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Population dynamics and development of the rhizocephalan *Sacculina carcini*, parasitic on the shore crab *Carcinus maenas*

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ABSTRACT: The ecologically important shore crab *Carcinus maenas* is commonly infected in its native range by the rhizocephalan *Sacculina carcini*. However, several aspects of this host–parasite interaction are poorly understood. Here, we analyse data from approximately 60 000 Danish crabs to unravel factors governing infection patterns in time and space, and according to host sex and size. Female crabs were more frequently infected (12.6%) than males (7.9%). Sites with high salinity supported the highest infection prevalence. Infection prevalence peaked in summer (10 to 15%) and winter (20 to 35%) due in part to emergence of virginal externae in summer (main outbreak) and autumn (minor outbreak) preceded by peaks in crabs with lost externa (scars). Younger externae and scars dominated among males, whereas adult externae were most frequent among females. Infection prevalence increased with size in females but decreased in males, and modified (feminized) males showed lower scar frequency than unmodified ones. Modified males occurred frequently among the smaller size classes, whereas unmodified males dominated the larger size classes. Externa size was positively related to host size in both genders (same linear relationship). Molecular analyses suggested that hosts below 16 mm in carapace width do not become infected. Dissections of infected hosts revealed marked reduction of ovaries, whereas testes were unaffected by sacculinization. Our study demonstrates great spatio-temporal variation in infection prevalence mainly related to the parasite's life history. *S. carcini* appears capable of infecting all host sizes except the smallest. Owing to incomplete feminization of males, infections are rapidly lost from the larger and highly profitable male hosts.

KEY WORDS: Parasitism · Infection prevalence · Phenology · Externa development · Sex-biased infection · Feminization · Host-size selection · Spatial distribution

INTRODUCTION

The shore crab *Carcinus maenas* (Linnaeus, 1759) occupies a central position in coastal marine food webs throughout its distributional range. By virtue of its abundance, size and wide prey spectrum, the shore crab qualifies as one of the most important invertebrate top predators, as well as being itself a significant prey item for an array of vertebrate predators (e.g. Crothers 1968, Reise 1985, Grosholz et al. 2000). The key ecological role played by *C. maenas* in coastal ecosystems even extends beyond the immediate top-down control of prey populations (see e.g. Matheson et al. 2016).

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Shore crabs also serve as host to a range of parasites (Torchin et al. 2001), among which the rhizocephalan *Sacculina carcini* Thompson, 1836 is one of the more spectacular. As a parasitic castrator, *S. carcini* may regulate its host population by reducing recruitment as well as reducing the biomass, survival and growth of its host (Høeg 1995, Torchin et al. 2001, Goddard et al. 2005, Mouritsen & Jensen 2006, Lafferty & Kuris 2009, Larsen et al. 2013). In addition, *S. carcini* often affects the phenotype of its host by altering behaviour and morphology (Mouritsen & Jensen 2006, Kristensen et al. 2012). All these effects, combined with the often considerable prevalence of parasites in native host populations (e.g. Lafferty & Kuris 2009, present study), emphasize the role of *S. carcini* as an ecosystem engineer by proxy; i.e. acting by mediating the ecological influence of its host. Given this potential, surprisingly little is known about the ecology of the host–parasite interaction.

Knowledge of the life history of *S. carcini*, mainly from the memoir of Delage (1884), has been complemented by Høeg (1987) and Glenner (2001), enabling the complete life cycle to be pieced together. Other studies have focused on other aspects of its biology in Scotland (Foxon 1940, Heath 1971), Scandinavia (Lützen 1984, Werner 2001), and southwest Europe (Costa et al. 2013). *S. carcini* is a morphologically strongly modified crustacean parasitizing a range of portunid crab species. The main body of females has developed into an absorptive root system (interna) branching into most of the host's tissues, and a sac (externa) containing the gonads energing externally on the ventral surface of the host's abdomen. The life cycle comprises several nauplius stages and 1 cyprid stage, the latter of which metamorphoses into a kentrogon stage that inoculates a vermiform body (the vermigon) into the interior of the host crab. Once inside, the vermigon branches into a mycelium-like organism (interna), enveloping the internal host organs. Then a specialized region approaches the ventral abdominal integument of the host from the inside and breaks through to the exterior as a small bean-shaped body, the externa. The interna and the externa connect via a short stalk.

During the life of the externa, 5 developmental stages can be distinguished. The virginal externa is a hyaline body, usually 2 to 5 mm in width, which only develops further if fertilized by pelagic male cyprids. The fertilized ovaries grow in size and add a milky white or yellow hue to the externa, usually 5 to 12 mm wide (immature externa). In the next, mature, stage, the up to 22 mm wide externa starts to breed, depositing the fertilized ova in a mantle cavity where they develop into pelagic nauplii that are released. During further growth, the colour of the mature externa changes from yellowish-brown through brown to dark brown. The distinction between the mature and the equally sized old externae is subjective, determined by the thickened, wrinkled and grey-brown to blackish cuticle of the old externa. Old externae may still be sexually active although egg production has often ceased. Death of the externa leaves a round, blackish scar on the host's abdomen where the stalk was placed (scarred crabs).

Crabs with externae or scars are collectively denoted as externally sacculinized crabs, whereas hosts bearing solely an interna are denoted internally sacculinized crabs.

Here we analyse a large sample of *C. maenas* hosts collected over several years in the northern end of the parasite's distributional range (Denmark). Focusing mainly (but not exclusively) on the externally sacculinized host population (see above), we aim at providing a clear picture of the factors governing the spatio-temporal distribution of the parasites as well as those leading to sex- and size-biased infection patterns in the host population.

MATERIALS AND METHODS

Sampling sites

Over 6 yr (2009 to 2014), a total of approximately 60 000 shore crabs *Carcinus maenas* were collected at 3 well separated sites (18 to 20 km apart) in the western and central part of the Limfjord, Jutland, Denmark (Fig. 1). The Limfjord is a shallow fjord system, reaching a maximum depth of typically 6 m, that transverses the northern part of Jutland, connecting the North Sea and the Kattegat. The southwestern site, Venoe Bay (56° 34′ 28″ N, 8° 40′ 39″ E), is protected from strong currents and prevailing westerly winds and has a sandy bottom covered with eelgrass *Zostera marina* Linnaeus, 1753 (>50% coverage). The average salinity practical salinity units (PSU) at the bottom is 30. The northern site, Livoe Broad (56° 49′ 25″ N, 8° 51′ 41″ E), is situated in the central part of the Limfjord and is likewise well protected from currents and westerly winds. It has a mixed sand/stone bottom without eelgrass and an average PSU of 28. The southeastern site, Lovns Broad (56° 38′ 04″ N, 9° 13′ 03″ E), is characterized by lower salinity (24 PSU on average) and a bottom consisting of mixed sand and mud with eelgrass (at 1 to 3 m) and patches of blue mussel *Mytilus edulis* Linnaeus,

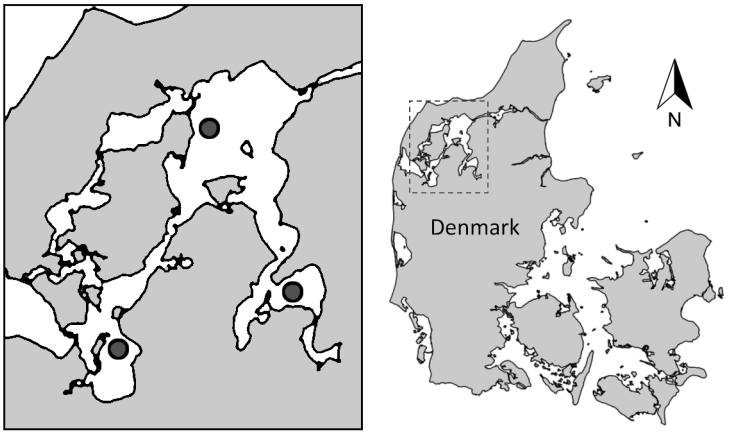

Fig. 1. Sampling sites in the Limfjord (grey dots with broken line): Livoe Broad (northern site), Venoe Bay (southwestern site) and Lovns Broad (southeastern site). See text for geographical positions

1758 banks at 6 m. At all sites, the water temperature varies between 1°C in February and March and 18°C in July and August, and the tidal amplitude is usually less than 0.5 m.

Field and laboratory procedures

Monthly samples were collected at each of the 3 study sites, except during winter (Dec–Feb) when crabs tend to be inactive and sampling was not always performed. Lovns Broad supported relatively few shore crabs and was only sampled during 2009 and 2010. Collection of crabs was performed using crayfish pots baited with cod. The pots measured 20 × 40 × 70 cm and were equipped with narrow gaps at both ends. At each sampling event, 5 pots were placed at each of the 3 depths (1, 3 and 6 m) at each site and emptied 2 d later. Sampling at different depths consecutively served the purpose of incorporating any depth dependent variation in crab size/gender and infection characteristics.

Collected crabs were kept wet in large buckets in a cold store room (4°C) at the Danish Shellfish Centre (DSC), Nykoebing Mors, until further processing within 2 d of capture. The gender of the crabs was determined according to abdominal shape and appendages (see Kristensen et al. 2012), and their carapace width (CW; distance between the 2 outermost anterior carapace spines) was measured to the nearest mm using a ruler. All crabs were inspected for signs of infection and, if externally parasitized by *Sacculina carcini*, categorized into 5 developmental stages: virginal externa, sexually immature externa, sexually mature externa, old externa and scar from lost externa (see 'Introduction'). The category of modified male crabs was also included. These were identified by their unusually broad abdomens (feminization; see Kristensen et al. 2012), and they often presented an externa or a scar as well.

Large catches were divided into 2 subsamples, one of which was treated according to the above general procedure, while only sex determination was carried out for the other. In the November 2011 sample, the CWs were not accurately measured but solely assigned to 5 mm intervals (lack of manpower).

Sacculinized male crabs collected from July through December 2011 were selected for a detailed study on the size–frequency distribution of morphologically modified males. During this period, the category of modified males was more rigorously defined as individuals having a relative abdominal width less than 2 (i.e. the ratio between the widths of the third and sixth abdominal segment; see Kristensen et al. 2012).

To investigate the effect of sacculinization on the fertility of the shore crab, the gonads were monitored in a limited number of infected (n = 31) and uninfected (n = 878) individuals collected during 2012 and 2014. Histological smears of testes and ovaries were inspected under a stereomicroscope and their developmental stage was recorded. Ovaries were assigned to 5 developmental stages: (1) extremely thin threads, invisible to the naked eye, (2) 1.0–1.5 mm wide translucent tubes, (3) 1.5–2.5 mm wide, creamy whitish to yellow and more or less straight tubes, (4) bulky rather than tubular, yellow to orange in colour, (5) voluminous, deep orange to red in colour. Testes were not categorized because no variation was evident among monitored individuals (16 uninfected and 8 infected males examined).

Molecular screening

Juvenile crabs are rarely reported externally infected, suggesting that they are not susceptible to *Sacculina* infection. However, absence of an externa does not exclude the presence of an interna and, in order to establish the frequency of infection in the smallest host size classes, molecular techniques were applied to stage 0–1 individuals (3 to 16 mm CW).

Juvenile crabs were sampled between December 2011 and April 2012 at Livoe Broad by means of a detritus sledge. Adult crabs with and without external *Sacculina* infection were also collected using

crayfish pots. Screening for *S. carcini* was performed using a diagnostic PCR-based approach, with *S. carcini*-specific primers used to target parasite DNA in combination with universal rRNA primers to act as an internal amplification control. Prior to genomic DNA extraction, hepatopancreata were removed from individual hosts and homogenized using a micro-pestle. An aliquot of the resulting homogenate was used for DNA extraction using the HotSHOT method (Montero-Pau et al. 2008). After assessing various volumes of hepatopancreas homogenate in the HotSHOT DNA extraction procedure and testing for subsequent PCR amplification of samples from hosts of known infection status (i.e. with externa present), smaller volumes (2 or 5 µl) were found to amplify better than larger volumes (5 and 10 µl). HotSHOT extraction consisted of the addition of 2 to 5 µl of homogenate to 75 µl of alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA, pH 12) followed by heating at 95°C for 30 min in a thermocycler. After cooling to room temperature, 75 µl of neutralization buffer (40 mM Tris-HCl, pH 5) was added before immediate use in PCR or storage at 4°C.

PCR-based screening was performed on the extracted DNA utilizing previously developed *S. carcini*-specific control region primers (Rees & Glenner 2014). PCR reactions were carried out in 25 µl final volumes, initially containing only the *Sacculina*-specific primers. After 10 cycles, the thermocycler was paused and universal 16S primers (Palumbi 1996) were added to the reaction (0.4 µmol l^{-1}). This step was undertaken to prevent the 16S primers from preferentially amplifying and dominating the PCR reaction. Additionally, each reaction contained 1× PCR buffer, 1.2 µl 2 mmol l^{-1} dNTPs, 0.75 units of TaKaRa *Taq* DNA polymerase, 2 µl of homogenate as template and ddH_2O up to 23 µl. PCRs were performed on a Bio-Rad C1000 thermocycler with the following cycling profile: initial denaturation at 94°C for 5 min, then 40 cycles of 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 2 min, with the program being paused after 10 cycles for addition of the 16S primers, then continued for a further 30 cycles before a final 72°C extension for 7 min. Amplification products were visualized on 1.5% agarose gels to confirm fragment presence and size.

The screening approach using *Sacculina*-specific PCR primers allowed a successful assessment of infection status in individual *C. maenas*. The inclusion of primers to amplify 16S rRNA as an internal reaction control meant that we reduced the risk of false negatives in our PCR screening. If using *Sacculina*-specific PCR primers only, while a PCR band of the correct size would be indicative of infection, absence of a band may be due to either absence of *Sacculina* or a failed amplification. However, inclusion of an easily amplified 16S fragment alongside the *Sacculina*-specific control region gives a clearer picture of infection status. Samples amplifying 16S only can be scored as negative, while the presence of the control region and 16S (or even just the control region) can be scored as positive; samples with no bands should be excluded.

Data processing and statistics

All data were analysed in IBM SPSS Statistics 24. Only standard parametric and non-parametric tests were applied and the summary statistics from the specific tests are all presented in the 'Results'.

Normally, only a single *S. carcini* externa was present on each externally infected crab. However, occasionally 2 or more were present, and in these cases the involved crab was statistically treated as having a single infection. In these cases, only the most developed externa was included in analyses of size and developmental stage relationships. Mention of infection prevalence refers solely to externally infected hosts (scars included).

If not otherwise specified, all analyses involve crabs having a CW ≥ 25 mm, which excluded 54 individuals of the total catch. Although crabs were collected from 3 different sites, data from Lovns Broad were generally excluded from the analyses due to the absence of *S. carcini* at this site, whereas data from Venoe Bay and Livoe Broad were generally combined due to similar patterns in infection characteristics at these 2 sites. Also, data from the 3 sampled depths were combined prior to analysis. Moreover, although several years of sampling were carried out, analyses of phenology are compressed into a single year cycle and monthly values may thus involve data from several years (2 to 4 yr depending on month). Full temporal resolution of the phenology data is given in Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d131p199_supp.pdf.

The main investigation ran from June 2009 to May 2012, during which systematic sampling and monitoring were conducted. Afterwards, during autumn 2012 and through 2013, sampling was less thorough and not all previously addressed parameters were measured consistently. In addition to the temporally limited campaigns focusing on specific issues, this means that sample size and period vary between the analyses.

RESULTS

Overall patterns in infection prevalence

The proportion of externally sacculinized *Carcinus maenas* across the entire data set was 9.4% (CW ≥ 25 mm). Female crabs were more frequently infected (12.6%) than males (7.9%) (Table 1). For both sexes, infection prevalence differed significantly among sites, attaining the highest value at the westernmost Venoe Bay site, whereas external *Sacculina carcini* infections were entirely absent in Lovns Broad (Table 1).

The average CW for both infected females and infected males was similar across sites, whereas the mean size of uninfected crabs tended to vary (Table 1). The latter was driven mainly by the occurrence of somewhat larger crabs in Lovns Broad. In both Venoe Bay and Livoe Broad, sacculinized females were significantly larger than uninfected ones, whereas the opposite was the case for male crabs (Student's *t*-tests, $t \geq 2.064$, $p \leq 0.039$). Quantitatively, however, these differences between groups are very minor and their statistical significance rests largely on the large sample sizes.

Seasonal development in infection prevalence

The seasonal pattern in prevalence of externally infected crabs was similar in Venoe Bay and Livoe Broad during the study period, and the combined data for these sites demonstrate a significant temporal pattern for both crab genders (Fig. 2A; chi-square test, $\chi^2_{10} \geq 516.358$, $p < 0.0005$), with the occurrence of 2 peaks: one during summer (10 to 15%) and a larger one during winter (20 to 35%). Otherwise, the proportion of sacculinized crabs remained low at around 5 and 10% for male and female hosts, respectively.

Infection prevalence according to host size

The infection prevalence was analysed according to different host size groups (Table 2). Overall prevalence tended to decline with increasing CW in the host population, driven largely by a similar pattern in the quantitatively dominant male crabs. In females, on the other hand, infection prevalence generally increased with host size, resulting in a 2- to 3-fold higher infection prevalence in the largest size group than that seen in males (Table 2). Although Table 2 only includes crabs having a CW ≥ 25 mm, the smallest encountered externally infected female and male crabs measured 20 and 23 mm, respectively. The largest infected female and male crabs measured 63 and 76 mm, respectively.

Prevalence of externa stages

Combining data from Venoe Bay and Livoe Broad, the relative frequency of the different external stages showed great seasonal variation in both host genders (chi-square test, $\chi^2_{40} \geq 769.909$, $p < 0.0005$; Fig. 2B,C). The virginal externae peaked in both females and

Table 1. Sample sizes, prevalence of infection by *Sacculina carcini* and mean carapace width (CW) (±SD) of female and male shore crabs *Carcinus maenas* collected at Venoe Bay, Livoe Broad and Lovns Broad in the Limfjord 2009–2012. Individuals with a CW less than 25 mm are excluded and only externally infected individuals possessing externae or scars are included among infected hosts. The p-values are for comparisons between sites

Site	n			Females			Males		
	Females	Males	Total	Prevalence (%)	CW (mm) Infected	CW (mm) Uninfected	Prevalence (%)	CW (mm) Infected	CW (mm) Uninfected
Venoe Bay	7800	16843	24643	16.5[a]	40.9 (7.1)	39.3 (6.4)	9.4[a]	47.2 (10.8)	48.0 (10.6)
Livoe Broad	6963	15739	22702	10.6[a]	40.3 (6.8)	39.6 (6.2)	6.8[a]	47.2 (10.5)	47.8 (10.0)
Lovns Broad	1363	974	2337	0	–	44.6 (5.0)	0	–	55.7 (9.3)
Total	16126	33556	49682	12.6[a]			7.9[a]		
p				<0.0005[b]	0.082[c]	<0.0005[d]	<0.0005[b]	0.996[e]	<0.0005[f]

[a]Female and male infection prevalence differ significantly (Fisher's exact test, $p < 0.0005$)
[b]Chi-square tests across sites, $\chi^2_2 \geq 163.114$; all pair-wise post hoc tests: Fisher's exact test, $p < 0.0005$
[c]Student's *t*-test, $t_{2021} = 1.740$
[d]Kruskal-Wallis test across sites, $K_2 = 896.124$; all pair-wise post hoc tests: $p < 0.0005$
[e]Student's *t*-test, $t_{2647} = 0.005$
[f]Kruskal-Wallis test across sites, $K_2 = 503.471$, post hoc tests: Venoe and Livoe are similar ($p = 0.642$), Lovns differs from the others ($p < 0.0005$)

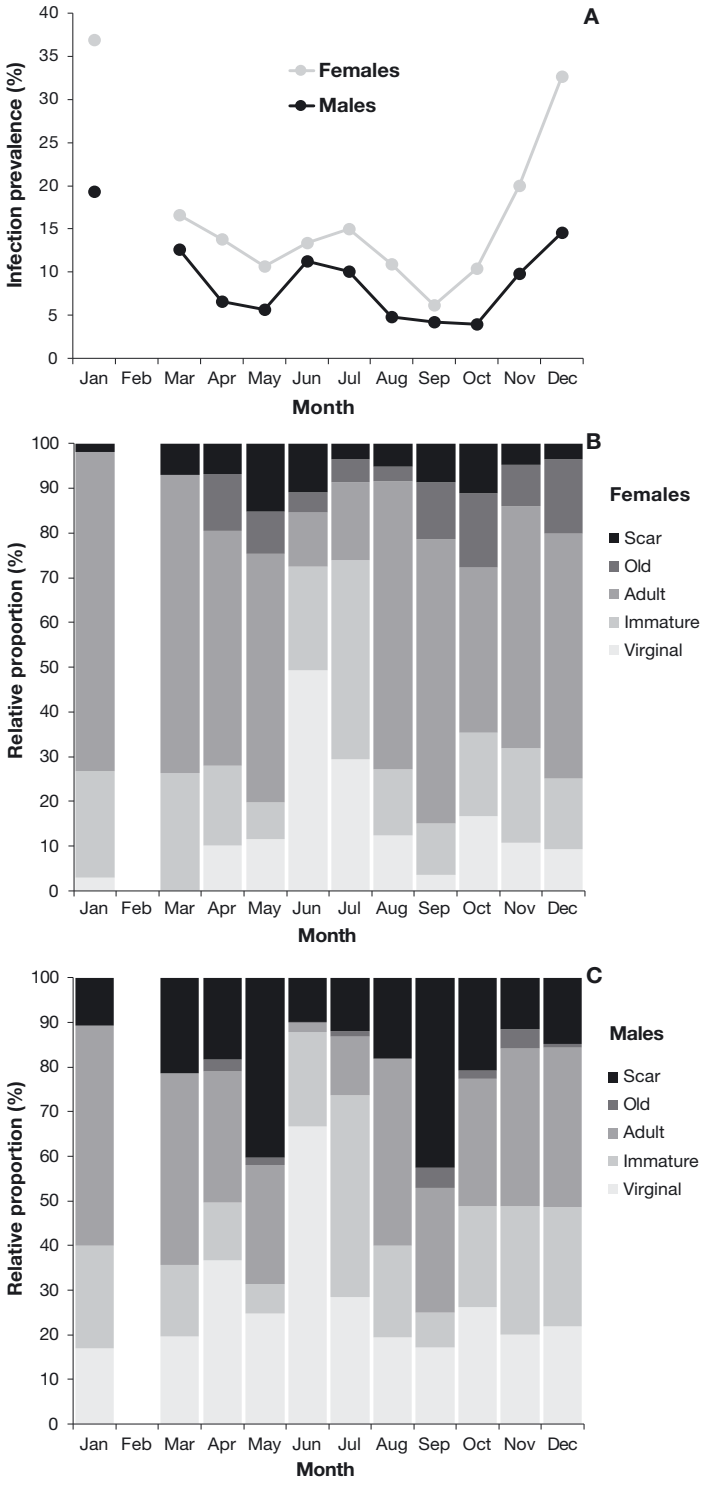

Fig. 2. Seasonal development of *Sacculina carcini* infections of female and male shore crabs *Carcinus maenas*. (A) Overall infection prevalence (%) by sampling month (n = 47345), (B) relative frequency (%) of externa developmental stages by sampling month in females (n = 2025) and (C) males (n = 2653). Data from Venoe Bay and Livoe Broad 2009–2012 combined (see Fig. S1 for data broken up into individual years). Note that no sampling was carried out in February during the 4 yr of investigation

Table 2. Infection prevalence (%; sample size in brackets) of *Sacculina carcini* distributed on size (carapace width) and gender of the shore crab *Carcinus maenas* in the Limfjord 2009–2012 (Venoe Bay and Livoe Broad combined)

Size class (mm)	Prevalence (%) Total	Females	Males	p[a]
25–35	11.7	12.8 (4160)	10.5 (4035)	0.001
36–40	10.1	10.8 (4631)	9.5 (4929)	0.035
41–45	9.5	14.7 (3270)	6.4 (5497)	<0.0005
46–50	9.9	18.4 (1899)	6.9 (5283)	<0.0005
>50	8.8	20.4 (802)	8.1 (12837)	<0.0005
p[b]	<0.0005	<0.0005	<0.0005	

[a]Fisher's exact test contrasting gender
[b]Chi-square test contrasting size groups, $\chi^2_4 \geq 48.517$

males in June with a smaller less well defined peak in October. On the other hand, old externae and scars occurred predominantly during May and September, preceding the outbreak of the new virginal infections in the host population.

Considering all seasons together, the relative proportion of the 5 externa stages differed significantly between sexes (Fig. 3; 2-way chi-square test, χ^2_4 = 454.516, p < 0.0005, n = 4675). Infected female crabs had relatively more adult and old externae than males, while males presented considerably more scars than females. Additionally, old externae were almost absent in male crabs. Virginal externae, in contrast, were 2-fold more frequent among infected

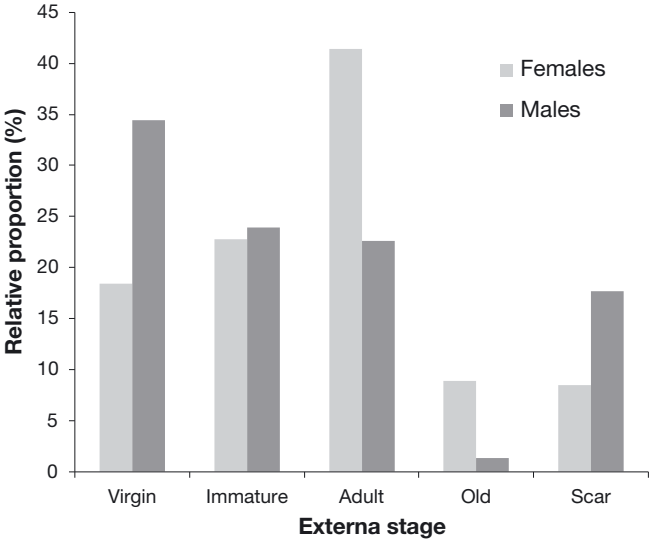

Fig. 3. Relative proportion of the different developmental stages of *Sacculina carcini* externa from female and male shore crabs *Carcinus maenas*. Data from Venoe Bay and Livoe Broad 2009–2012 combined (n = 4678)

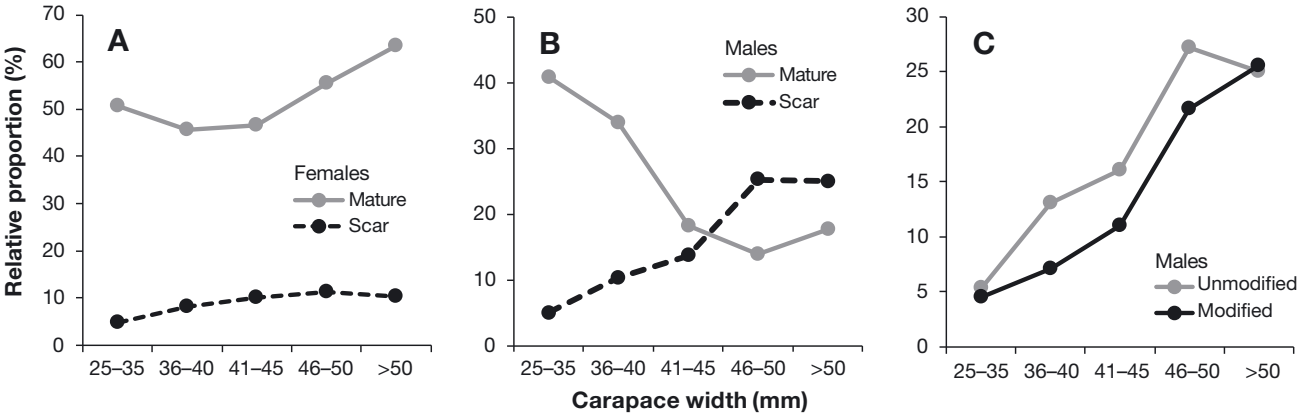

Fig. 4. Relative proportion (%) of different developmental stages of *Sacculina carcini* externae and scars as a function of host size classes (carapace width) of female (n = 2029) and male (n = 2649) shore crabs *Carcinus maenas* from Venoe Bay and Livoe Broad 2009–2012 (all crab sizes included). (A) Contrasting mature externae (adult and old) and scars on female crabs; (B) contrasting mature externae and scars on male crabs; (C) contrasting scar frequency of abdominally unmodified and modified male crabs. See 'Materials and methods' for definition of externa stages. All shown trends across size groups are statistically significant (chi-square tests, $\chi^2_4 \geq 14.775$, $p \leq 0.005$)

males than females. As opposed to female hosts, males therefore showed a more or less steady decline in the relative proportion of externae as the externae become older and larger. For all externa stages, the difference in relative proportion between genders was statistically significant (Fisher's exact test, $p \leq 0.008$). The much higher proportion of virginal externae among male than female crabs (Fig. 3) cannot be interpreted as evidence for a higher initial infection rate in males. The overall prevalence of virginal externae in the host population during the study period was 2.5 and 2.8% for female and male crabs, respectively. Despite the large sample size (n = 47345) this difference is statistically insignificant (Fisher's exact test, $p = 0.088$).

The relative proportion of sexually mature externae (adult and old) tended to increase with the size of the female host, while the proportion of scars by comparison was low and rather constant with only a weak tendency to rise with host size (Fig. 4A). In contrast, the proportion of sexually mature externae on male crabs decreased rapidly with increasing CW, particularly in intermediate size groups (Fig. 4B). Conversely, the proportion of scarred males increased with host size. This pattern is almost inversely proportional to the combined prevalence of the mature externae. Separating sacculinized males into abdominally modified and unmodified individuals, the latter had a consistently higher scar frequency than modified hosts for all size groups but the largest (Fig. 4C).

The relationship between morphologically modified males with or without externae and normal, unmodified males with externae was further explored according to their size (Fig. 5). Among externally infected males, modified individuals were particularly frequent in the smaller size classes and decreased rapidly in relative proportion with increasing size, whereas unmodified individuals showed the opposite pattern (2-way chi-square test, $\chi^2_7 = 164.779$, $p < 0.0005$). The group of modified males without an externa followed a pattern roughly similar to that of the modified males with an externa. However, the

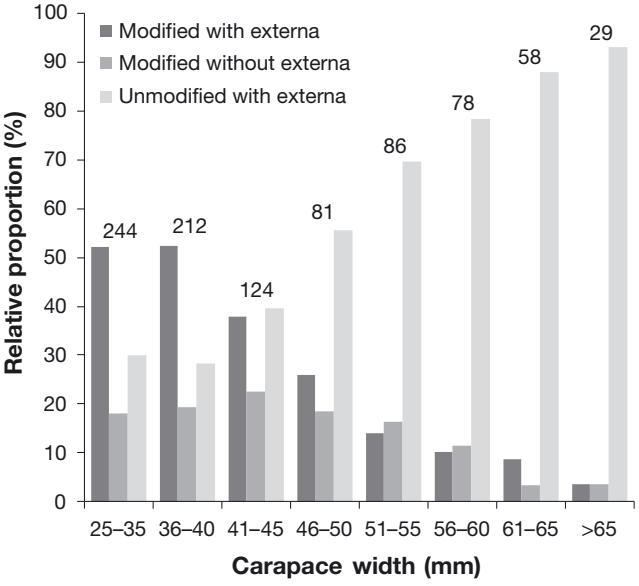

Fig. 5. Relative proportion (%) of male shore crabs *Carcinus maenas* abdominally modified and bearing a *Sacculina carcini* externa, abdominally modified without externa and normal, unmodified with externa according to size class (carapace width). Data from Venoe Bay and Livoe Broad combined, July to December 2011. Sample size for each size class is given above columns ($n_{tot} = 912$)

somewhat lower frequencies of the former group in the smallest size classes also made this contrast statistically significant (2-way chi-square test, χ^2_7 = 15.811, p = 0.027). The epicaridean *Portunion maenadis* (Giard, 1886) may also modify the abdomen of male crabs to some degree (Rasmussen 1973). However, this parasitic isopod occurs infrequently in the studied crab population (<6% prevalence; authors' unpubl. data), therefore modified males without externae likely represent mainly sacculinized hosts in which the externae have not yet erupted.

Externa size according to host gender and size

The size of mature (adult and old) *S. carcini* externae was a significantly positive function of host size for both crab sexes (Fig. 6, Table 3). Interestingly, the relationships were statistically similar in female and male crabs (no interaction and no main effect, Table 3), demonstrating that host gender has no impact on the development of the externa. Host size, on the other hand, appears to determine the final size that mature externae can achieve.

Molecular screening

Of 79 screened juvenile crabs with 3 to 6 mm CW, all PCR analyses came out negative. This was also the case for 49 juveniles with a CW between 7 and 10 mm and 37 juveniles with a CW between 11 and 16 mm. In contrast, adult crabs that otherwise showed no visible/morphological signs of infection (no interna or rootlets noted during dissection), the DNA extractions of the hepatopancreas yielded positive PCR results (*Sacculina*-specific PCR bands) in 12 out of the 37 individuals tested. This amounts to a hidden infection rate of over 30%. Overall, the PCR screening assays suggest that *Sacculina* generally does not infect crabs below approximately 20 mm CW. Our analyses also indicate that more adult crabs are infected than can be demonstrated morphologically, and hence, infection prevalence based on externa presence alone may seriously underestimate the true prevalence of *Sacculina* infection in the host population.

Table 3. Summary statistics of a full model ANCOVA including rank-transformed *Sacculina carcini* externa size (diameter) as dependent variable, crab (*Carcinus maenas*) size (carapace width) as covariate and crab gender as fixed factor. Preceding Levene's test for equality of error variance demonstrated no heterogeneity of error variance ($F_{1,1325}$ = 0.019, p = 0.498). See Fig. 6 for graphical presentation of the analysed data

Source	df	Mean square	*F*	p
Gender	1	30 460.8	0.287	0.593
Carapace width	1	5.177×10^7	487.006	<0.0005
Interaction	1	2064.771	0.019	0.889
Error	1323	1.063×10^5		
Total	1327			

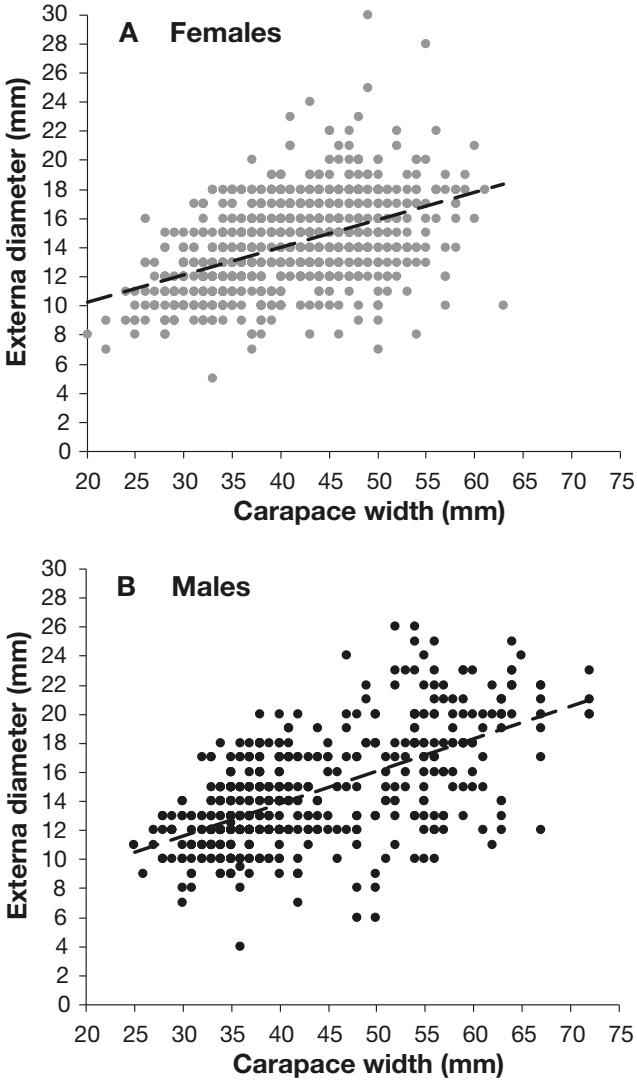

Fig. 6. Relationship between mature (adult and old) *Sacculina carcini* externa size (diameter, mm) and host size (carapace width) for (A) female and (B) male shore crabs *Carcinus maenas*. Data from Venoe Bay and Livoe Broad 2009–2012 combined and all crab sizes included. The linear regression (dashed line) is shown for females (y = 0.189x + 6.466; r^2 = 0.219, p < 0.0005, n = 486) and males (y = 0.224x + 4.898; r^2 = 0.373, p < 0.0005, n = 841)

Sacculinization and host fertility

Judging from histological smears of the testes, males had intact and normal sexual tissues whether or not they were parasitized. In contrast, females had ovaries that were generally smaller and less developed in sacculinized than in uninfected crabs (Table 4; 2-way chi-square test, $\chi^2_4 = 31.635$, $p < 0.0005$). No simultaneously egg-carrying and sacculinized females were encountered during the 6 yr study.

Table 4. Relative proportion (%) of sacculinized and uninfected female *Carcinus maenas* according to ovarian developmental stage (1–5) in Venoe Bay and Livoe Broad 2012–2014. The host size ranges (carapace width) are also given. Ovarian stages: (1) invisible to the naked eye; (2) 1.0–1.5 mm broad translucent tubes; (3) 1.5–2.5 mm broad yellowish tubes; (4) bulky and orange; (5) voluminous and reddish (see 'Materials and methods' for details)

Stage	Sacculinized crabs			Uninfected crabs		
	Size (mm)	%	n	Size (mm)	%	n
1	33–55	41.9	13	30–57	25.3	223
2	38–53	25.8	8	30–54	7.9	69
3	31–49	22.6	7	31–54	9.9	87
4	37–53	9.7	3	30–58	33.0	290
5	–	0	0	31–57	23.8	209

DISCUSSION

Spatial infection pattern

In agreement with previous reports from other crab populations (e.g. Robertson 1894, Veillet 1941), the present results show strong small-scale spatial variation in the prevalence of *Sacculina carcini* infection (hereafter *Sacculina*) in the sampled Limfjord shore crab population (Table 1). The infection prevalence decreased significantly from 9.4% in males and 16.5% in females at the westernmost site (Venoe) to nil at the southeastern site (Lovns), while the northern site (Livoe) attained intermediate values (6.8 and 10.6% for males and females, respectively). This spatial pattern reflects a similar trend in the salinity at the 3 study sites. There is a pronounced salinity gradient along the Limfjord with the highest values occurring in the western broads (30 PSU at Venoe) decreasing in an eastward direction (28 and 24 PSU at Livoe and Lovns, respectively), and hence, *Sacculina* may be sensitive to low salinities. This is supported by Mathieson et al. (1998), who studied the occurrence of *Sacculina* infections in shore crabs from the Forth Estuary, Scotland. In this study, the infection prevalence increased progressively seaward with 38 to 47% of *Carcinus maenas* parasitized at the mouth of the Forth (24 to 35 PSU) in contrast to only 6% further upstream (12 to 31 PSU). Similarly, Waser et al. (2016) found a tendency of increasing infection prevalence with salinity (0 to 3% at 18 to 29 PSU) in the Dutch Wadden Sea, while in the low salinity Danish fjord, Isefjord (18 to 20 PSU), *Sacculina* has been found only irregularly and in moderate prevalence when present (2 to 3% depending on host gender; Rasmussen 1973, Lützen 1984, J. Lützen unpubl. data). Thus, although scattered records are also available from a few brackish-water localities in the Inner Danish Seas bordering the Baltic (Lützen 1984), a salinity around 20 PSU appears to be the lower limit for maintaining a permanent population of *Sacculina*. The reason for this is unknown, but Ramult (1935) found that although able to survive at lower salinities, the eggs of *S. carcini* develop optimally at salinities from 26 to 41 PSU. We suspect that the larvae of *S. carcini*, in accordance with other sacculinid rhizocephalan species (Walker et al. 1992), are far more sensitive to low salinities than the adults, implying that it is the physiology of the larvae that is responsible for the observed salinity distribution.

Temporal infection pattern

For both host genders, there was a similar and distinct pattern in the seasonal development in infection prevalence: a summer peak (June and July) followed by a markedly higher winter peak (Fig. 2A). These peaks in *Sacculina* prevalence were preceded by outbreaks of virginal externae during spring/early summer (major outbreak) and autumn (minor outbreak) (Fig. 2B,C). Hence, the emergence of externae mainly took place in the first of these 2 seasons, as was also found by Lützen (1984) in the Isefjord, Denmark (but note that virginal infections may occur throughout the year). It requires 3 to 5 wk for a virginal externa to reach maturity (Lützen 1984, authors' unpubl. data) and the peaks in occurrence of immature and adult externae were delayed more or less accordingly, relative to the virginal outbreaks. In August and the following 9 or 10 mo, mature externae (adult and old) tended to dominate numerically but disappeared almost entirely in June during the major virginal outbreak. In addition, the relative proportion of scarred crabs that had lost the externae also peaked prior to the virginal outbreaks in sum-

mer and autumn, logically coinciding with the minima in overall infection prevalence (Fig. 2). Together this puts the longevity of an externa at a maximum of 12 mo, as also suggested by Mathieson et al. (1998). On average, however, the life span may be considerably less, particularly among *Sacculina* individuals infecting male hosts (see Lützen 1981 and below).

The considerable rise in the prevalence of *Sacculina* infections during winter (November to March, see Fig. 2A) is puzzling because relatively few outbreaks of virgin externae take place at that time of the year. Possibly, sacculinized crabs of both sexes migrate to off-shore waters during the winter period to a lesser extent than uninfected crabs (Broekhuysen 1937, Rasmussen 1973), in turn increasing the infection prevalence in our near-coastal catches. Total monthly catches of uninfected and infected crabs support this contention (see Fig. S2 in the Supplement).

The 2 outbreaks of virginal externae in male crabs are intimately connected with the regular moults performed by the male crabs during June and July and again in autumn (authors' unpubl. data). The relative proportion of scarred crabs found in the Limfjord is only partly in agreement with the limited data available from other studies. Figures presented by Werner (2001) show that scars occur without any regularity (May to October) in western Sweden, and Heath (1971) found scarred male crabs to peak in May to June in western Scotland.

Host gender and infection

Aside from the spatio-temporal patterns addressed above, the most striking feature of the studied host–parasite system is that *Sacculina* infections were strongly female biased: the proportion of female crabs found infected was consistently higher than that of males regardless of host size, location and season (Tables 1 & 2, Fig. 2A). This female bias is in general agreement with previous reports. Rasmussen (1973) found females to exhibit higher infection prevalence than males in shallow waters of the Isefjord (3.1 and 1.8%, respectively), and Costa et al. (2013) found a 3.3:2.2% predominance of parasitized females in the Mondega Estuary, Portugal (excluding scarred crabs). Likewise, Mathieson et al. (1998) found higher infection rates in females than males in the largest size classes of crabs from eastern Scotland. However, approximately similar levels of sacculinization among female and male crabs (3.0 and 2.9%, excluding scarred crabs) was observed in a population from western Sweden (Werner 2001).

Two not mutually exclusive processes may explain this pattern: (1) female crabs are more frequently infected by *Sacculina* than males crabs, and (2) males lose their infections at a higher rate than females. Our data do not support the former possibility, as the relative proportion of initial external infections (virginal externae) is similar in the 2 genders (2.8 and 2.5% of all males and females, respectively). This presumably means that sexes are equally exposed to being infected. Instead, our data suggests that males lose their external infections at a much higher rate than females, as evidenced by the rapid decline in the relative proportion of externae as they mature (Fig. 3). This results in fewer adults and scarcely any old externae in the male host population and, in turn, a high relative proportion of scars (Fig. 3). Female hosts, on the contrary, show a clear dominance of adult externae, indicating high survival of younger stages. This pattern of relatively more scars at the expense of adult externae in males than in females is clearly evident across all seasons (Fig. 2B,C). Because externa loss generally leads to the death of the parasite and often also the host (Veillet 1945, Lützen 1981), the higher rate of externa loss in male compared to female hosts immediately creates a female-biased population of infected hosts. This finding collaborates well with data extracted from previous studies of *C. maenas* as well as *C. aestuarii* Nardo, 1847 showing greater externa survival in female than in male hosts (Veillet 1945, Heath 1971, Mathieson et al. 1998, Werner 2001).

Why do male hosts lose their externae more often than females? Maybe male crabs are more effective than female crabs in launching the internal defence system, which can result in failed reproductive development and death of the parasite (Goddard et al. 2005). Budvytyté (2010) found significantly more defensive haemocytes in the blood of externally infected males than females. However, male and female hosts appear to support the growth of the externa equally well, because the development into mature externae across host sizes is similar between sexes (Fig. 6, Table 3). This would not be expected if the male defence system is markedly better than that of females at fighting the infection. Rather, the morphology of the hosts' abdomen that protects the externa against physical stress may be involved and Kristensen et al. (2012) found that sacculinization in *C. maenas* males causes a broadening of the abdomen (feminization) to a varying degree. This study further documents that the frequency of infected male crabs with scars decreases with degree of feminization, strongly indicating that the *Sacculina*-

induced broadening of the host abdomen increases the protection to the externa. In *C. maenas*, however, feminization of male crabs is incomplete, and even among the most modified individuals the abdomen never reaches the same width as in adult females. Externae of male crabs are, regardless of the degree of host modification, at a higher risk of being damaged and ultimately lost than those infecting females. Kristensen et al. (2012) found that this risk obviously increases as the externa grows larger during maturation. Interestingly, Kristensen et al. (2012) further showed that the rate by which the crab's abdomen enlarges as a function of CW is considerably greater in females than in males. Because the relationship between mature externa size and host size is similar in both sexes (Fig. 6), this means that the male abdomen, relative to the female abdomen, becomes increasingly poorer at protecting the externa as a function of host size. This contention is supported by the increasing proportion of mature externa with host size in females, while the proportion of scarred individuals remains low and rather constant (Fig. 4A). In males, on the other hand, the proportion of mature externae decreases rapidly with host size, while the proportion of scarred individuals increases correspondingly (Fig. 4B). A further indication that the host's abdominal size is important for sustained external *Sacculina* infection is that abdominally modified males generally show fewer scars than unmodified ones regardless of crab size (Fig. 4C).

Host size and infection

The smallest encountered externally sacculinized crab was a female with a CW of 20 mm, whereas the largest was male and measured 72 mm CW. In the smallest externally parasitized crabs, which are hardly 2 or 3 yr old, Delage (1884) assumed infection to have taken place when they were 5 to 12 mm large. Veillet (1941) has demonstrated that infection can occur even in newly settled *C. aestuarii*. In spite of this, our molecular screening showed that none of the 165 individuals between 3 and 16 mm CW that were analysed contained an internal stage of *Sacculina*.

On average, uninfected and externally sacculinized specimens barely differ in size (<4%), which is consistent across genders and sites (Table 1). This suggests that *S. carcini* does not infect any specific host size, in general agreement with studies by Werner (2001) and Waser et al. (2016). However, reports of both smaller and larger sizes of infected compared to uninfected hosts do exist (Veillet 1945, Mouritsen & Jensen 2006). Nevertheless, a closer look at the mean sizes of infected and uninfected crabs (Table 1) reveals a consistent pattern across sites: infected females are larger than uninfected ones and infected males are smaller than those that are uninfected. Although these differences are quantitatively very small, they are statistically significant. Breaking the prevalence data up into host size classes (Table 2) shows why: external *Sacculina* infections tend to increase in relative frequency with size of the female host, whereas the opposite appears to be the case in male hosts. Veillet (1945) and Werner (2001) observed similar patterns. This strongly indicates that the life span of an externa on a female crab is much longer than that on a male.

This sex bias is puzzling and may not relate to a single process. If *Sacculina* shows no or limited host size preference, the *a priori* expectation is that externally infected shore crabs are smaller than uninfected ones. This is because at least one moult inevitably separates an externally parasitized crab from the preceding state of solely internal infection, and these moults result in less host size increment (10 to 15%) than seen in uninfected specimens (20 to 30%) (Broekhuysen 1937, Veillet 1945, Berrill 1982, authors' unpubl. data). Additionally, it has been experimentally demonstrated that recently moulted crabs are significantly more vulnerable to infection than intermoult crabs (Glenner & Werner 1998), implying that smaller sized crabs with higher moulting frequencies will attain a higher prevalence of the parasite. A third explanation is that externally sacculinized shore crabs stop moulting (Høeg 1995, Høeg & Lützen 1995). Together with the rapidly increasing rate of externa loss with increasing host size in males (see 'Host gender and infection' above), this readily explains the overall pattern of decreasing infection prevalence in males with increasing size (Table 2). Although females are not subject to the same size-dependent externa loss as males, they are similarly influenced by the effect of changed moulting behaviour. Still, infection prevalence increases with size in females (Table 2). Two additional processes may combine to explain this. Firstly, *Sacculina* could in fact predominantly infect larger female hosts, as they constitute larger targets for the infective cyprid larvae (passive selection). Secondly, because small crabs are exposed to a high predation risk (Reise 1985, Thiel & Dernedde 1994, Moksnes et al. 1998), and because sacculinized crabs in particular may be exposed due to changed burying behaviour and overall reduced condition (Mouritsen & Jensen 2006), then small sacculinized crabs are likely

to be removed from the population more rapidly than larger ones. These 2 processes will also operate on males but may be entirely overridden by the substantial size-dependent externa loss. Hence, the size-dependent prevalence pattern seen in female hosts may more closely reflect the parasite's immediate host size selection, whereas the male pattern is the odd one, strongly modified by secondary processes.

Host fertility and infection

It is generally assumed that sacculinization restrains the development of the sexual organs in the crab hosts (Høeg 1995). Zetlmeisl et al. (2011) showed accordingly that sacculinized male shore crabs have smaller testes than uninfected specimens. Our study, however, shows that the testes of infected males appeared normal and healthy and the spermatophores equally abundant when compared to uninfected males. Whether they score a similar fertilization success or if sacculinized males engage in mating behaviour at all remains unknown. It is clear that infection retards ovarian development in comparison to that in uninfected females of similar size as the initial ovarian stages (1 to 3) are considerably more frequent in sacculinized than healthy females (Table 4). Stage 5, which includes sexually ripe females ready to spawn, does not occur at all among sacculinized females. This explains why parasitized females in berry were entirely absent in our collections, and why such individuals have never been recorded in other studies of *C. maenas* (but see Veillet 1945 for a possible exception in *C. aestuarii*).

Host modification and the sacculinid infection strategy

The positive relationship between mature externa size and host size (Fig. 6) suggests that the parasite extracts energy from the host proportional to the size of the latter. This can be viewed as a parasite strategy to avoid overexploitation of the host. It also suggests that *Sacculina* individuals infecting larger host individuals attain greater fitness, under the assumption that a larger externa results in a larger number or quality of larval offspring. The latter appears to be the case in other crustacean–rhizocephalan systems (Nagler et al. 2017). This should place male crabs, and particularly the very largest ones, in the position of being the preferred host individuals due to their superior size alone. This is evidently not the case as *Sacculina* infects a wide range of host sizes, and targets the 2 host genders in equivalent proportions as judged by the frequency of virginal externae. Possibly, a parasite cannot afford to reject a susceptible host when encountered unless hosts are superabundant. Moreover, infecting large males entails a great risk of being eliminated due to the less protective abdomen of males. Together, this emphasizes the selective forces behind the evolution of the significant parasite-induced modification (or feminization) of the male host that, among other changes, results in a broader and more protective abdomen (Kristensen et al. 2012). However, this modification is not complete in terms of feminization (Kristensen et al. 2012) and the parasites are further challenged by the fact that large and potentially profitable males are difficult to modify morphologically. Because several moults may pass before an internally infected male host reach maximum feminization (e.g. Veillet 1945) and because the frequency of moults decreases with size/age of the crab (Crothers 1968), modified infected males are mainly found among smaller individuals, whereas unmodified infected males predominate among the larger individuals (Fig. 5; see Day 1935, Foxon 1940, Werner 2001 for similar pattern). This further stresses that infection of large males is a high risk, high reward strategy, whereas small male hosts support a low risk, low reward strategy.

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