TRENDS IN AQUATIC ECOLOGY III

Are *Cardinium* infections causing asexuality in non-marine ostracods?

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Abstract Endosymbiotic bacteria manipulating host biology and reproduction, and sometimes also causing parthenogenesis, are known from many metazoan taxa. Three recent studies have reported *Cardinium* endosymbionts in non-marine ostracods with different reproductive modes. Here, we test with all available data which (a)biotic factors could possibly shape infection patterns in these crustaceans. The presence of *Cardinium* in non-marine ostracods differs significantly between genders and between species with different reproductive modes. We observed more infections in females and found *Cardinium* only in ostracods with mixed and asexual reproduction. There is a significant positive correlation between latitude and *Cardinium* infection, which might be linked to geographic parthenogenesis, a common phenomenon in non-marine ostracods with mixed reproduction. We suggest that the observed patterns best fit a polymorphic equilibrium between endosymbionts and their hosts. Ostracods with mixed reproduction often produce young asexual lineages, implying that *Cardinium* infections might have occurred more recently, and are widespread. In contrast, putative ancient asexual darwinulid ostracod shows less frequent occurrence of *Cardinium*. Loss of endosymbionts in these asexual ostracods during their long evolutionary histories of millions of years seems a more likely explanation. Which factors influence *Cardinium* prevalence in non-marine ostracods needs to be further tested in life history experiments.

Keywords Endosymbiont · Parthenogenesis · Mixed reproduction · 16S

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Introduction

The paradox of sex remains one of the last open evolutionary questions. All present-day asexual animal populations or species have originated from sexual ancestors at one point in their history (Schön et al., 2009a). How asexuality originates and is maintained is central for our understanding of genetic diversity and adaptability in asexuals, which in turn

determines the evolutionary fate of asexuals in the long and short terms. Infections with endosymbionts are one of the major drivers for the transition from sex to asexuality in invertebrates (Hilgenboecker et al., 2008; Engelstädter & Hurst, 2009; Zug & Hammerstein, 2012; Ma & Schwander, 2017) and can induce parthenogenesis or cause female-biased sex ratios through feminization and male killing. Likewise, endosymbionts have been shown to maintain the co-existence of sexual (non-infected) and asexual (infected) host strains, thus causing reproductive polymorphisms or mixed reproduction (Ma & Schwander, 2017). Endosymbiotic infections can have profound effects on the microevolution and reproductive ecology of their hosts (Engelstädter & Hurst, 2009). Some of the best studied endosymbionts are bacteria of the genus *Wolbachia*, which are known to be particularly widespread in terrestrial arthropods and can manipulate the host's reproductive system (Werren et al., 2008). A review (Ma & Schwander, 2017) revealed that 124 host species from seven different arthropod taxa showed evidence for endosymbiont-induced parthenogenesis; *Wolbachia* was with 65–75% occurrence the most frequent endosymbiont. *Rickettsia*, *Arsenophonus*, *Cardinium* and *Spiroplasma* can also affect reproductive modes and sex ratios in various arthropods (Engelstädter & Hurst, 2009; Vanthournout et al., 2011; Ma & Schwander, 2017) but the exact mechanisms are less well understood for those endosymbionts. The currently available data on the presence of parthenogenesis-inducing endosymbionts in invertebrates are still biased towards studies on terrestrial insects, especially Hymenoptera, and probably underestimates the real prevalence of these bacteria in other (arthropod) groups (Ma & Schwander, 2017).

Non-marine ostracods show a high incidence of obligate parthenogenesis and multiple origins of asexuality in different lineages (Chaplin et al., 1994; Martens, 1998; Butlin et al., 1998). Although ancient asexuality of the non-marine Darwinulidae is still debated (Smith et al., 2006; Martens & Schön, 2008; Schön et al., 2009b), these ostracods currently remain one of the few examples of putative ancient asexual scandals (Heethoff et al., 2009; Mark Welch & Ricci, 2009; Schön et al., 2009a, b; Schwander et al., 2011; Schwander, 2016), having possibly reproduced exclusively asexually for up to 200 myr (Martens et al., 2003).

Mixed reproduction is particularly common in another group of non-marine ostracods, the family Cyprididae (Martens, 1998), which comprises half of the more than 2300 known extant non-marine ostracod morphospecies (Meisch et al., 2019). Mixed reproduction in ostracods is very different from cyclic or facultative parthenogenesis as known from cladocerans (Decaestecker et al., 2009), rotifers (Serra & Snell, 2009) or aphids (Simon et al., 2002) because mixed reproduction in ostracods usually involves three "genders": males, sexual females and asexual females (Martens, 1998). In contrast to cyclic parthenogenesis, asexuality in non-marine ostracods is irreversible (Martens, 1998). Asexual and sexual females, which are morphologically indistinguishable, can occur sympatrically (Adolfsson et al., 2010; Bode et al., 2010; Schmit et al., 2012). In Europe, mixed reproduction is frequently combined with geographic parthenogenesis, where asexual ostracod populations have a wide distribution, while sexuals are restricted to areas around the Mediterranean (Horne & Martens, 1999; Adolfsson et al., 2010; Schmit et al., 2012).

Schön et al. (2018) were the first to report *Cardinium* in non-marine ostracods, investigating more than 300 specimens from 22 morphospecies, followed by Mioduchowska et al. (2018) who found *Cardinium* in the ostracod *Heterocypris incongruens* and Çelen et al. (2019) who screened 64 non-marine ostracod populations from Turkey representing 24 morphospecies. In light of these observations, we further investigate here to what extent these endosymbiotic infections could be the cause of parthenogenesis in non-marine ostracods. For this aim, we statistically reanalyse all existing data of Mioduchowska et al. (2018), Schön et al. (2018) and Çelen et al. (2019) to test specifically for (1) differences in infection prevalence between female and male non-marine ostracods; (2) differences in the frequency of *Cardinium* infections according to reproductive modes and type of habitat; (3) correlations between *Cardinium* infections and latitude as a proxy for temperature effects.

Materials and methods

Sampling and screening for *Cardinium* was done differently in the three published reports on endosymbionts of non-marine ostracods. Two studies (Schön et al., 2018; Çelen et al., 2019) focused on non-marine

ostracods only, whereas Mioduchowska et al. (2018) investigated a wider range of aquatic crustaceans for endosymbionts from temporary ponds and only one ostracod species, *Heterocypris incongruens*. Schön et al. (2018) screened non-marine ostracods from all over the world, while Çelen et al. (2019) limited their study to Turkey; both studies investigated non-marine ostracods from four different types of habitats: springs, permanent water bodies (lakes, ponds, creeks and leaf litter), streams and temporary aquatic habitats (pools, wetlands, ditches or troughs). Schön et al. (2018) also compared results of classic PCRs and Sanger sequencing with data of high-throughput sequencing, while neither of the other two studies used such an approach. Furthermore, Schön et al. (2018) and Mioduchowska et al. (2018) screened individual ostracods by classic PCR for the presence of *Cardinium* but Çelen et al. (2019) pooled up to ten individuals for each PCR reaction. The latter study can thus only provide minimum or maximum estimates of infection prevalence per series of pooled individuals. For example, if a sample of ten pooled females shows evidence for a *Cardinium* infection, this implies that at least one of the ten females was infected. These differences require specific approaches to combine the results of the three studies for statistical analyses as outlined below.

Statistical tests

We summarized *Cardinium* infections per morphospecies (Table 1) from the studies by Mioduchowska et al. (2018), Schön et al. (2018) and Çelen et al. (2019). We could not estimate infection patterns per cryptic genetic species using data of all three studies, as such data are only available from Schön et al. (2018)). To test for the effect of gender on *Cardinium* infection, we added up records of infected females and males per locality and morphospecies. Here, we could not include the data from Mioduchowska et al. (2018) as no information on the gender of the screened specimens was provided. We applied two procedures to avoid pseudoreplication in our statistical analyses. Firstly, concerning gender, we considered all individuals from the same gender, geographic locality and morphospecies as a single data point for the categories of infected or not infected ostracods (see Table S1), respectively. For example, when Çelen et al. (2019) reported a sample of ten

pooled females as being infected, we counted this sample as a single data point for infected females. Secondly, we defined the reproductive mode at the morphospecies level relying on the information in Wouters (1988), Rossetti & Martens (1998), Meisch (2000), and Martens (2001). We distinguished between species with mixed reproduction and fully sexual and asexual species. Species were regarded as fully sexual when males were present in all known populations. Similarly, species without any male records were considered as fully asexual. The presence of males in a population was regarded as evidence for sexual reproduction of that population (Martens, 1998). Species with male records from a single geographic location only or species with geographic parthenogenesis (where both sexual and asexual populations are known) were regarded as having mixed reproduction. Thirdly, we analysed infection patterns per type of aquatic habitat using the four categories described above (permanent, temporary, springs and streams) and only one data point per morphospecies and geographic location to avoid pseudoreplication (see Table S1 for more details). We applied Chi-squared tests in PAST v3.0 (Hammer et al., 2001) to test if gender, reproductive mode or type of habitat statistically affected infection patterns.

To test for geographic variation of infection patterns (latitude as proxy for temperature) and reproductive modes, we analysed infection patterns per unique geographic locality and morphospecies (again to avoid pseudoreplication) from the ostracod datasets of Mioduchowska et al. (2018), Schön et al. (2018) and Çelen et al. (2019) (see Table S1 for details). Çelen et al. (2019) only provided information on the Turkish provinces in which the samples were taken. We therefore used the website https://latitude.to/articles-by-country/tr/turkey/ to obtain central coordinates of each province. Because infection patterns can vary between individuals from the same morphospecies or genetic species and geographic locality, we included both data points, presence and absence of *Cardinium*, for the same species at a given location (if such variation occurred) (Table S1) in the Pearson's linear r correlation analyses in PAST v3.0 (Hammer et al., 2001) to test for possible correlations between *Cardinium* infection, reproductive mode, and latitude.

Table 1 Prevalence of *Cardinium* in non-marine ostracod species

Morphospecies	Infected	Reproductive mode	# Screened individuals	# Infected males (# screened)
Superfamily Cypridoidea				
Candona weltneri Hartwig, 1899	+	Mixed[&]	Çelen et al. (2019): 1 × 6	
Candona neglecta G.O. Sars, 1887	+	Mixed[&]	Çelen et al. (2019): 1 × 10	
Cypria ophthalmica (Jurine, 1820)	−	Sexual[1]	Schön et al. (2018): 6	
Cypridopsis vidua (O.F. Müller, 1776)	+	Asexual[1]	Schön et al. (2018): 9	
Cypris pubera O.F. Müller, 1776	+	Mixed[1]	Çelen et al. (2019): 2 × 3; 4x1	6 (1 × 3, 3 × 1)
Eucypris virens (Jurine, 1820)	+	Mixed[1]	Schön et al. (2018): 116	0 (43)
Eucypris pigra (Fischer, 1851)	+	Mixed[1]	Schön et al. (2018): 1 (1)	
Herpetocypris brevicaudata (Kaufmann, 1900)	+	Mixed[1]	Çelen et al. (2019): 1 × 5	
Herpetocypris chevreuxi (Sars, 1896)	+	Mixed[1]	Schön et al. (2018): 4	
Herpetocypris intermedia (Kaufmann, 1900)	−	Asexual[1]	Çelen et al. (2019): 1 × 2	
Herpetocypris reptans (Baird, 1835)	+	Mixed[1]	Çelen et al. (2019): 1 × 5, 1 × 2, 2 × 1	
Heterocypris barbara (Gauthier & Brehm, 1928)	+	Mixed[1]	Çelen et al. (2019): 1 × 5	
Heterocypris incongruens (Rahmdor, 1808)	+	Mixed[1]	Schön et al. (2018): 51	
			Mioduchowska et al. (2018): 2	
			Çelen et al. (2019): 2 × 3, 6 × 5, 1 × 4,	
Heterocypris reptans (Kaufmann, 1900)	+	Mixed[1]	Çelen et al. (2019): 1 × 5	
Heterocypris rotundata (Bronstein, 1928)	+	Asexual[1]	Çelen et al. (2019): 1 × 5	
Heterocypris salina (Brady, 1868)	+	Mixed[1]	Schön et al. (2018): 1	
			Çelen et al. (2019): 2 × 3, 7 × 5, 1 × 10	
Heterocypris spec.1	−	Mixed[2]	Schön et al. (2018): 3	0 (3)
Heterocypris spec.2	+	Mixed[2]	Schön et al. (2018): 11	0 (11)
Ilyocypris bradyi (Sars, 1890)	+	Asexual[1]	Çelen et al. (2019): 6 × 5	
Ilyocypris inermis (Kaufmann, 1900)	+	Mixed[1]	Çelen et al. (2019): 3 × 5, 1 × 3	0 (1 × 3, 1 × 5)
Ilyocypris gibba (Ramdohr, 1808)	+	Mixed[1]	Çelen et al. (2019): 1 × 5	
Potamocypris fallax (Fox, 1967)	+	Asexual[1]	Çelen et al. (2019): 1 × 8	
Potamocypris villosa (Jurine, 1820)	+	Mixed[1]	Çelen et al. (2019): 1 × 7	
Potamocypris arcuata (Sars, 1903)	+	Mixed[1]	Çelen et al. (2019): 1 × 5	
Potamocypris variegata (Brady & Norman, 1889)	+	Asexual[1]	Çelen et al. (2019): 1 × 5	
Prionocypris zenkeri (Chyzer & Toth, 1858)	+	Mixed[1]	Çelen et al. (2019): 1 × 5	
Psychrodromus olivaceus (Brady & Norman, 1889)	+	Mixed[1]	Çelen et al. (2019): 3 × 5, 1 × 10	
Stenocypria fischeri (Lilljeborg, 1883)	+	Mixed[1]	Çelen et al. (2019): 3 × 5, 3 × 3	
Stenocypris major (Baird, 1859)	+	Asexual[3]	Çelen et al. (2019): 1 × 3	
Tonnacypris lutaria (Koch, 1838)	−	Mixed[1]	Schön et al. (2018): 10	
Trajancypris clavata (Baird, 1838)	−	Asexual[1]	Çelen et al. (2019): 1 × 3	
Superfamily Darwinuloidea				
Darwinula stevensoni (Brady & Robertson, 1870)	+	Asexual[1]	Schön et al. (2018): 37	
Vestalenula paglioli (Pinto & Kotzian, 1961)	+	Asexual[4]	Schön et al. (2018): 2	

Table 1 continued

Morphospecies	Infected	Reproductive mode	# Screened individuals	# Infected males (# screened)
Vestalenula cornelia Smith, Kamiya & Horne, 2006	+	Mixed[5]	Schön et al. (2018): 2	
Vestalenula cylindrica Straub, 1952	–	Asexual[4]	Schön et al. (2018): 2	
Vestalenula molopoensis Rossetti & Martens, 1998	–	Asexual[4]	Schön et al. (2018): 1	
Penthesilenula brasiliensis (Pinto & Kotzian, 1961)	–	Asexual[1]	Schön et al. (2018): 5	
Penthesilenula aotearoa (Rossetti, Eagar & Martens, 1998)	–	Asexual[4]	Schön et al. (2018): 1	
Microdarwinula nov. spec.	–	Asexual[6]	Schön et al. (2018): 2	
Alicenula inversa Rossetti & Martens, 1998	–	Asexual[4]	Schön et al. (2018): 1	
Superfamily Cytheroidea				
Cytherissa lacustris[$] Sars, 1925	+	Mixed[1$]	Schön et al. (2018): 4	
Cyprideis torosa (Jones, 1850)	–	Sexual[1]	Schön et al. (2018): 7	0 (2)
Romecytheridea ampla Wouters, 1988	–	Sexual[7]	Schön et al. (2018): 10	0 (10)

Data are summarized from Schön et al. (2018), Mioduchowska et al. (2018), and Çelen et al. (2019). # indicates number. The mode of reproduction is derived from 1: Meisch (2000); 2: Pieri et al. (unpublished); 3: Martens (2001); 4: Rossetti & Martens (1998); 5: Smith et al. (2006); 6: Pinto et al. (unpublished); 7: Wouters (1988). Because Çelen et al. (2019) mostly pooled individuals for PCR screening, the number of PCR reactions and number of individuals per PCR reaction is shown; for example, 1x3 indicates one PCR reaction, where three individuals were screened simultaneously. [$] *Cytherissa lacustris* is only fully asexual outside of Lake Baikal (Schön & Martens, 2012; Schön et al., 2017). [&] According to Meisch (2000) males are always present in these species, but the specimens used by Çelen et al. (2019) are reportedly from all-female populations

Results

Prevalence of *Cardinium* in non-marine ostracods: correlation with sex/asex?

The total prevalence of *Cardinium* in all investigated ostracod morphospecies was 70%. This number is somewhat biased because this endosymbiont was (with one exception) detected only in females and only in species with either asexual or mixed reproduction (Table 1). If we tested for the effect of the reproductive mode, we found 22 of 24 species with mixed reproduction being infected (92%), while eight out of 16 asexual species showed *Cardinium* infections, and none of the three sexual species were infected (Table 1). These differences in infection frequency per reproductive mode were statistically significant (Chi-square test: $\chi^2 = 14.131$, $P = 0.0004$; Table 2). We found 90 females from unique localities being infected compared to one data point for infected males from one species (*Cypris pubera*) and a single locality (if we used the results of four PCR screenings by Çelen et al. (2019), from single and pooled males,

respectively; Tables 1, 3 and S1). This difference in the presence of *Cardinium* in males as compared to females is significant (Chi square test: $\chi^2 = 17.13$, and $P = 0.0001$; Table 3). If the *Cardinium* patterns in females of species with mixed reproduction were compared at the population level, females from asexual populations were significantly more often infected than females from sexual populations (Chi square test: $\chi^2 = 6.2325$, and $P = 0.0125$; Table 4).

Statistical analyses further demonstrated a significant positive correlation of *Cardinium* infection with latitude (Pearson's linear $r = 0.23731$, $P = 0.001$; see

Table 2 Number of infected and non-infected morphospecies from Table 1 and their reproductive modes

	Mixed	Asexual	Sexual	χ^2	P
Infected	22	8	0	14.131	0.0004
Not infected	2	8	3		

The Chi-squared test was conducted with PAST (Hammer et al., 2001)

Table 3 Number of infected female and male ostracods

Gender	Female	Male	χ^2	P
infected	90	1	17.13	0.0001
not infected	59	15		

To avoid pseudoreplication, we only used one data point per gender, morphospecies and unique geographic locality from the published studies of Schön et al. (2018) and Çelen et al. (2019). Table S1 provides more details. Populations with males are regarded as sexual, female-only populations as asexual (Martens, 1998). The Chi-squared test was conducted with PAST (Hammer et al., 2001)

Table S1 for details of the analysed data) but no correlation of reproductive mode with latitude (Pearson's linear $r = -0.091$, $P = 0.226$). We found that infection patterns differed significantly between habitat types (Chi-square test: $\chi^2 = 20.981$, and $P = 0.0002$; Table 5) with a majority of ostracods from temporary aquatic habitats being infected.

Cardinium was very frequent in the superfamily Cypridoidea, where only five out of 31 morphospecies showed no infection. Infections were less frequent within the Darwinuloidea where *Cardinium* was present in three morphospecies of the investigated nine species (including *Vestalenula cornelia* for which three rare males have been reported (Smith et al., 2006), whose functionality is still doubtful (Martens & Schön, 2008; Schön et al., 2009a, b)). *Cardinium* was least frequent in the Cytheroidea; it was detected in only one of three screened morphospecies (Table 1).

Discussion

Prevalence of *Cardinium* in non-marine ostracods

We found large differences in *Cardinium* infection frequencies between the three superfamilies of non-

marine ostracods (see Fig. 1), with the Cypridoidea having the highest frequencies (Table 1), and the Cytheroidea the lowest, although we only investigated three morphospecies of the latter. Infections in the Darwinuloidea were less frequent than in the Cypridoidea without any obvious phylogenetic patterns as shown in Schön et al. (2018). If *Cardinium* would be the sole cause of parthenogenesis in non-marine ostracods, we might expect complete fixation in all asexual lineages. Other studies found similar (incomplete) infection patterns of *Cardinium* in mites (Ros & Breeuwer, 2009) and spiders (Stefanini & Duron, 2012) and described them as a state of "polymorphic equilibrium between parasite and host".

Several mechanisms could have caused the absence of complete prevalence of *Cardinium* in some ostracod species, but not all of these are equally likely. The previous study by Schön et al. (2018) might have underestimated the prevalence of *Cardinium* in species with known low population densities (e.g. the Darwinulidae: *Vestalenula molopoensis*, *V. cylindrica*, *V. paglioli*, or *Alicenula inversa*), but this does not apply to all investigated ostracod species (Table 1). We can largely rule out technical difficulties, at least in the data from Schön et al. (2018), given that their results on *Cardinium* presence/absence were totally congruent between classic, PCR-based and novel 16S high-throughput amplicon sequencing techniques while also using different primers. Large genetic distances between *Cardinium* strains from different ostracod hosts and the genetic variability within the host species themselves as estimated by Schön et al. (2018) both provide evidence against recent, incomplete *Cardinium* infections in the darwinulids and some cyprids.

For *Wolbachia*, the best studied endosymbiont, several factors and their interactions are known to influence bacterial density in host tissues and effects on hosts (Hoffmann et al., 2015). These include

Table 4 Female infection patterns per sexual and asexual population

Females	Populations with males	Female-only populations	χ^2	P
infected	7	83	6.2325	0.0154
not infected	13	46		

To avoid pseudoreplication, we only used one data point per morphospecies and unique geographic locality from the published studies of Schön et al. (2018) and Çelen et al. (2019). Table S1 provides more details. Populations with males are regarded as sexual, female-only populations as asexual (Martens, 1998). The Chi-squared test was conducted with PAST (Hammer et al., 2001)

Table 5 Infection patterns per habitat type

	Permanent aquatic habitats	Streams	Springs	Temporary aquatic habitats	χ^2	P
Infected	15	2	6	144	26.554	0.0001
Not infected	22	0	8	41		

To avoid pseudoreplication, we only used one data point per morphospecies and unique geographic locality from the published studies of Schön et al. (2018) and Çelen et al. (2019). Creeks, lakes, ponds and leaf litter were regarded as permanent aquatic habitats; wetlands, pools, ditches and troughs were regarded as temporary aquatic habitats. Table S1 provides more details. The Chi-squared test was conducted with PAST (Hammer et al., 2001)
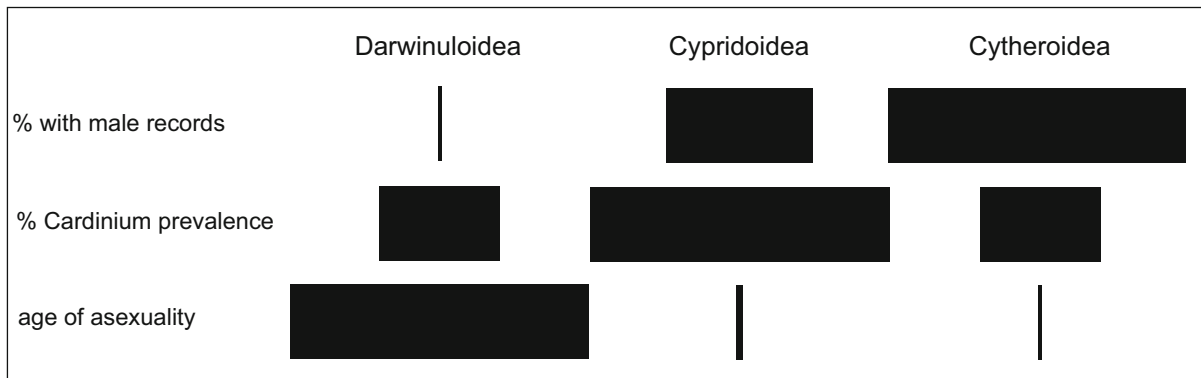
Fig. 1 Reproductive mode, *Cardinium* prevalence and age of asexuality of the three non-marine ostracod superfamilies. Reproductive modes are inferred from the frequency of morphospecies with at least one male record in %. Prevalence of infections with *Cardinium* per morphospecies is derived from Table 1 and calculated in %. Age of asexuality in myr is based on Martens (1998), Martens et al. (2003), Schön et al. (2009b) and Bode et al. (2010)

temperature (Hurst et al., 2001; Mouton et al., 2007; Bordenstein & Bordenstein, 2011; Morag et al., 2012), age (Unckless et al., 2009; Tortosa et al., 2010) and the occurrence of natural antibiotics, for example from fungi (Ho et al., 2003). Schön et al. (2018) only investigated adults; no age information is provided by Mioduchowska et al. (2018) and Çelen et al. (2019), suggesting that potential age effects in the infection patterns should be further investigated.

We observed a significant correlation between latitude and infection, which could indicate the presence of a temperature effect. However, the observed correlation is positive, thus, with increasing latitude, infection frequency increases while ambient temperatures decrease. This pattern is the opposite of the pattern that has been described in midges which were infected with *Wolbachia* (Morag et al., 2012) and also differs from the study of Bordenstein & Bordenstein (2011) where both extreme cold and hot temperatures reduced *Wolbachia* densities in wasps. It is thus more likely that our correlation between

latitude and infection is related to the phenomena of geographic parthenogenesis in certain ostracods as described below. In any case, the extent to which external factors such as temperature and antibiotics can influence *Cardinium* infection patterns in non-marine ostracods needs to be further investigated experimentally. We also observed that non-marine ostracods from temporary aquatic habitats were significantly more infected than ostracods from other habitats (Table 5). This result can best be explained by the high frequency of *Cardinium* infections in the Cypridoidea, which typically occur in temporary aquatic habitats. Also, most of the analysed samples came from temporary aquatic habitats (see Table 5). To test conclusively for the effect of habitat type, more samples from the other habitat types would also need to be screened for *Cardinium* infections.

During long histories of coevolution, endosymbiont genomes can get significantly reduced in size, as reviewed in for example McCutcheon & Moran (2011) and Wernegreene (2015). This can happen through

gene loss (see for example Latorre & Manzano-Marín, 2017) and lateral gene transfer to host genomes (e.g. Nikoh et al., 2008) where bacterial genes are pseudo-genized, which will obstruct their molecular detection. Incomplete coevolution between *Cardinium* and its ostracod hosts and loss of infection are alternative explanations for the observed low prevalence of *Cardinium* in darwinulid ostracods and the absence of *Cardinium* in some cyprid species. Several studies have reported the loss of *Wolbachia* during evolutionary histories of different ants (Ahrens & Shoemaker, 2005; Frost et al., 2010) including an invasive species (Reuter et al., 2005) or full or partial replacement of endosymbionts in the long history of certain insects (Wilson & Duncan, 2015). Reversible evolution of selfish elements (including *Wolbachia*) results in a gradual weakening of sterilizing effects (Hurst & McVean, 1996), eventually leading to the complete loss of infections (Weeks et al., 2001). Given the old age of non-marine ostracod lineages of more than 400 myr in general (Martens, 1998) and of asexuality in certain darwinulids of 200 myr in particular (Martens et al., 2003), evolutionary time spans could have been long enough for such a reversible evolution and the subsequent loss of *Cardinium* infections in old asexual lineages of non-marine ostracods. With this reasoning, we would expect that prevalence of *Cardinium* frequency in the older Darwinuloidea is lower than in the younger asexual Cypridoidea, which is indeed the case (Table 1). With asexuality being older, at least in certain lineages, non-marine ostracods are different from the most frequently used asexual study organisms for endosymbiont bacteria, which are usually of young evolutionary age and have haplo-diploid sex determination (Ma & Schwander, 2017).

Relationship of *Cardinium* to reproductive mode

Cardinium is associated with the reproductive mode as we found a significant difference between infection frequencies in ostracod species with mixed, asexual and sexual reproduction (see Table 2), and between infected females from asexual and sexual populations (Table 4). The observed high prevalence of *Cardinium* in ostracod species with mixed reproduction matches general patterns of endosymbionts causing reproductive polymorphisms and mixed reproduction as described by Ma & Schwander (2017). With the

exception of (a) putative male(s) of *Cypris pubera* from a single locality (Çelen et al., 2019), *Cardinium* furthermore only occurred in females (Table 3). The male records of Çelen et al. (2019) need to be further verified by obtaining DNA sequences of *Cardinium* from these samples and checking their taxonomic identity and genetic similarity to *Cardinium* from female ostracods of this and closely related ostracod species. Also, more sexual ostracod species need to be screened for possible *Cardinium* infections. In any case, the observed patterns suggest that the presence of *Cardinium* is at least partly associated with asexuality in non-marine ostracod species with mixed reproduction and female transmission of *Cardinium*.

The significant positive correlation of *Cardinium* to latitude appears to link infections to geographic parthenogenesis in non-marine ostracods across Europe (Martens, 1998; Horne & Martens, 1999). In species with mixed reproduction, asexuals have a wide geographic distribution including western, central and southern Europe, while the distribution of sexual populations is limited to the area surrounding the Mediterranean Sea as in the examples of *E. virens* (Martens, 1998; Adolfsson et al., 2010; Schmit et al., 2012) or *H. incongruens* (Martens, 1998).

A review by Ma & Schwander (2017) concluded that in 13% of all investigated arthropod species to date, *Cardinium* is the confirmed cause of parthenogenesis. *Cardinium* has also been shown to cause feminization in mites (Zchori-Fein et al., 2004) and in parasitic wasps (Giorgini et al., 2009). Our present study detects associations of *Cardinium* infections with asexual reproduction and female (vertical) transmission in non-marine ostracods as described above.

Whether *Cardinium* effectively causes feminization and/or parthenogenesis in non-marine ostracods still needs to be confirmed by life history experiments of different non-marine ostracod species with exposure to antibiotics and cytogenetic studies (Ma & Schwander, 2017). The case of *V. cornelia* belonging to the putatively ancient asexual Darwinulidae deserves special consideration for future studies. To test more conclusively if (incomplete) *Cardinium* infections are the most likely explanation for the production of rare males in *V. cornelia*, several populations of this species need to be screened for genetic diversity of the ostracod hosts and their *Cardinium* symbionts, followed by culturing experiments at different temperatures and with antibiotic

exposure. Under high temperatures and antibiotic exposure, we would predict that *Cardinium* infections are cured and rare males could be produced similarly to what Weeks et al. (2001) and Chigira & Miur (2005) reported from mites.

Conclusions

This study analysed infection patterns of *Cardinium* in non-marine ostracods from all available data to date. It should be followed up by studies on the genetic identity of all ostracod host species and genomic and karyological investigations on the mechanisms and effects of *Cardinium* infections on its hosts under different environmental conditions. Also, the occurrence of *Cardinium* in other crustaceans with mixed reproduction and other putative ancient asexuals remains to be further explored.

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