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## To cite this version:

Ambre Ribardière, Julia Centanni, Arnaud Dano, Jérôme Coudret, Claire Daguin-Thiébaut, et al.. Female-biased sex ratios unrelated to Wolbachia infection in European species of the Jaera albifrons complex (marine isopods). Journal of Experimental Marine Biology and Ecology, 2018, 509, pp.91-98. 10.1016/j.jembe.2018.09.002 . hal-02006763

**HAL Id: hal-02006763**

**https://hal.sorbonne-universite.fr/hal-02006763v1**

Submitted on 4 Feb 2019

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# Female-biased sex ratios unrelated to *Wolbachia* infection in European species of the *Jaera albifrons* complex (marine isopods)

Ambre Ribardière[1], Julia Centanni[1], Arnaud Dano[1], Jérôme Coudret[1], Claire Daguin-Thiebaut[1], Céline Houbin[2], Elise Kerdoncuff[1], Ségolène Jambut[1], Richard Cordaux[3], Thomas Broquet[1]

1- CNRS & Sorbonne Université, UMR 7144, Station Biologique de Roscoff, 29680 Roscoff, France.

2- CNRS & Sorbonne Université, FR2424, Station Biologique de Roscoff, 29680 Roscoff, France.

3- Laboratoire Ecologie et Biologie des Interactions, Université de Poitiers, UMR CNRS 7267, 86000 Poitiers, France

Corresponding author: Thomas Broquet, thomas.broquet@sb-roscoff.fr, tel +33 298 292 351, fax +33 298 292 324

**Abstract**

Female-biased sex ratios and reproductive isolation in arthropods can be caused by endosymbiotic *Wolbachia* bacteria that manipulate the reproductive system of their host. *Wolbachia* is particularly common in terrestrial host species, but its frequency in marine arthropods is less well known. Here we asked whether *Wolbachia* bacteria are accountable for the female-biased sex ratio and variation in reproductive isolation levels observed in the four European species of the *Jaera albifrons* complex (marine intertidal isopods). We analysed the sex ratio in young adults reared in the laboratory (indicative of the sex ratio at birth), compared it with the adult sex ratio in natural populations, and performed a molecular survey of *Wolbachia* infection based upon amplification of three gene targets using 11 different protocols tested in 817 individuals from all species of the *Jaera albifrons* complex. One species (*J. ischiosetosa*) had a female-biased sex ratio at birth but showed no sign of infection by *Wolbachia* bacteria. This species, together with two others (*J. albifrons* and *J. forsmani*) also displayed female-biased sex ratio in adults in nature, while the adult sex ratio in the fourth European species (*J. praehirsuta*) was unbiased. A new *Wolbachia* strain was identified in *J. albifrons* and *J. praehirsuta*, albeit at very low frequency in populations. We conclude that *Wolbachia* bacteria are present in at least two species of the *Jaera albifrons* complex, but their prevalence is too low to have any effect on sex ratio and reproductive isolation. A sex ratio distorter other than *Wolbachia* may be acting in some *J. ischiosetosa* populations, and we hypothesize that the adult female excess seen in most species results from habitat-dependent, male-biased mortality in natural conditions.

Keywords: female biased sex ratio, *Wolbachia* endosymbionts, marine crustaceans, *Jaera albifrons*

## 1. Introduction

Sex allocation theory predicts that frequency-dependent selection favours equal investments into male and female offspring (Fisher, 1999). If production costs are equivalent between offspring of the two sexes then the number of males and the number of females in a population are expected to be balanced. However, there are many constraints to the regulation of this equilibrium (West and Sheldon, 2002), and biased sex ratios are common in nature (Hardy, 2002). Causes of departure from Fisher's null model include in particular local mate competition between individuals for access to sexual partners (e.g. Werren, 1983), meiotic drive on sex chromosomes (reviewed in Jaenike, 2001) and cytoplasmic sex ratio distorters (Cordaux et al., 2011; Taylor, 1990) such as *Wolbachia*.

*Wolbachia* is a group of cytoplasmic endosymbiotic bacteria found in a large number of invertebrates (Werren et al., 2008). It is predominantly transmitted via female gametes (Werren et al., 2008) and it often promotes its transmission by interfering in the process of reproduction of its host (Rousset et al., 1992; Werren, 1997). *Wolbachia* is notably known to be involved in the conversion of genetic males into functional phenotypic neo-females, a process called feminization (Bouchon et al., 2008; Cordaux and Gilbert, 2017; Rigaud et al., 1999; Werren et al., 2008). It can also induce parthenogenesis in several otherwise sexually reproducing species, or specifically kill the male offspring of infected females (Hurst et al., 1999). Therefore, in absence of compensatory mechanisms (which would be readily favoured by sex ratio selection), *Wolbachia* can cause an increase in the proportion of females in infected populations.

In addition, these bacteria can also play a role in speciation by inducing interspecific cytoplasmic incompatibilities (Rousset et al., 1992; Telschow et al., 2007; Werren, 1997; Werren et al., 2008). For example, studies on wasps of the genus *Nasonia* (Bordenstein et al., 2001; Bordenstein and Werren, 1998; Bordenstein and Werren, 2007) have shown that *Wolbachia* can cause reproductive isolation between two closely related species by bidirectional incompatibility of two strain types. Furthermore, *Wolbachia* can also drive the reinforcement of behavioural isolation between infected and uninfected species (Jaenike et al., 2006).

Although *Wolbachia* is essentially maternally transmitted, its capacity to move into new hosts via horizontal transmission has promoted its spread to different invertebrate clades (Cordaux et al., 2001; Duron and Hurst, 2013; Turelli et al., 2018; Vavre et al., 1999). *Wolbachia* is particularly frequent in arthropods, infecting mites (Breeuwer and Jacobs, 1996), crustaceans (Bouchon et al., 1998) and insects (Werren and Windsor, 2000). Although the prevalence of *Wolbachia* is difficult to quantify, it was estimated to infect 20 to 60% of insects (Sazama et al., 2017; Werren and Windsor, 2000). Few species of marine crustaceans are known to be infected: *Wolbachia* was found in a handful of marine amphipods, isopods, and cirripedes (Bouchon et al., 1998; Cordaux et al., 2012).

In this study, we are interested in the *Jaera albifrons* complex, a group of small (2-5 mm) marine isopods where there are both (i) observations of strongly female-biased sex ratios and (ii) variations in the level of reproductive isolation between species. The *Jaera albifrons* complex includes five intertidal species that live on the shores of the temperate and cold waters of the North-Atlantic Ocean (Bocquet, 1953; Bocquet, 1972; Naylor and Haahtela, 1966). Three of these species (*J. albifrons*, *J. praehirsuta*, and *J. ischiosetosa*) have an amphi-atlantic distribution, while the other two species are restricted either to the North-American East coast (*J. posthirsuta*) or the European coasts (*J. forsmani*).

Nearly all demographic analyses of the four species that are found on the European coasts have reported female-biased sex ratios in adults in natural populations (Cléret, 1966b; Jaźdźewski, 1969; Jones and Naylor, 1971; Naylor and Haahtela, 1967; Naylor et al., 1961; Piertney and Carvalho, 1996; Solignac, 1976) with male proportions locally as low as 0.07 (*J. ischiosetosa* in the UK, Jones and Naylor, 1971). To our knowledge no such estimations have been reported for *J. posthirsuta*.

Two studies examined the sex ratio in offspring reared in the laboratory (with low mortality, sex ratio at maturity is informative of sex-ratio at birth). In contradiction with the results from adults in natural populations, these two studies found unbiased offspring sex ratio in species *J. albifrons* (Cléret, 1966b; Piertney and Carvalho, 1996), *J. forsmani*, and *J. ischiosetosa* (Cléret, 1966b). Solignac (1978, p. 89) also reported that he did not observe a biased sex ratio in young adults reared in the

95 laboratory. However, the figures reported by this author suggest a slight excess of females at birth in
96 *J. ischiosetosa* ($r$=0.42, binomial test $p$=0.02, from table 4.1 in Solignac 1978: 89). In addition, a
97 notable exception concerns a population from Normandy, France, where a subset of *J. praehirsuta*
98 females were shown to produce an excess of female offspring (Cléret, 1966a). The author concluded
99 that a genetic factor lethal for the males was segregating in this population.

100 Observations of even numbers of males and females at birth are compatible with the genetic
101 sex-determination system that was identified for the four European species (males ZZ, females
102 ZW1W2, Staiger and Bocquet, 1954; Staiger and Bocquet, 1956, again to our knowledge this system
103 has not yet been confirmed in *J. posthirsuta*). Accordingly, all four European species of the *Jaera*
104 *albifrons* group have long been proven to be strictly gonochoric (no sex-change, which is a strategy
105 found in some other isopods, as reviewed in Policansky, 1982; Poore and Bruce, 2012). Moreover,
106 genetic sex determination has been confirmed through repeated karyotype studies (Lécher, 1967;
107 Lécher and Prunus, 1971) and molecular linkage maps (AFLP: Mifsud, 2011, and RAD-seq genotyping:
108 Ribardière et al. unpublished). One study also found sex determination in species *J. albifrons* to be
109 unaffected by strong variations in temperature conditions (Piertney and Carvalho, 1996).

110 Several ideas have been put forward to explain why adult females are systematically more
111 abundant than adult males in natural populations. First, males are smaller and less conspicuous than
112 females, meaning that they could simply be missed during collection in the field (Cléret, 1966b).
113 However, sex ratio investigations based on a careful dedicated sampling consistently found female
114 biases (Jaźdźewski, 1969; Naylor et al., 1961; Piertney and Carvalho, 1996). Second, it has been
115 suggested that males have a shorter lifespan than females (Jaźdźewski, 1969; Solignac, 1976; Steele
116 and Steele, 1972), although the only data available, obtained in laboratory conditions, suggest
117 otherwise (Solignac, 1976, *J. albifrons* and *J. ischiosetosa* males both had better survival than
118 females). Finally, strong sex ratio biases could be driven by sex ratio distorters such as mitochondrial
119 variants or cytoplasmic microorganisms (reviewed in Duron et al., 2008; Duron and Hurst, 2013;
120 Jaenike, 2001; Werren et al., 2008). In isopods in particular, *Wolbachia* is the most frequent inherited

121 bacterium that can manipulate reproduction of its host. Yet an effect of sex ratio distorters would be
122 visible not only at the adult stage in natural populations but also in young adults reared in the
123 laboratory. Such effects have not yet been observed (Cléret, 1966b; Piertney and Carvalho, 1996)
124 except in some specific populations of *J. praehirsuta* (Cléret, 1966a) and perhaps *J. ischiosetosa*
125 (Solignac, 1978).

126 In summary, sex ratio biases in the *Jaera albifrons* complex are intriguing, and the causes of
127 female excesses in adults and perhaps in some cases in offspring are unresolved. In addition to sex
128 ratio biases, another reason to investigate whether *Wolbachia* endosymbionts are present in the
129 *Jaera albifrons* complex is that they could play a role in reproductive isolation. Species in this
130 complex are isolated by a combination of ecological isolation, sexual isolation, and genetic
131 incompatibilities (reviewed in Mifsud, 2011; Ribardière et al., 2017; Solignac, 1981) so that they
132 generally coexist without hybridization. But in some instances, local populations undergo
133 hybridization (Solignac, 1969a, b; Solignac, 1978). Species *J. albifrons* and *J. praehirsuta* are
134 particularly interesting in this respect as replicate mixed populations show varying degrees of
135 introgressive hybridization (Ribardière, 2017; Ribardière et al., 2017). It would be important to know
136 if *Wolbachia* may play any role in this variation. Interestingly, the *J. praehirsuta* broods showing
137 aberrant sex ratios as observed by Cléret (1966a) came from a population with introgressive
138 hybridization between *J. praehirsuta* and *J. albifrons* (Solignac 1969a, Ribardière et al. 2017).

139 Bouchon et al. (1998) found no *Wolbachia* in *J. albifrons*, but it could have been due to
140 sampling insufficiency (three individuals were tested). Recently, Wenzel et al. (2018) performed an
141 analysis of the microbiome of the four European species of the *Jaera albifrons* complex using
142 bacterial 16S rDNA sequencing in populations from Scotland. None of the bacterial 16S sequences
143 that they identified belonged to the *Wolbachia* group. Here we had two objectives. Our first
144 objective was to compare sex ratios at birth *vs* adult stage in the four European species of the *Jaera*
145 *albifrons* complex. For that, we reanalysed data from a large number of broods reared in the
146 laboratory as part of an ongoing project on speciation in the *Jaera albifrons* complex (Ribardière et

147 al., 2017). Although adult sex ratios have been well documented by others, for comparison with our
148 brood data we also estimated sex ratios in natural populations using a dedicated sampling effort in
149 the region where most of our brood data came from. Our second objective was to screen all five
150 species of the complex with a targeted assay for the presence of *Wolbachia* endosymbionts using
151 several gene targets and populations of various geographic origins. We focused particularly on the *J.*
152 *albifrons* / *J. praehirsuta* pair in regions with varying degrees of reproductive isolation. With this, our
153 overarching goal was to test whether *Wolbachia* may or may not be involved in sex ratio biases and
154 reproductive isolation in the *Jaera albifrons* complex.
155

156 **2. Methods**

157 *2.1 Study system and general strategy*

158 The five species of the *Jaera albifrons* group are found mid- to upper-shore, where they can
159 locally show some differential preferences in substrate (under pebbles or on seaweeds), position in
160 the intertidal zone, salinity, and exposure (Bocquet, 1953; Jones, 1972; Naylor and Haahtela, 1966).
161 Individuals from the different species have the same general morphology, with males (2-3 mm)
162 smaller than females (5 mm). Males from the five species differ only in the number and position of
163 spines and setae that they use to court females (Bocquet, 1953), and females cannot be
164 distinguished based on morphology. Development is direct, there is no pelagic larval stage, and
165 offspring measure ca. 0.5 mm when they are released. Individuals become sexually mature within 4
166 to 5 weeks and can then be sexed based on praeoperculum differentiation (e.g. Solignac, 1979). We
167 define sex ratio ($r$) as the proportion of males in a sample.

168 Our sex ratio analyses are based on two classes of data. First, we determined the sex of adult
169 individuals sampled in natural populations. These analyses, based on a small number of sampling
170 sites, were used to check if our study populations conform to the well-described sex ratio biases
171 reported in the literature. Second, we used a much larger dataset to analyse sex ratios in broods
172 reared in the laboratory. When survival in the laboratory is high (see results and discussion), then the

173 sex ratio measured at least five weeks after birth is a good proxy for the sex ratio at birth, which has
174 been less well documented than adult sex ratios and brings complementary information. In addition
175 to sex ratio analyses we used a multi-target molecular approach to search for *Wolbachia* in a large
176 number of individuals from different species and geographic origins.
177
178 *2.2 Adult sex ratio in natural populations*
179 Because males of all four European species of the *Jaera albifrons* group are smaller than
180 females and can thus be more easily missed during field sampling, we selected four sampling
181 sites/habitats (Table 1) where we carefully sampled all individuals in a delimited area. In our primary
182 study region (Brittany, western France), species *J. albifrons*, *J. ischiosetosa*, and *J. forsmani* live under
183 rocks, while *J. praehirsuta* is found mainly on *Fucus vesiculosus* and *Ascophyllum nodosum* seaweeds
184 (Bocquet 1953, Ribardière et al. 2017). To estimate sex ratio we thus sampled all individuals found on
185 a limited number of rocks or algae in each site (5-15 pebbles or stones of different sizes or a random
186 sample of algae typically contained within 10 $m^2$, depending on local density). All individuals were
187 brought back to the laboratory where they were sexed and males were identified based on their
188 secondary sexual characters. The four sampling site/habitat combinations were previously known to
189 shelter a single species, meaning that there would be little risk that the females (for which species
190 cannot be identified from morphological characters) could belong to two different species (see
191 results). Binomial tests were used to test for a departure from even sex ratio (all analyses performed
192 in R, R Core Team, 2016).
193
194 *2.3 Sex ratio in broods reared in the laboratory.*
195 Raising individuals from birth to sexual maturity in the laboratory gives access to sex ratio
196 under controlled conditions, and, if survival has been high enough (see results), to estimate sex ratio
197 at birth. In the course of an ongoing project on the *Jaera albifrons* complex we raised a large number
198 of broods in standardized laboratory conditions. These data were used to count the number of males

199 and females in each brood and compare sex ratio estimates across broods and populations. Rearing
200 conditions are detailed in supplementary material.

201 All broods were produced by females maintained in the laboratory, but within two different
202 experimental frameworks. First, we reared broods produced by females that came directly from the
203 field. This is possible because females store sperm and can thus produce offspring in the laboratory
204 in absence of males (each female was kept in an separate well and was thus never in contact with
205 males). Within this framework, we have no information on the father of the offspring, the age of the
206 mother, and little *a priori* information, if any, on the female's species (this was later determined from
207 the morphological characters borne by male offspring of each female).

208 Second, we reared broods produced through controlled experimental crosses featuring
209 parents that had been reared in captivity. In that case both the mother and father of each brood are
210 known and identified, and the parents were virgin before the experiment (i.e. in most cases we
211 analysed their first brood, or we knew how many broods were produced before). Such controlled
212 crosses were run only with *J. albifrons* and *J. praehirsuta*. Since we did not detect any significant
213 difference in sex ratio between the broods produced by females caught in the wild *vs* those from
214 controlled experimental crosses (supplementary material Figure S1), we did not separate these two
215 types of data for sex ratio analyses (unless stated otherwise).

216 Overall, we used data from 375 broods reared in the laboratory between 2013 and 2016. A
217 large fraction of the data came from a region of France where we have been studying natural
218 hybridization between *J. albifrons* and *J. praehirsuta* (region Normandy, Ribardière et al., 2017).
219 These data (243 broods) were analysed separately (next section). The other fraction corresponds to
220 132 broods from four species that were originally sampled in region Brittany, where no hybridization
221 has been detected (Ribardière et al., 2017): *J. albifrons* ($n$ = 72 broods), *J. praehirsuta* ($n$ = 25), *J.*
222 *ischiosetosa* ($n$ = 12), and *J. forsmani* ($n$ = 23). We used these data to estimate brood sex ratio and
223 compare it with adult sex ratio estimated in natural populations from Brittany (as described above).
224

225 *Sex ratio in case of introgressive hybridization between J. albifrons and J. praehirsuta*

226 Two analyses were performed to investigate the consequences of introgressive hybridization
227 on sex ratio in *J. albifrons* and *J. praehirsuta*. First, we compared the sex ratio in broods from each
228 species in regions Brittany (no hybridization) *vs* Normandy (hybridization). For that we used broods
229 from females sampled in the wild (*J. albifrons* in Brittany, *n* = 34 broods, Normandy, *n* = 22; *J.*
230 *praehirsuta* in Brittany, *n* = 15, Normandy, *n* = 39). Second, we compared the sex ratio in broods
231 resulting from controlled intra- and inter-specific crosses. For that we used intra-specific *J. albifrons*
232 crosses (*n* = 131 broods), intra-specific *J. praehirsuta* crosses (*n* = 134), first-generation hybrids (*n* =
233 17), and backcrosses (F1 hybrid females crossed with either *J. albifrons* or *J. praehirsuta* males, *n* =
234 58). Generalized linear models with a logit link function and binomial errors were used to test for
235 differences in sex ratio amongst categories.

236

237 *2.4 PCR detection of Wolbachia*

238 The presence of *Wolbachia* endosymbionts can be detected by PCR amplification using host
239 DNA extracts (potentially containing co-extracted DNA from endosymbionts) with *Wolbachia* specific
240 primers (Simoes et al., 2011).

241 A total of 817 individuals belonging to the five species of the *Jaera albifrons* complex,
242 including 321 females and 496 males, were tested. We used samples from 9 populations from
243 France, 2 populations from Quebec (Canada), 1 population from the Isles of Scilly (United-Kingdom),
244 and 1 population from Tromsø (Norway), as detailed in supplementary Table S1. The samples from
245 France included *J. albifrons* and *J. praehirsuta* from sites with and without hybridization, as described
246 above for the sex ratio analyses. In addition, because the presence of *Wolbachia* has been
247 investigated in few marine species, we also analysed 34 individuals of 6 marine isopod and amphipod
248 species (Table S2).

249 Total DNA (from *Jaera spp.* and potential *Wolbachia sp.*) was extracted using either a
250 NucleoSpin Tissue kit (Macherey–Nagel) or a salt protocol (detailed in supplementary material). To

251 check the general quality of DNA extracts, we used a subset of 90 randomly chosen individuals to
252 amplify 24 *Jaera albifrons* microsatellites (Ribardière et al., 2015). In addition, a DNA extract from
253 *Oniscus asellus* (common woodlouse) known to be infected by *Wolbachia* was used as positive
254 control to check if *Wolbachia* was correctly detected by the different methods described below.
255 Three different loci were targeted to increase our detection ability and confidence (details in
256 Table 2). First, we used the *wsp* gene (*Wolbachia* surface protein), which encodes a major cell surface
257 coat protein and is one of the most common targets for *Wolbachia* PCR detection (Braig et al., 1998;
258 Zhou et al., 1998). We used four distinct *wsp* protocols (*wsp*-1, *wsp*-2, *wsp*-L, and *wsp*-J, Table 2) with
259 three different pairs of primers, including newly developed primers
260 JwspF 5' CGTTCGTTTACAATACAACGGTGA 3' and JwspR 5' AACCGAAGTAACGAGCTCCA 3'. These new
261 primers were designed from a *Wolbachia* sequence that we obtained in this study from two
262 individuals of the *Jaera albifrons* complex (see results). Second, the ribosomal 16S-2 marker was also
263 chosen because it is a universal barcoding locus for bacteria identification that is expected to
264 produce a strong signal (Simoes et al., 2011). Finally, we also used the *fbpA* primers that amplify a
265 region of the fructose-bisphosphate aldolase gene (Baldo et al., 2006). This method lacks specificity
266 but it can be more sensitive (Simoes et al., 2011). Polymerase Chain Reaction (PCR) conditions are
267 detailed in Tables S3 and S4. PCR products were electrophoresed in 1.5% agarose gels stained with
268 ethidium bromide and visualized under UV light. Some individuals were tested several times with
269 different primers, bringing the total number of tests to 1692 (or 2445 when including individuals
270 tested several times with the same protocol). A re-amplification based on a subset of PCR products
271 for the *wsp* gene was also performed for 423 samples to enhance detection ability. Details of
272 analysed samples (primers used, species, sex, origin) are given in supplementary materials (Table S1).
273 The large majority of these tests produced no amplified products (see results), but all
274 amplifications resulting in a single band around the expected size were directly sequenced. Overall,
275 98 PCR products (in addition to the positive control) were sent for sequencing to Eurofins Genomics
276 (Ebersberg, Germany). The sequences obtained were assembled and checked with the CodonCode

277 Aligner software (CodonCode corporation, Centerville, MA) and then compared to the GenBank
278 Nucleotide database using BLAST (Basic Local Alignment Search Tool, NCBI).
279 To determine the phylogenetic position of the *Jaera*-specific *Wolbachia* sequence that we
280 found in this study (see results), we built a neighbour-joining phylogeny using a sample of related
281 sequences deposited in GenBank (with a particular focus on strains found in marine crustaceans,
282 Cordaux et al., 2012).
283
284 **3. RESULTS**
285 *3.1 Adult sex ratio in natural populations*
286 Between 64 and 115 adult individuals were sampled in each of the four populations chosen for
287 sex ratio estimates. Three out of the four European species of the *Jaera albifrons* group presented a
288 female-biased sex ratio (Table 1 and Figure 1), with *J. ischiosetosa* showing the strongest bias (20
289 males, 94 females, binomial test $p$-value < 0.001). *J. albifrons* and *J. forsmani* were also characterized
290 by a significant excess of females, while *J. praehirsuta* did not reveal any bias (30 males, 34 females,
291 $p = 0.71$). All males sampled in a site/habitat combination belonged to a single species, except for
292 three *J. praehirsuta* males that were found in the *J. albifrons* sample (that is, under rocks), in
293 agreement with the level of habitat overlap between these two species in the region sampled
294 (Ribardière et al., 2017). These males were removed from all analyses but there is a small possibility
295 that some of the females used to estimate the *J. albifrons* sex ratio were in fact *J. praehirsuta* (see
296 discussion).
297
298 *3.2 Sex ratio in broods reared in the laboratory.*
299 Using 132 broods from four species that were originally sampled in the French region Brittany
300 (i.e. where species are reproductively isolated), we raised 1349 progenies (Table 4). The average
301 survival rate in the laboratory (that is, from birth to the date when each individual was sexed) was
302 77.5%, meaning that 1046 individuals could be sexed successfully across the four species. We

observed a strong bias towards females in *J. ischiosetosa* ($r$ = 0.29, binomial test $p$-value < 0.001). The other three species did not show any significant sex bias (Table 3 and Figure 1). The distribution of sex ratio per brood is shown in supplementary material Figure S2.

In the case of *J. ischiosetosa*, we raised 130 offspring from 12 broods with a survival rate of 85%. With 20 dead out of 130 individuals, sex-biased mortality cannot account for the sex ratio bias that we observed. Even if all dead individuals were males, then the sex ratio at birth would still be significantly biased (52 theoretical males, 78 females, binomial test $p$-value = 0.028). The same reasoning applies to all four species and we consider laboratory sex ratio as a good proxy for sex ratio at birth (Figure 1).

*3.3 Sex ratio in case of introgressive hybridization between J. albifrons and J. praehirsuta*

First, we compared the sex ratio in broods produced by females *J. albifrons* and *J. praehirsuta* in Brittany (no hybridization) and Normandy (introgressive hybridization). For this analysis we had the sex of 798 individuals (out of 1070 offspring isolated) and found no significant bias in sex ratio within each species in each region (all binomial test $p$-values > 0.05) and no significant differences across species and regions (GLM $p$-values > 0.05 for both factors and their interaction). These results are shown in supplementary material Figure S3.

We came to the same conclusion when analysing broods from experimental crosses featuring *J. albifrons*, *J. praehirsuta*, F1 hybrids, and backcross hybrids (Figure 2, 1902 sexed individuals out of 2744 reared offspring). Again we found no significant deviation from even sex ratio within each class (all binomial test $p$-values > 0.05) and no significant difference between classes (GLM $p$-value > 0.05).

*3.4 PCR detection of Wolbachia*

We searched for *Wolbachia* in the *Jaera albifrons* complex by conducting 2,445 tests involving 817 individuals (see supplementary material, Table S1). Overall, 731 individuals were tested more than once, among which 232 have been tested with at least 2 different protocols. None of our

329 negative controls showed amplification signals, whilst our positive control (DNA extract from a
330 *Wolbachia*-infected woodlouse) was correctly and constantly amplified by all methods. Our *Jaera*
331 microsatellite markers amplified well, suggesting that there was no obvious problem with the
332 general quality of the DNA extraction protocols (linked e.g. with PCR inhibitors).

333 With the *fbpA* and 16S-2 protocols no amplification of expected size was detected for any of
334 the tested individuals (*n*=47 individuals tested for each marker). The wsp-1 and wsp-2 protocols
335 produced a weak amplification signal for 6% (*n*= 6 out of 95) and 3% (*n*=8 out of 252) of the tested
336 individuals, respectively. Re-amplification attempts for 272 of these PCR products (details in Table
337 S1) did not produce any amplicon corresponding to a *Wolbachia* sequence. By contrast, the sequence
338 obtained with our positive control hit the expected *Wolbachia* sequence (accession number
339 **AJ276601.1**).

340 The more sensitive wsp-L protocol, tested on 615 individuals, produced 62 PCR products
341 showing a single band with amplification size close to that expected. These 62 PCR products were
342 directly sequenced, and two of them were identical and showed >99% identity with the top
343 *Wolbachia wsp* hit in Genbank (3 differences out of 540 nucleotides). We deposited this new
344 *Wolbachia* sequence in Genbank with accession number **MH121068**. Here again, re-amplification
345 attempts of 102 individuals did not lead to the discovery of further *Wolbachia* hosts.

346 The new primers specifically designed from the *Wolbachia* sequence that we isolated as
347 described above were used with 129 individuals (protocol *wsp*-J). None of them produced
348 amplification signals (including the two individuals that were previously found to be infected with
349 *Wolbachia*). Re-amplification of a subset of 40 PCR products did not yield any additional result.

350 Finally, we used the newly designed primers to re-amplify 53 PCR products previously
351 amplified with the *wsp*-L primers (chosen because they produced a signal of expected size). Twelve
352 of these re-amplifications produced a strong band at the expected size, and eight of them appeared
353 to be *Wolbachia* sequences, including the 2 individuals previously detected as positives. All eight
354 individuals shared the same *Wolbachia* sequence (accession number **MH121068**). They were all

355 females: 1 supposed *J. praehirsuta* (because found on seaweeds in region Brittany, Ribardière et al.,
356 2017), and 4 *J. albifrons*, 2 *J. praehirsuta* and 1 female of unknown species from French region
357 Normandy.

358 The phylogeny that we built using closely related sequences indicated that our discovered
359 *Wolbachia* strain belongs to the Rug group of *Wolbachia*, within the B supergroup (Figure 3, Cordaux
360 et al., 2001). Interestingly, the Rug group happens to be the main *Wolbachia* group known to include
361 strains infecting marine arthropods (Cordaux et al., 2012).

362 Finally, the presence of *Wolbachia* was not detected in any of the other isopod and amphipod
363 species that were tested in this study (Table 3).

364

365 **4. DISCUSSION**

366 Unexpectedly, the four European species of the *Jaera albifrons* complex followed one of three
367 distinct sex ratio regimes. Two species (*J. albifrons* and *J. forsmani*) were found to have balanced sex
368 ratios at birth (as deduced from the sex ratio in young adults and low mortality in laboratory
369 conditions), but a female-biased adult sex ratio in natural populations (Table 1 and Figure 1). This
370 result confirms previous findings for these two species (Cléret, 1966b; Piertney and Carvalho, 1996).
371 More surprising results came from *J. ischiosetosa*, which showed a strong bias towards females both
372 at the offspring and adult stages, and *J. praehirsuta*, which showed no bias at any stage (Table 1 and
373 Figure 1).

374 The case of *J. ischiosetosa* is the most interesting, as it indicates that there is a mechanism in
375 this species that drives a female-biased sex ratio very early in the life cycle. Moreover, this result
376 disagrees with the only other report of sex ratio at birth in this species (Cléret, 1966b), suggesting
377 that there is variation in the sex ratio at birth between populations, or through time (see also
378 Solignac, 1978, p. 89). This points towards endogenous factors such as mitochondrial variants,
379 meiotic drive sex ratio distorters, or reproductive parasites. Here we have performed an intensive
380 survey for the presence of *Wolbachia* endosymbionts, which were an obvious candidate for

381 reproductive manipulation in isopods showing an excess of females. We did not find any trace of
382 *Wolbachia* in 77 *J. ischiosetosa* individuals using several different protocols that targeted the *wsp*
383 region (Table S1). This targeted assay involved the mothers of seven of the 12 broods that were
384 analysed for that species (brood sex ratio from 0.08 to 0.43, Figures 1 and S2). We conclude from
385 these observations that *Wolbachia* is either absent or has a low prevalence in *J. ischiosetosa* and thus
386 cannot be accountable for the excess of females observed in this species.

387 Other endosymbiotic manipulators of sex (such as bacteria from groups *Cardinium*, *Rickettsia*,
388 and *Spiroplasma*, or microsporidia) could be involved. In their microbiome study, (Wenzel et al.,
389 2018) found evidence for the presence of *Rickettsiacea* in *J. ischiosetosa* females (and not in males),
390 but in minute quantities (1.16% of the 16s sequences obtained for a sample of *J. ischiosetosa*
391 females, M. Wenzel pers. comm.). The best way to test for this hypothesis will be to estimate sex
392 ratio in broods reared in laboratory with and without broad-spectrum antibiotic treatment
393 (Bordenstein et al., 2001; Breeuwer and Jacobs, 1996). Alternatively, the hypothesis of sex ratio
394 distorters borne on the sex chromosomes could be tested using controlled crossing experiments.
395 Finally, although it seems an unlikely hypothesis (ruled out in species *J. albifrons*, Piertney and
396 Carvalho, 1996), an effect of the environment on sex determination could be further checked
397 specifically in species *J. ischiosetosa*.

398 The three other species analysed here did not show any bias in sex ratio at birth, but two of
399 them (*J. albifrons* and *J. forsmani*) had female-biased sex ratios at the adult stage in natural
400 populations, in agreement with all previous sex ratio reports for these two species. Hence for these
401 two species at least, males must have lower survival than females in nature. This sex-linked
402 difference in survival is not seen in the laboratory (Piertney and Carvalho, 1996; Solignac, 1978, p.
403 89), which suggests that it depends on environmental conditions encountered in natural settings.
404 Excluding environmental effects on sex determination (Piertney and Carvalho, 1996), the earlier
405 mortality of males could be due to a difference in costly activities between males and females. In
406 particular, one can imagine that males wander more than females to search for potential mates.

Moving from one shelter to another could be a risky business in species that live under pebbles and stones on coarse sand or gravel. This sex-specific behaviour could also be linked to a more general pattern of male-biased dispersal. In both cases, it could result in a higher mortality of males seen in nature but not in the laboratory. Mortality risks associated with sex-specific behaviours such as mate seeking or dispersal have been found to impact adult sex ratios in other animal groups (e.g. reviewed in Donald, 2007; Székely et al., 2014).

Interestingly, *J. ischiosetosa* not only showed a female bias at birth but it was also the species with the strongest bias in adults from natural populations in this study (Table 1 and Figure 1) and other works (e.g. Jones and Naylor, 1971). Here we found that the bias in adults ($r$=0.18) was significantly stronger than in offspring reared in the laboratory ($r$=0.29, binomial proportions test $p$=0.03), suggesting that the factor that causes the female bias at the adult stage in other species may also be acting in *J. ischiosetosa*.

Finally, the fourth species studied here (*J. praehirsuta*) did not show any bias at the offspring and adult stages (Table 1 and Figure 1). The most obvious difference between this species and others is that it lives essentially on seaweeds (in our study area). It is thus possible that the male-biased mortality discussed above is habitat-dependent. Since ecological conditions vary widely across the distribution of all species of the *Jaera albifrons* complex, one straightforward way to test this hypothesis is to compare sex ratios in populations in different ecological contexts (especially different substrate types where one can hypothesize that exploratory movements are more or less facilitated). For instance, while *J. praehirsuta* is dwelling on seaweeds in our study area (French region Brittany), it is found under pebbles in other areas such as region Normandy. There, this species is found mixed with *J. albifrons*, with which it hybridizes (Ribardière et al., 2017), making it more difficult to estimate specific sex ratios in nature (also because females cannot be identified unless they are kept in the laboratory until they produce offspring and these offspring are reared for long enough that the males can be identified based on their secondary sexual traits). Nonetheless, the available data for such mixed populations occupying stones and pebbles strongly suggest that the

433 sex ratio is female-biased in *J. praehirsuta* (e.g. 61 males for 138 females of a mixed *J. albifrons*/*J.*
434 *praehirsuta* population, Ribardière et al. 2017, and the same type of observation was made in
435 populations from the UK where there seems to be no hybridization, M. Wenzel pers. comm.). Hence
436 it seems that the adult female excess is dependent upon ecological conditions, at least in species *J.*
437 *praehirsuta*.

438 Our *Wolbachia* survey involved a large number of individuals (*n*=817) from the five species of
439 the *Jaera albifrons* complex sampled in different geographic regions and tested with a variety of
440 protocols (11 protocols involving 5 different primer pairs). This effort led to the discovery of a new
441 *Wolbachia* strain (*wsp* sequence deposited in Genbank under accession number **MH121068**) that
442 was detected in *J. albifrons* and *J. praehirsuta*. This adds these two species to the limited list of
443 marine crustaceans known to be infected by *Wolbachia* (Bouchon et al., 1998; Cordaux et al., 2012).
444 Our negative controls always came back negative, no *Wolbachia* gene amplification was carried in
445 our laboratory before this study, and the new sequence that we found was different from that of our
446 positive control and related to other known marine arthropod-infecting strains. Yet this result must
447 be considered with caution, as this new *Wolbachia* sequence was amplified from only eight DNA
448 extracts, nearly only using a double amplification protocol (*wsp*-L followed by *wsp*-J, see Tables 2 and
449 S1), and could not be replicated using simple-amplification protocols (even using newly designed
450 primers). We conclude that *Wolbachia* bacteria are present in at least two species of the *Jaera*
451 *albifrons* complex in French regions Brittany and Normandy, but most likely with a very small
452 prevalence (both in terms of the number of individuals infected and the concentration of bacteria
453 within infected individuals). This finding consolidates the simultaneous result obtained by Wenzel et
454 al. (2018), where bacterial 16s rDNA sequencing did not reveal any trace of *Wolbachia* in Scottish
455 populations from the four European species of the *Jaera albifrons* complex.

456 Taken together, our observations that i) *Wolbachia* was not found in the only population
457 showing a female-biased sex ratio at birth (*J. ischiosetosa*), ii) it was detected in very few individuals
458 in other species, and iii) a single strain was detected in species *J. albifrons* and *J. praehirsuta* based

on the *wsp* locus (and in regions with and without hybridization) lead to the conclusion that *Wolbachia* bacteria most likely have no feminization or male killing effects in the *Jaera albifrons* complex, and that they have also no bearing upon reproductive isolation between species within the complex. Sex ratio analyses prompted us to hypothesize that some other sex ratio distorter is acting in a subset of populations (such as the *J. ischiosetosa* population studied here, and perhaps the *J. praehirsuta* population reported by Cléret 1966b), and that the ubiquitous excess of adult females in nature may be due to substrate-dependent male-biased mortality.

**Acknowledgements**

We thank Marius Wenzel for sharing ideas and submitted or unpublished results. We thank Elsa Pabion for her help with the maintenance of animals in the laboratory, and Olivier Timsit and Caroline Broudin for helping us collecting individuals in the field. This work benefited from access to the Biogenouest genomic platform at Station Biologique de Roscoff and was supported by the French Agence Nationale de la Recherche (grant ANR-13-JSV7-0001-01 to TB).

**Research data**

All data used for the brood sex ratio analyses presented in this article will be available on Dryad upon acceptance

**Author contributions**

Conceptualization and methodology: AR, CD-T, RC, and TB. Sex ratio data acquisition: AR, JC, JeC, CH, EK, SJ and TB. Molecular investigations: AR, JC, AD, and CD-T. Data curation: AR, JC, and TB. Writing – original draft: AR, CD-T and TB. Writing – review and editing: AR, CD-T, RC, and TB. Supervision, project administration and funding acquisition: TB. All authors have approved the final article.

Declarations of interest: none

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628

**Tables**

Table 1. Number of males and females in natural populations of the four European species of the *Jaera albifrons* complex. Departure from an even sex ratio (proportion of males) was tested using a binomial test within each site (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

| Species | Site | Habitat | Coordinates | Males | Females | Sex ratio |
|---|---|---|---|---|---|---|
| *J. albifrons* | Lingoz | rocks | 48°39’12.31"N 3°57’0.43”W | 15 | 49 | 0.23*** |
| *J. praehirsuta* | Lingoz | algae | 48°39’12.31"N 3°57’0.43”W | 30 | 34 | 0.47 |
| *J. ischiosetosa* | Pempoul | rocks | 48°41'16.9"N 3°57'27.6"W | 20 | 94 | 0.18*** |
| *J. forsmani* | Pointe Grande Grève | rocks | 48°42'22.6"N 3°58'18.7"W | 41 | 74 | 0.36** |

Table 2. Primers used in the six protocols tested in this study. Protocols *Wsp*-1 and -2 use the same primers with different PCR conditions (see supplementary Tables S3 and S4).

| Name | Primer (F/R) | Product size (bp) | Reference |
|---|---|---|---|
| *Wsp-1* | 81F/691R | 610 | Zhou et al. (1998) |
| *Wsp-2* | 81F/691R | 650 | Braig et al. (1998) |
| *Wsp-L* | LongF/LongR | 657 | Jeyaprakash and Hoy (2000) |
| *Wsp-J* | JwspF/JwspR | 464 | This study |
| *16S-2* | Wspecf/Wspcr | 438 | Werren and Windsor (2000) |
| *fbpA* | FbpA_F1/FbpA_R1 | 509 | Baldo et al. (2006) |

Table 3. Number of males and females obtained from broods reared in the laboratory for the four European species of the *Jaera albifrons* complex. Column "sexed" gives the number of individuals that survived and were sexed, out of the total number of offspring isolated at birth (*n*). Departure from an even sex ratio (proportion of males) was tested using a binomial test within each site (*** p<0.001).

| Species | *n* | sexed | males | females | sex ratio |
|---|---|---|---|---|---|
| *J. albifrons* | 655 | 497 | 240 | 257 | 0.48 |
| *J. praehirsuta* | 211 | 116 | 61 | 55 | 0.53 |
| *J. ischiosetosa* | 130 | 110 | 32 | 78 | 0.29*** |
| *J. forsmani* | 353 | 323 | 171 | 152 | 0.53 |

**Figure legends**

Figure 1: Sex ratio (proportion males) in the four European species of the *Jaera albifrons* complex. Grey bars indicate 95% binomial confidence intervals around the observed sex ratio. All species but *J. praehirsuta* were found to be significantly female-biased at the adult stage in natural populations (sample sizes in tables 1 and 6). Only *J. ischiosetosa* presented a female-biased sex ratio in young adults reared in the laboratory (a good proxy for the sex ratio at birth when mortality is low, as in this study).

Figure 2: Distribution of offspring sex ratio per brood in *Jaera albifrons* (*n*=131 broods), *J. praehirsuta* (*n*=134), first-generation hybrids (*n*=17), and backcross hybrids (first generation female hybrids crossed with either *J. albifrons* or *J. praehirsuta* males, *n* = 58). All broods were obtained from experimental crosses where both the mother and the father were known.

Figure 3: Neighbour joining tree of some Supergroup-B *Wolbachia* sequences, based on Kimura 2-parameters distances. Strains infecting marine hosts are represented in black, and the *Wolbachia* sequence that we found in *J. albifrons* and *J. praehirsuta* is highlighted. Other *Wolbachia* sequences identified in non-marine hosts are represented in grey. The strain that we used as a positive control is identified with an asterisk. The definition of the "RUG" group follows Cordaux et al. (2012). Bootstrap support inferred from 1000 replicates is shown when it is greater than 50%.

Figure 1

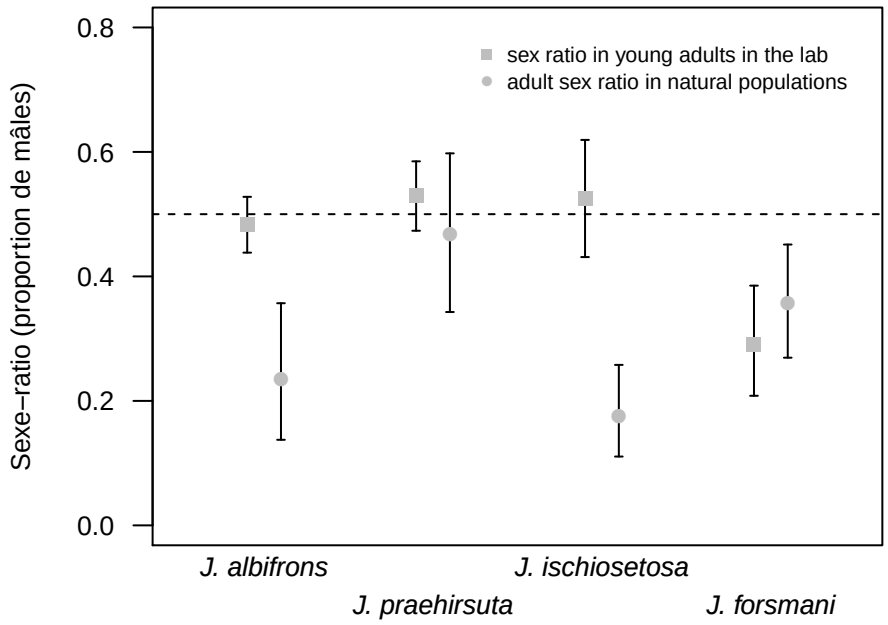

0.8
0.6
0.4
0.2
0.0
Sexe−ratio (proportion de mâles)
sex ratio in young adults in the lab
adult sex ratio in natural populations
J. albifrons
J. praehirsuta
J. ischiosetosa
J. forsmani

Figure 2

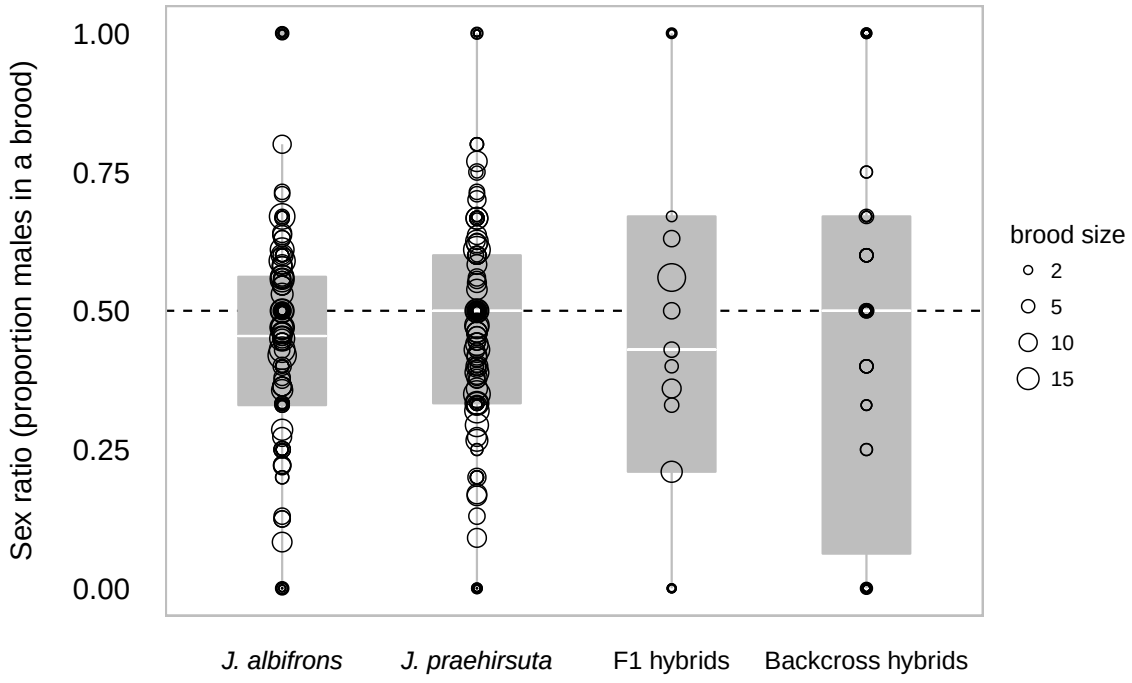
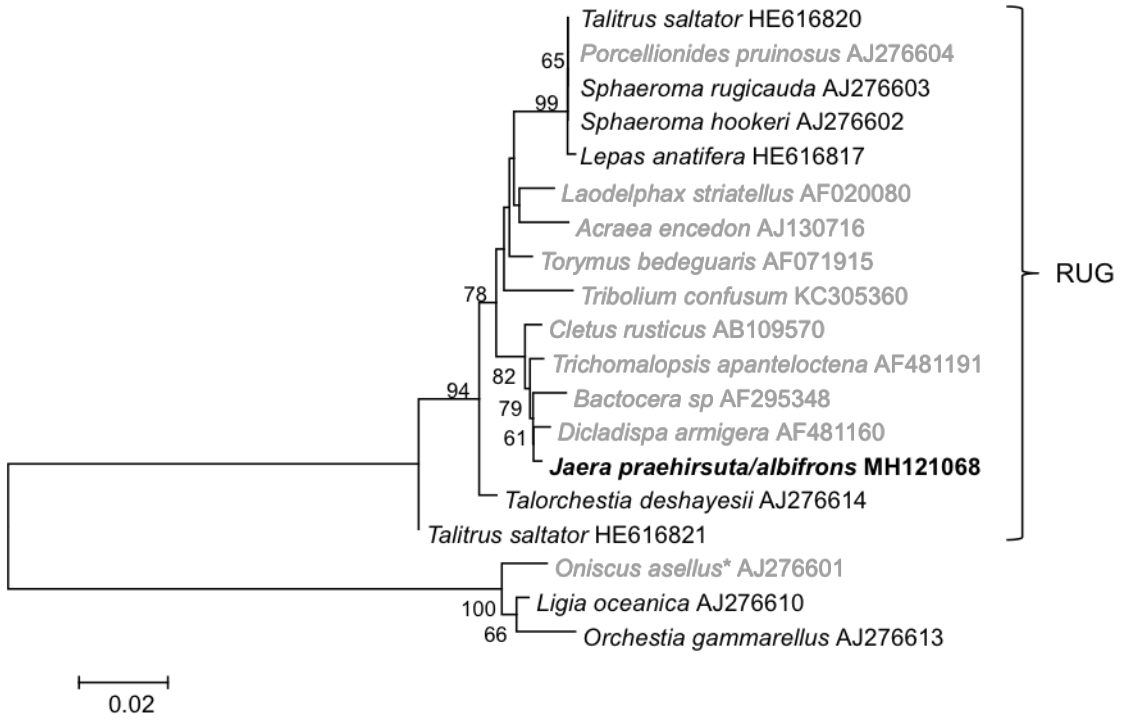

Sex ratio (proportion males in a brood)
1.00
0.75
0.50
0.25
0.00
J. albifrons
J. praehirsuta
F1 hybrids
Backcross hybrids
brood size
2
5
10
15

Figure 3


Talitrus saltator HE616820
Porcellionides pruinosus AJ276604
Sphaeroma rugicauda AJ276603
Sphaeroma hookeri AJ276602
Lepas anatifera HE616817
Laodelphax striatellus AF020080
Acraea encedon AJ130716
Torymus bedeguaris AF071915
Tribolium confusum KC305360
Cletus rusticus AB109570
Trichomalopsis apanteloctena AF481191
Bactocera sp AF295348
Dicladispa armigera AF481160
Jaera praehirsuta/albifrons MH121068
Talorchestia deshayesii AJ276614
Talitrus saltator HE616821
Oniscus asellus* AJ276601
Ligia oceanica AJ276610
Orchestia gammarellus AJ276613
RUG
65
99
78
94
82
79
61
100
66
0.02

**Supplementary material**

**Rearing conditions**

Lab conditions were as follows. All individuals were reared individually in 6-well plates (one individual per well, each well has a diameter of approximately 3.5 cm and contains ca. 10 mL of 3 µm-filtered seawater). Following the conditions described in Bocquet (Bocquet 1953: 212-213), each well contained small pieces of green algae (*Enteromorpha* sp.) and a small piece of elm leaf (elm leaves provide shelter and food, and have been used successfully for several decades by previous researchers). The plates were kept in thermostat cabinets at 17°C with an 11h/13h light/obscurity cycle and seawater was changed once a week (together with algae and leaves when needed). Embryos develop in a marsupium (brood pouch containing typically around 15 offspring) until the female releases them. At that stage the offspring measure approximately half a millimetre and closely resemble the adults (direct development, no pelagic larval stage). Offspring were isolated one by one within a few days after their release and reared individually in the same conditions as the adults, with two exceptions: seawater was not changed during the first week, and elm leaves were added only during the second week. Under these conditions, individuals can usually be sexed around the age of 4 to 5 weeks but all experiments lasted for a longer time. Since each individual was reared individually (and thus the number of offspring per brood was counted) the survival rate could be estimated for each brood.

**Salt extraction protocol**

Entire individuals were mashed then tissues were soaked into 500 µl of NaCl buffer (containing 1% SDS, 150 mM NaCl, 1 mM EDTA and 20 mM TRIS) with 1 mg of proteinase-K (Macherey-Nagel, Duren, Germany) and incubated at 56°C during 3h30 under agitation at 120 rpm. 0.5 mg of ribonuclease A (Macherey-Nagel, Duren, Germany) was added to each tube and incubated for 20 min under agitation at room temperature. 200 µl of NaCl buffer (5M) was then added and the tubes were

centrifuged at 16,000 g for 10 min. Supernatant was pipeted and mix to an equal volume of cold isopropanol and stored overnight at -20 °C. After centrifugation at 16,000 g for 15 min, the DNA pellet was washed with 500 µl of 80% cold ethanol and with 70% cold ethanol subsequently. DNA pellets were then dried and again suspended in 100 µl of TE 0.1X.

**Table S1.** List of the samples tested with the different protocols

| location | species | sex | num. of indiv. | *16S-2* | *Fbp-A* | *Wsp-1* | *Wsp-2* | *Wsp-L* | *Wsp-J* | *Wsp-1 Wsp-2* | *Wsp-2 Wsp-2* | *Wsp-L Wsp-L* | *Wsp-J Wsp-J* | *Wsp-L Wsp-J* | *TOTAL* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| France - Brittany (5 sites) | *J. albifrons* | F | 32 | 15 | 2 | 8 | 16 | 25 | 0 | 8 | 1 | 0 | 0 | 0 | 75 |
| | *J. albifrons* | M | 86 | 6 | 0 | 13 | 5 | 74 | 45 | 13 | 1 | 3 | 0 | 0 | 160 |
| | *J. praehirsuta* | F | 25 | 1 | 16 | 8 | 8 | 25 | 9 | 8 | 0 | 4 | 9 | 1 | 89 |
| | *J. praehirsuta* | M | 55 | 3 | 0 | 9 | 0 | 51 | 26 | 9 | 0 | 9 | 0 | 0 | 107 |
| | *J. ischiosetosa* | F | 33 | 0 | 0 | 0 | 6 | 32 | 0 | 0 | 2 | 29 | 0 | 30 | 99 |
| | *J. ischiosetosa* | M | 39 | 0 | 0 | 3 | 2 | 39 | 8 | 3 | 0 | 28 | 0 | 16 | 99 |
| | *J. forsmani* | F | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| | *J. forsmani* | M | 15 | 0 | 0 | 8 | 0 | 15 | 8 | 8 | 0 | 3 | 0 | 0 | 42 |
| | NA | F | 7 | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 14 |
| France - Normandy (2 sites) | *J. albifrons* | F | 23 | 1 | 0 | 15 | 16 | 17 | 15 | 15 | 0 | 4 | 15 | 4 | 102 |
| | *J. albifrons* | M | 163 | 3 | 13 | 6 | 0 | 161 | 0 | 6 | 0 | 12 | 0 | 0 | 201 |
| | *J. praehirsuta* | F | 20 | 15 | 0 | 1 | 12 | 16 | 16 | 1 | 0 | 2 | 16 | 2 | 81 |
| | *J. praehirsuta* | M | 79 | 0 | 7 | 11 | 0 | 77 | 0 | 11 | 0 | 2 | 0 | 0 | 108 |
| | *J. albifrons* / *J. praehirsuta* inter. morph. | M | 23 | 2 | 8 | 0 | 0 | 23 | 0 | 0 | 0 | 1 | 0 | 0 | 34 |
| | *J. forsmani* | M | 2 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 4 |
| | NA | F | 74 | 1 | 0 | 0 | 73 | 4 | 0 | 0 | 70 | 0 | 0 | 0 | 148 |
| England - Scilly Islands | *J. albifrons* | M | 7 | 0 | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 1 | 0 | 0 | 8 |
| | *J. praehirsuta* | M | 12 | 0 | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 12 |
| | NA | F | 10 | 0 | 0 | 0 | 8 | 10 | 0 | 0 | 0 | 3 | 0 | 0 | 21 |
| Norway (1 site) | NA | F | 43 | 0 | 0 | 0 | 43 | 5 | 0 | 0 | 43 | 0 | 0 | 0 | 91 |
| Canada - East coast (2 sites) | *J. albifrons* | M | 2 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 4 |
| | *J. ischiosetosa* | M | 5 | 0 | 0 | 5 | 2 | 5 | 0 | 5 | 0 | 0 | 0 | 0 | 17 |
| | *J. posthirsuta* | M | 8 | 0 | 0 | 8 | 0 | 8 | 0 | 8 | 0 | 0 | 0 | 0 | 24 |
| | NA | F | 53 | 0 | 0 | 0 | 53 | 4 | 0 | 0 | 53 | 0 | 0 | 0 | 110 |
| TOTAL | | | 817 | 47 | 47 | 95 | 252 | 615 | 129 | 95 | 177 | 102 | 40 | 53 | |

**Table S2.** Marine isopod and amphipod species tested for *Wolbachia*

| Species | Order | Number of individuals |
|---|---|---|
| *Jaera hopeana* | Isopoda | 8 |
| *Jaera nordmanni* | Isopoda | 4 |
| *Janira maculosa* | Isopoda | 5 |
| *Sphaeroma serratus* | Isopoda | 1 |
| *Haploops nirae* | Amphipoda | 8 |
| *Ampelisca spinipes* | Amphipoda | 8 |

**Table S3.** PCR mix composition for the six protocols used in this study.

| Name | MgCl2 (mM) | primers (µM) | dNTPs (mM) | Taq (U) | Template DNA (µl) | Final Volume (µl) |
|---|---|---|---|---|---|---|
| *16S-2* | 1.5 | 0.28 | 0.2 | 1 | 3 | 20 |
| *fbpA* | 1.5 | 1 | 0.2 | 1 | 3 | 20 |
| *Wsp-1* | 2.5 | 0.5 | 0.25 | 1 | 3 | 20 |
| *Wsp-2* | 1.5 | 0.28 - 0.8 | 0.05 | 0.75 | 2 | 15 |
| *Wsp-L* | 2 | 0.5 - 1 | 0.2 | 1 | 0.6 | 20 |
| *Wsp-J* | 2 | 0.5 - 1.33 | 0.2 | 1 | 0.6 | 20 |

**Table S4.** PCR program details; T: Temperature (°C); D: Duration (sec). A touchdown program was used for 16S-2 (presented on two lines.

| Name | Initial denaturation | | Denaturation | | Annealing | | Elongation | | Nb of cycles | Final elongation | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | T | D | T | D | T | D | T | D | | T | D |
| 16S-2 | 95 | 120 | 95 | 120 | 60 | 60 | 72 | 60 | 2 | NA | NA |
| | | | 95 | 30 | 60 | 60 | 72 | 45 | 35 | 72 | 300 |
| fbpA | 94 | 120 | 94 | 30 | 59 | 45 | 72 | 90 | 36 | 72 | 600 |
| Wsp-1 | 94 | 120 | 94 | 60 | 55 | 60 | 72 | 60 | 35 | 72 | 600 |
| Wsp-2 | 94 | 180 | 94 | 30 | 55 | 30 | 72 | 60 | 35 | 72 | 600 |
| *Wsp-L* | 98 | 30 | 98 | 15 | 55 | 20 | 72 | 60 | 50 | 72 | 600 |
| *Wsp-J* | 98 | 30 | 98 | 15 | 54 | 20 | 72 | 60 | 35 | 72 | 600 |

**Figures**

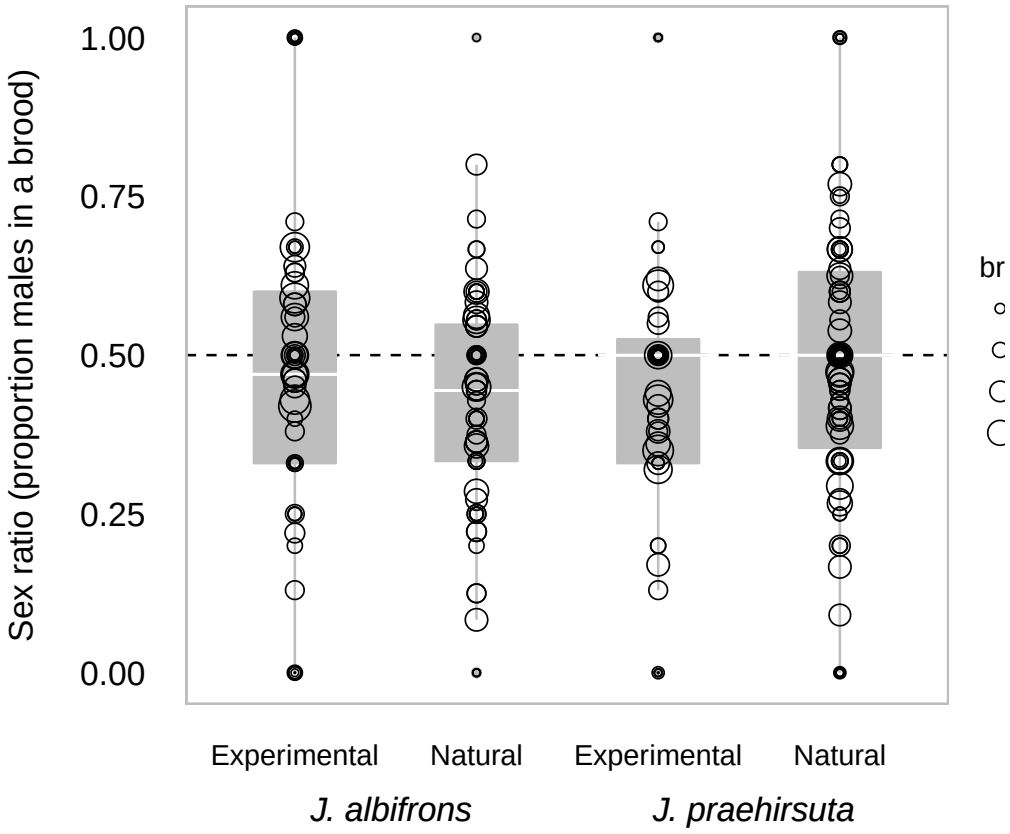



**Figure S1.** Distribution of offspring sex ratio per brood in *Jaera albifrons* (59 experimen natural broods) and *J. praehirsuta* (47 and 87). Each brood was reared in the lab until in were sexed, but the brood came either from a female sampled in the wild ("Natural") o female that was itself born and reared in the lab until maturity and used in a controlled experiment ("Experimental"). This figure combines data from the two regions where *J.* *J. praehirsuta* were studied (Brittany and Normandy, see maintext).

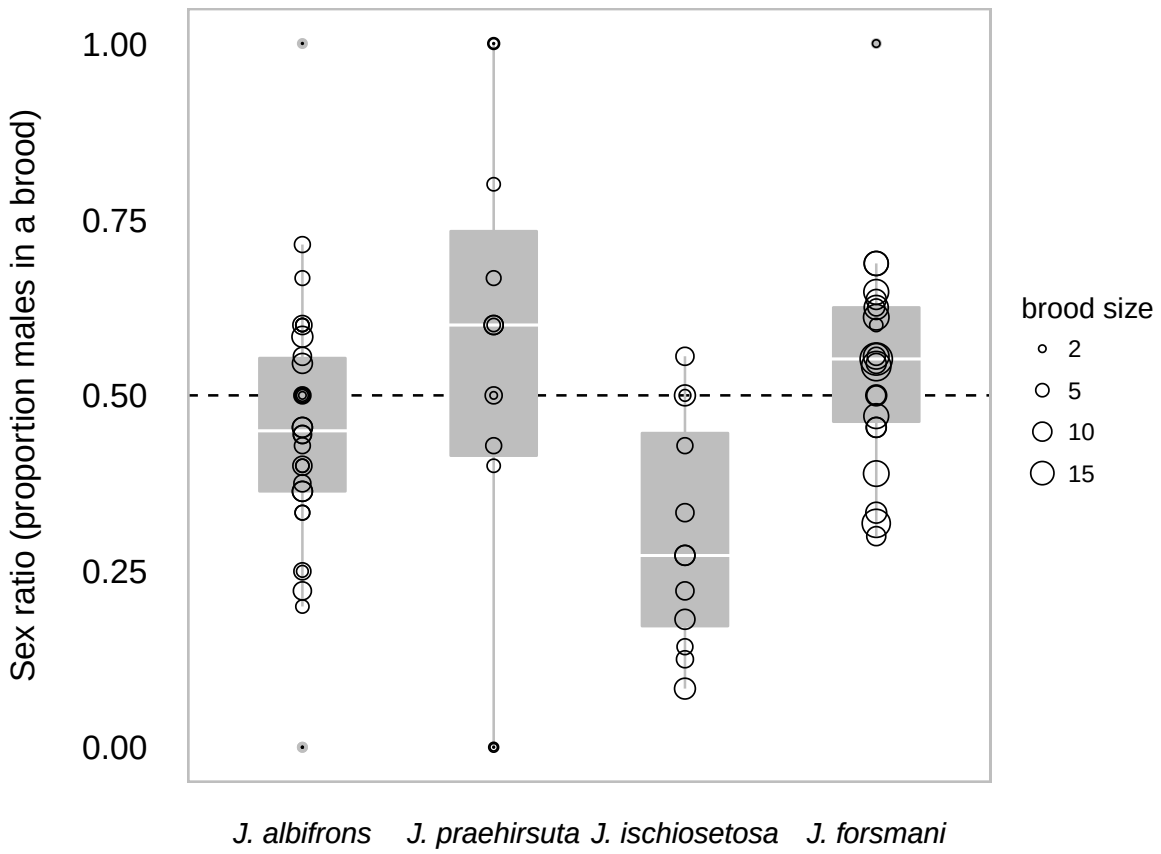



**Figure S2.** Distribution of offspring sex ratio per brood in *Jaera albifrons* (*n*=34 broods), *J. praehirsuta* (*n*=15), *J. ischiosetosa* (*n*=12), and *J. forsmani* (*n*=23). These broods were produced by mothers sampled from natural populations in Brittany (i.e. uncontrolled mating, fathers unknown). The average brood sex ratio in *J. ischiosetosa* is significantly biased towards females ($r$ = 0.29, binomial test $p$-value < 0.001).

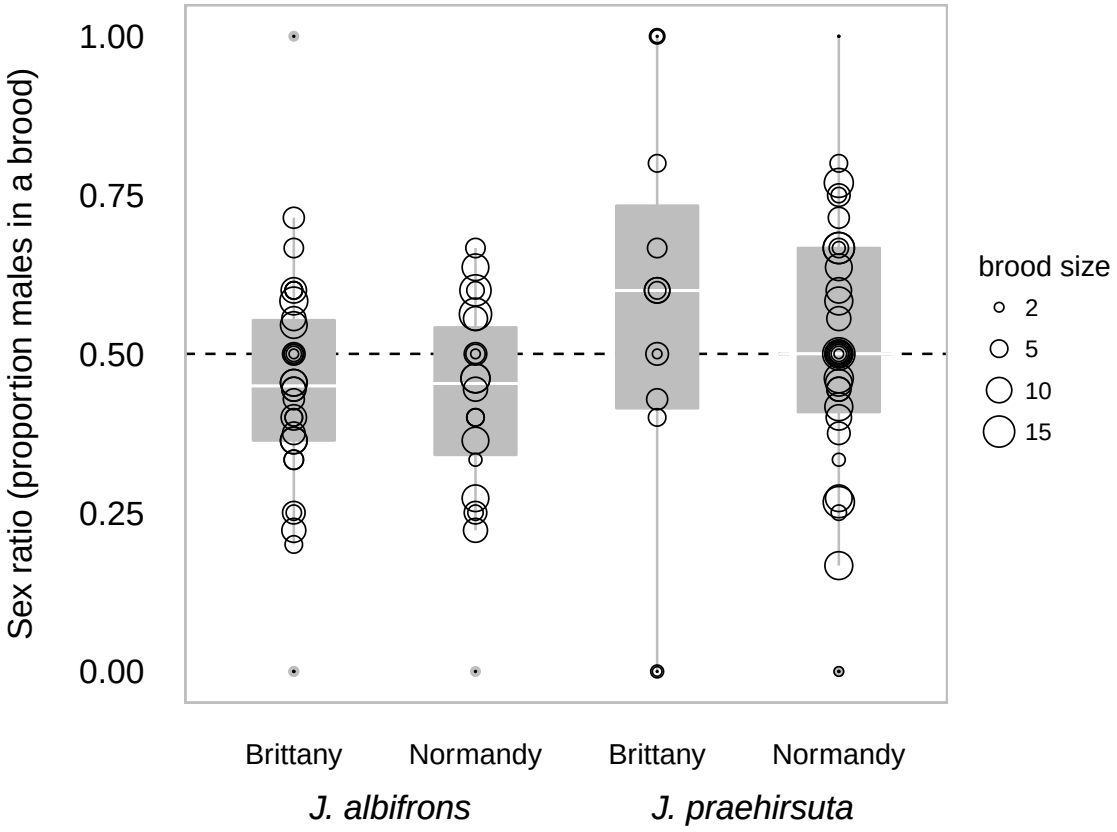



**Figure S3.** Distribution of offspring sex ratio per brood in *Jaera albifrons* and *J. praehirsuta* in a region where the two species are reproductively isolated (Brittany, $n_{albifrons}$= 34 broods, $n_{praehirsuta}$= 15) and in a region with introgressive hybridization (Normandy, $n_{albifrons}$= 22, $n_{praehirsuta}$= 39). These broods were produced by mothers sampled from natural populations (i.e. uncontrolled mating, fathers unknown).

**References**

Bocquet, C., 1953. Recherches sur le polymorphisme naturel des *Jaera Marina* (Fabr.)(Isopodes Asellotes) : Essai de systématique évolutive. Centre national de la recherche scientifique.