generally differed between sexes (see "Results" section below), we separated females and males in further analyses. Statistical differences in prevalence of rhizocephalan infections in crabs among different sampling months as well as between different tidal heights were tested using two-factorial ANOVAs with logit transformed prevalence values. When testing for temporal patterns, we lumped data from all tidal height stations, and when testing for spatial patterns, we lumped seasonal data. For this lumping, we considered the three line transects at each site as replicates, resulting in 3 replicate values of prevalence per tidal height station and month per site. Sampling site served as a second (fixed) factor in both analyses, and we tested for factor effects as well as interactions. At some tidal height-site and month-site combinations, there were no crabs of a specific sex present, resulting in slightly unbalanced datasets. To cope with the unbalance, ANOVAs with Type III sums of squares were applied.

The relationship between the prevalence of ovigerous females and the prevalence of infected female *L. exaratus* was tested with a logistic regression (quasi-binomial error

fix, because of overdispersion). As ovigerous females were only observed in the summer period (Al-Wazzan 2017), we confined the data for this analysis to the period May 2014 to September 2014, when a relatively high portion of females was bearing eggs. All analyses were performed using the R platform.

Results

Species identification

The morphological study of the rhizocephalic parasites from Kuwait in the present study indicates that the species is *Parasacculina leptodiae*. DNA sequence data from the ribosomal core gene 18 s and the mitochondrial protein coding gene CO1 differ greatly from the *Parasacculina leptodiae* sequences deposited in the gene bank for the species (see SM 2). Genetic p-distance between the CO1 sequences of *Parasacculina leptodiae* ORIG from Kuwait and the other *Parasacculina* species in the analysis including *P. leptodiae* NEW sp. obtained from GenBank were extremely large (see SM 1). Since the GenBank data probably are derived from a parasite of the Southeast Asian crab (*Leptodius affinis*) and not from the type-host (*Leptodius exaratus*), from which *P. leptodiae* was originally described (Djibuti, Gulf of Aden), we assume that the sequence in GenBank belongs to another presumable undescribed *Parasacculina* species. A study of this relationship will be the subject of a future study. A phylogenetic maximum likelihood analysis of the new DNA sequences including 23 rhizocephalan species (SM 1. in the online resources) places the original *P. leptodiae* (ORIG) from Kuwait basally in a monophyletic *Parasacculina* clade, while the species from GenBank (*P. leptodiae* NEW SP.) is positioned distally in the clade.

Field results

Of the 4749 crabs investigated at the 6 sites between November 2013 and December 2014 along the shores of Kuwait, 664 crabs carried an externa of *Parasacculina leptodiae*. The size of infected crabs ranged from 9 to 32 and 10 to 28 mm CW in male and female crabs, respectively (Fig. 3a, b), and were similar in size to ovigerous females (10–30 mm CW; Fig. 3c). Two hundred fifty-five crabs were juveniles and smaller than 9 mm CW and were thus smaller than the minimum detection limit of 9 mm for parasites (i.e. crabs with an externa). These juveniles were excluded from further analysis, resulting in 4494 crabs that were considered for more detailed analysis. Out of these 4494 crabs, the prevalence of *P. leptodiae* was significantly different between sexes (G-test, $G = 48.96$, $p < 0.001$), with a prevalence in female crabs of 18%
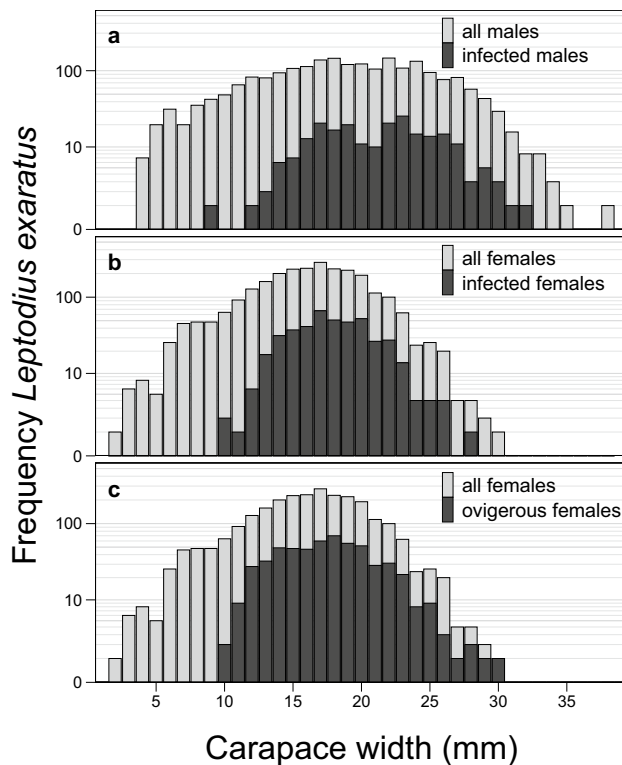
Fig. 3 Size frequency distributions of crabs *Leptodius exaratus* (on a log-scale) combined for all sampling sites and dates showing; **a** male crabs infected with *Parasacculina leptodiae* in comparison to all male crabs caught; **b** female crabs infected with *P. leptodiae* and the total of female crabs caught; **c** ovigerous female crabs compared to all female crabs caught

compared to a prevalence of 11% in male crabs. For more detailed general information on sex ratios and crab densities depending on site and season, see AL-Wazzan (2017) and AL-Wazzan et al. (2020).

The prevalences for both sexes were generally significantly different between sites (Tables 1 and 2; Figs. 4 and 5). While there was no temporal difference in parasite prevalence for male crabs, prevalence in female crabs varied significantly between months, depending on the site as indicated by a significant interaction term (Table 1; Fig. 4). At some sites, prevalence slightly increased over the sampling period (site 5), while at other it slightly decreased (sites 2 and 4). Concerning tidal height, there were significant differences in parasite prevalence for both male and female crabs, independent of the sampling site (Table 2; Fig. 5). Prevalence was generally lowest at the uppermost sampling locations (Fig. 5).

The prevalence of ovigerous females significantly decreased with an increase in the prevalence of infected female crabs ($R^2 = 0.26$, $p = 0.004$; Fig. 6).

Table 1 Results of two-factorial ANOVAs (Type III sums of squares) on the effect of month and site on prevalence (logit transformed) of *Parasacculina leptodiae* in adult female and male *Leptodius exaratus* between November 2013 and December 2014. Table is showing degrees of freedom (DF), sum of squares (SS), mean squares (MS), and *F* and *p* values

| Source of variation	DF	SS	MS	*F*	*p*
Female crabs					
Month	13	37.42	2.88	1.879	0.037
Site	5	215.06	43.01	28.075	<0.001
Month × Site	65	146.04	2.25	1.466	0.029
Residuals	153	234.41	1.53		
Male crabs					
Month	13	22.89	1.76	0.868	0.588
Site	5	162.44	32.4982	16.007	<0.001
Month × Site	65	95.83	1.47	0.726	0.928
Residuals	155	314.58	2.03		

Discussion

Our study is the first to verify the presence of the rhizocephalan *Parasacculina leptodiae* in the rocky shore crab *Leptodius exaratus* in the Persian Gulf. The species has probably also been recorded at a few localities along the Iranian coast, but the species was not identified by the authors (Afkhami et al. 2016).

Our 14-month study at 6 sites along the shores of Kuwait indicated that *P. leptodiae* is a common parasite of *L. exaratus*, with a slightly higher prevalence in females (18%) than in males (11%). A higher rhizocephalan prevalence in female compared to male crab hosts has also been observed in other rhizocephalan species, sometimes also with even more

Table 2 Results of two-factorial ANOVAs (Type III sums of squares) on the effect of tidal height and site on prevalence (logit transformed) of *Parasacculina leptodiae* in adult female and male *Leptodius exaratus* along different tidal height levels. Table is showing degree of freedom (DF), sum of squares (SS), mean squares (MS), and *F* and *p* values

Source of variation	DF	SS	MS	*F*	*p*
Female crabs					
Tidal height	7	24.96	3.57	4.425	<0.001
Site	5	105.57	21.11	26.198	<0.001
Tidal height × Site	35	39.28	1.12	1.392	0.109
Residuals	88	70.92	0.81		
Male crabs					
Tidal height	7	19.27	2.75	2.833	0.010
Site	5	88.01	17.60	18.112	<0.001
Tidal height × Site	35	36.48	1.04	1.072	0.385
Residuals	92	89.41	0.972		

Fig. 4 Monthly prevalence (± SE) of *Parasacculina leptodiae* in female and male *Leptodius exaratus* between November 2013 and December 2014 at 6 sampling sites along the shores of Kuwait. For a general overview, also the total monthly prevalence of the crab population along the Kuwait coast during the entire sampling period is shown
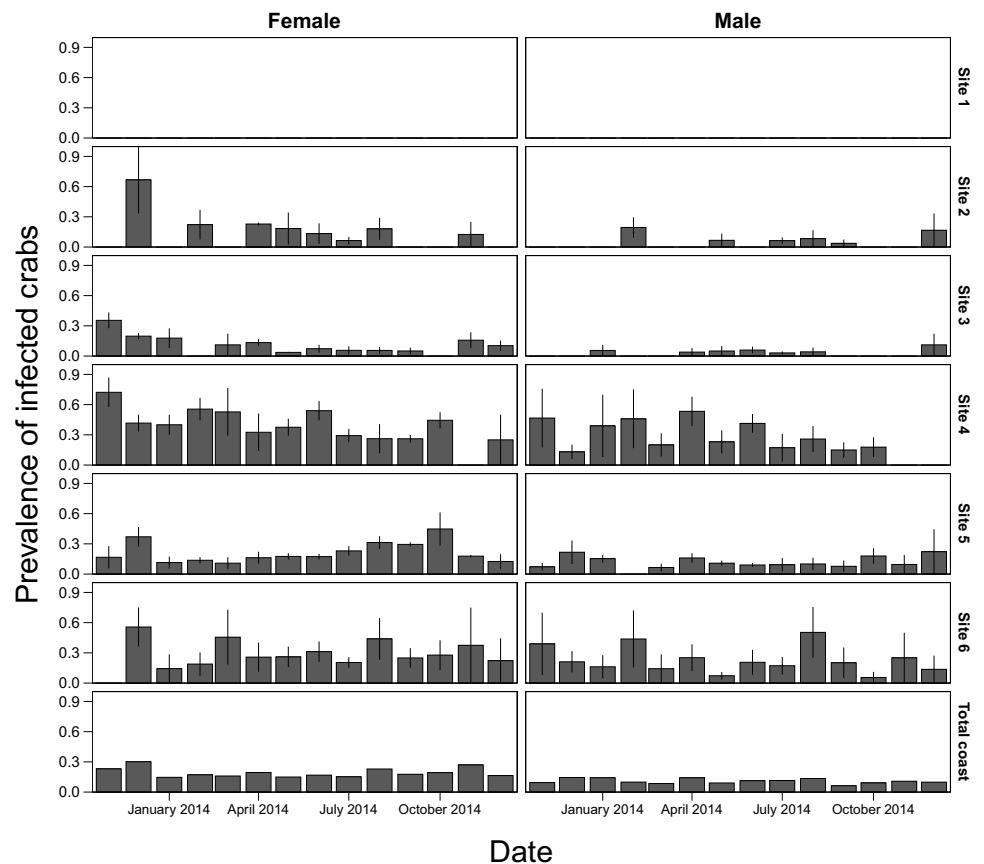
marked differences (Rasmussen 1973; Høeg and Lützen 1995; Mouritsen et al. 2018). It has been suggested that this difference in prevalence does not result from a preferred settlement of infective stages on female crabs but rather stems from a higher survival of parasites in females as their broader abdomen gives better protection for the parasite and its externa (Sloan 1984, 1985; Reisser and Forward 1991; Høeg and Lützen 1995; Kristensen et al. 2012). However, further studies are necessary to investigate whether this is also the case in *P. leptodiae*.

Differences in rhizocephalan prevalence between male and female hosts were also observed in our study in regard to season, with prevalence in female crabs showing significant differences among months, depending on the site, while there was no significant effect of season on prevalence in male crabs. We can only speculate about the underlying mechanisms, but potential differences between males and females in their moulting cycle may play a role. Host penetration might be easier on soft skinned host crabs, and it has been documented that cypris larvae of several rhizocephalan species prefer to settle on recently moulted crabs (Veillet 1947; Walker 1992; Glenner and Werner 1998; Tindle et al. 2004). Moulting in *L. exaratus* was reported all year round with higher growth rate during the warmer season (March and September) with males and females have

a similar seasonal growth pattern which are highly driven by seasonal environmental changes (Al-Wazzan 2017). Thus, rhizocephalan penetration into hosts could be expected to be higher during growth season. However, it is hard to predict the seasonal effect without understanding the time needed from infection to the emergence of the externa. For that, further investigations will be needed to understand the observed difference in seasonal effects between male and female crabs.

In regard to spatial variations in *P. leptodiae* prevalence, both sexes showed similar patterns. In general, there were significant differences in prevalence among the studied sites. Crabs from sites in the more sheltered part of Kuwait Bay (sites 1–3) showed lower infection levels than crabs from more exposed sites in the Persian Gulf (sites 4–6). Similar differences in prevalence among sites have also been observed in other rhizocephalan species such as in *Sacculina carcini* infecting shore crabs *Carcinus maenas* (Werner 2001; Waser et al. 2016; Mouritsen et al. 2018) and are considered to result from differences in abiotic conditions such as salinity, water depth, and exposure (Høeg and Lützen 1995; Werner 2001; Waser et al. 2016). Exposure may play a particularly important role in our system as lowest infections, or even a complete absence of *P. leptodiae* was observed at the more sheltered sites inside Kuwait

Fig. 5 Prevalence (± SE) of
Parasacculina leptodiae in
female and male *Leptodius
exaratus* at each of the 8
sampling stations along a tidal
gradient (stations 1 and 2, high
intertidal; stations 3 to 5, mid
intertidal; stations 6 to 8, low
intertidal) at 6 sampling sites
along the shores of Kuwait.
For a general overview, also
the total prevalence of the crab
population along the Kuwait
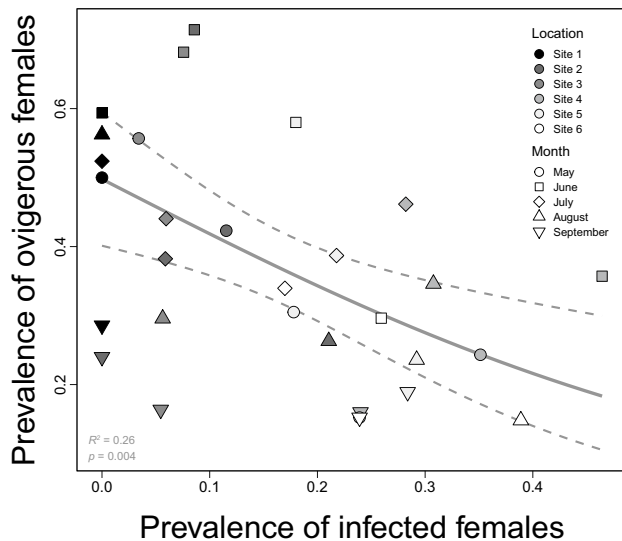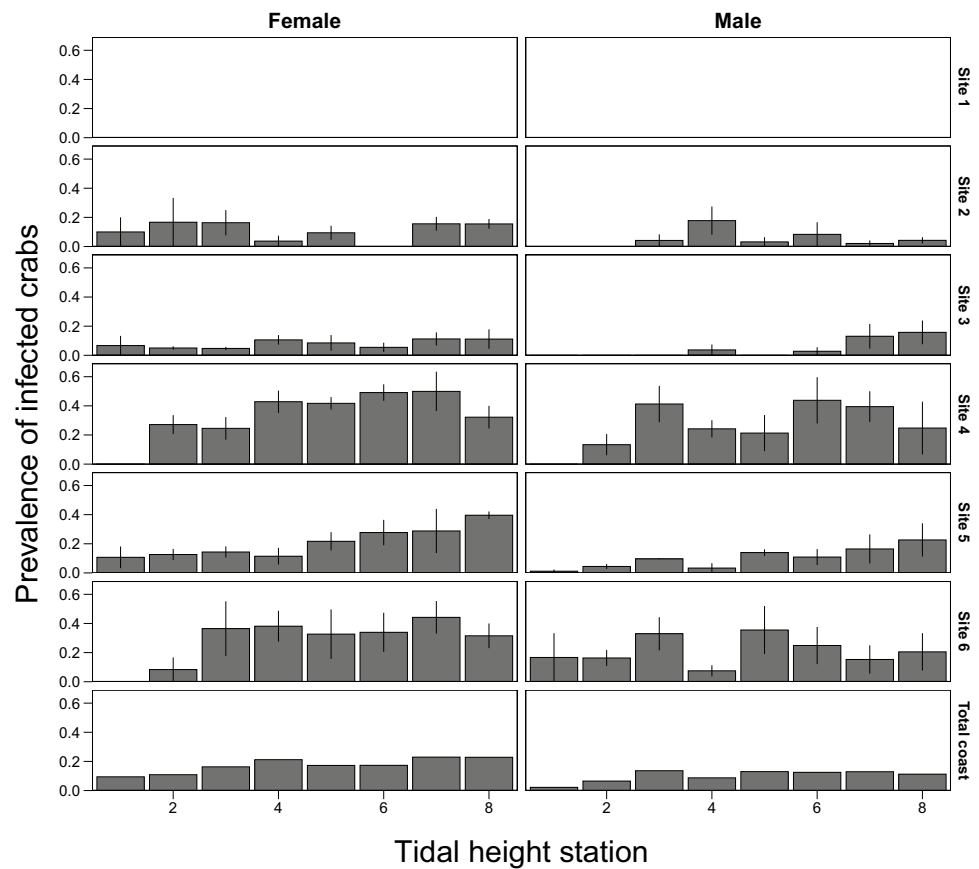coast during the entire sampling
period is shown

Fig. 6 Relationship between the prevalence of female *Leptodius
exaratus* infected with *Parasacculina leptodiae* and the prevalence of
ovigerous females at the 6 sampling sites during the period May 2014
to September 2014. The plot shows observed values (symbols) and
fitted values of the logistic regression model (solid line) with 95%
confidence intervals (dashed lines)

Bay. This pattern may point to differences in exposure, e.g.
infective stages of rhizocephalans could be more limited in
sheltered sites due to the counter clockwise current pattern
which is dominant in most of Kuwait's waters (Alosairi and
Pokavanich 2017). Another possible explanation could relate
to the high-water temperature and salinity in Kuwait Bay
waters (Al-Yamani et al. 2004), which could act as limiting
factors for *P. leptodiae* to flourish. However, it is also pos-
sible that exposure is similar at the different sites, but that
susceptibility of crabs differs due to environmental stress.
In the intertidal porcelain crab *Petrolisthes cabrilloi*, host
defences against infections include the removal of infective
parasite stages from the gills by grooming appendices on
the limbs. Crabs in high exposure environments show sig-
nificant higher damage of the grooming appendages (the
modified fifth pair of thoracic legs walking legs of porcelain
crabs), making them more susceptible to infections, while
conspecifics from sheltered environments have low infection
levels (Høeg et al. 2005). Similar mechanisms may explain
the lower prevalence of *P. leptodiae* at the sheltered sites in
our study.

However, a differential susceptibility of crabs depend-
ing on exposure cannot explain the lower infection levels
of crabs observed at the higher tidal locations in our sur-
vey, as crabs most likely will experience more wave action

at these locations. An alternative explanation for the lower prevalence at high tidal levels may be a lower exposure of crabs to infective stages due to shorter immersion times and thus shorter time windows for infection in the high intertidal. In addition, infected crabs may migrate down the shore to a more protected depth, as part of a parasite induced behavioural changes of their hosts, which has been observed in European shore crabs (*Carcinus maenas*) infected with *S. carcini* (Rasmussen 1959; Rainbow et al. 1979; Lützen 1984; Waser et al. 2016). Alternatively, crabs weakened by rhizocephalan parasites might avoid the competition with healthy conspecifics in unpredictable and energy demanding habitats as the high intertidal.

Besides affecting the behaviour of their hosts, rhizocephalans can also affect the physiology and reproduction of their hosts (Høeg 1995; Høeg and Lützen 1995). Infections with rhizocephalans usually lead to the castration of their hosts with severe fitness consequences for individual hosts (Høeg 1995). Such an infection-mediated reduction in individual fitness may also have carry-over effects on the crab population level, as high infection levels in a crab population may remove a significant number of crabs from the pool of reproducing individuals, thus leading to lower production of offspring. Our data suggest that this may indeed be a relevant scenario for infections with *P. leptodiae*, as the proportion of ovigerous female crabs declined with increasing infection levels. Whether this decline in ovigerous females has any consequences for local population dynamics will depend on the level of recruitment from local stocks. In any case, the observed relationship suggests that the effects of *P. leptodiae* infections may be significant on the host population level.

In conclusion, our results indicate that the rhizocephalan *P. leptodiae* is commonly parasitized by the rocky shore crab *L. exaratus* along the shores of Kuwait and that it may have the potential to affect the crab's population dynamics. Further studies will be helpful in verifying the underlying mechanisms and population-level consequences of infections.

Declarations

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