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Sexual isolation with and without ecological isolation in marine isopods *Jaera albifrons* and *J. prae-hirsuta*.

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Abstract

Sexual barriers associated with mate choice are often found to be associated with some level of ecological isolation between species. The independence and relative strength of sexual isolation are thus difficult to assess. Here we take advantage of a pair of marine isopod species (*Jaera albifrons* and *J. prae-hirsuta*) that show sexual isolation and coexist in populations where they share the same microhabitat or not (i.e. without or with ecological isolation). We estimated the strength of sexual isolation between *J. albifrons* and *J. prae-hirsuta* using no-choice trials and a multiple-choice experimental population. We found that sexual isolation is strong in both the presence and absence of ecological isolation, but that it is asymmetric and fails to prevent interspecific gene flow entirely. First-generation intrinsic post-zygotic barriers were low, and there was no sexual isolation within *J. prae-hirsuta* across habitats. The *J. albifrons* / *J. prae-hirsuta* species pair thus provides an example where the role of sexual isolation as a barrier to gene flow i) does not depend upon current ecological isolation, ii) seems to have evolved independently of local ecological conditions, but iii) is insufficient to complete speciation entirely on its own.

30 Introduction

Sexual isolation resulting from divergence in mating choice is common between closely related animal species, and the evolution of this type of barrier is considered to be a major component of speciation (Coyne & Orr, 2004). However, sexual barrier effects are often found in conjunction with some level of ecological isolation, raising the following questions (discussed e.g. in Ritchie, 2007; 35 Maan & Seehausen, 2011): What are the current relative contributions of sexual and ecological barrier effects in reproductive isolation between animal species? How often, if ever, has sexual isolation initiated speciation rather than evolving secondarily to ecological isolation? And may sexual isolation be an independent driver of speciation or is the evolution of sexual barriers necessarily linked with that of ecological isolation? Similar questions extend to other isolating barriers as well, 40 but we focus here on the relationship between sexual and ecological isolation, and more precisely on the last question, pertaining to the interdependence of these two isolating barriers.

Sexual isolation may be tightly linked with ecological isolation for several reasons (see Butlin & Smadja, 2018 for a discussion of coupling mechanisms and their importance in speciation). A direct form of coupling happens when some genes or traits affect ecological and sexual barriers at once 45 (reviewed in Servedio *et al.*, 2011). In *Heliconius* butterflies, for example, wing colour patterns affect both mimicry and mating signals, resulting in a strong combination of ecological and sexual barrier effects (Jiggins *et al.*, 2001). Along the same line, sexual and ecological barriers will coevolve when they involve overlapping metabolism networks, or sets of genes that are physically linked on the genome.

50 Furthermore, when there is no such intrinsic interdependence between barrier effects, other forms of coupling can happen if environmental conditions promote ecological isolation and simultaneously affect sexual isolation mechanisms. Such situations occur when the environment has an effect on male phenotypes, mortality costs associated with sexual display or choosiness, or the transmission of sexual signals (e.g host-dependent sexual signalling in *Enchenopa* treehoppers 55 McNett & Coccoft, 2008). All these cases may lead to the simultaneous evolution of sexual and

ecological isolation (reviewed in Maan & Seehausen, 2011; Nosil, 2012; Safran *et al.*, 2013; Boughman & Svanback, 2017; Servedio & Boughman, 2017). This list should even be extended if one considers not only the behavioural aspects of sexual isolation but also gametic isolation. The impact of ecological differentiation appears ubiquitous and one may wonder in what conditions can sexual
60 isolation evolve independently from ecological isolation.

Sexual selection mechanisms such as the Fisher-Lande process of coevolution between arbitrary male traits and female preferences can theoretically drive reproductive isolation largely independently of environmental heterogeneity and ecological barrier effects. This is also perhaps possible in some cases when good genes or compatible genes systems drive sexual isolation between
65 populations. An objective of empirical research is thus to explore how sexual isolation mechanisms are connected to ecological conditions and preferences, and evaluate to what extent sexual isolation may act as an independent driving force in speciation.

Hybrid zones provide good opportunities to investigate the interplay between different types of isolating barriers, and the most detailed information should come from situations where one can
70 investigate the role of sexual isolation in different ecological contexts. This is possible in mosaic or otherwise replicated hybrid zones (reviewed in Harrison & Larson, 2016) where hybridizing taxa meet repeatedly in different locations. In such cases, when sexual isolation was proven to be a critical barrier to gene flow it appeared to be nonetheless strongly dependent upon ecological conditions. This is either because sexual and ecological isolation involved the same traits, or sexual isolation
75 mechanisms were linked with ecological conditions. Habitat heterogeneity may then have driven ecological isolation and sexual isolation simultaneously (e.g. speciation in *Gasterosteus* sticklebacks and *Pundamilia* cichlids driven by adaptation of female perceptual sensitivity to ambient light combined with sexual selection on male colour, Boughman, 2001; 2002; Seehausen *et al.*, 2008). In these examples, sexual isolation is likely to break down when ecological conditions are homogenized
80 (Seehausen, 2009, see also Taylor *et al.*, 2006). There are also many other cases of mosaic or replicated hybrid zones where sexual isolation is not fully understood but where ecological isolation

or spatial segregation, regardless of other isolating barriers, appeared fundamental to the maintenance of reproductive isolation (e.g. field crickets, Harrison & Rand, 1989; marine mussels, Bierne *et al.*, 2003; swordtail fish, Culumber *et al.*, 2011; river herrings, Hasselman *et al.*, 2014; lampreys, Rougemont *et al.*, 2015).

There are comparatively few cases of replicated hybrid zones where sexual isolation appears to be strong and essentially independent of ecological isolation. A potential example is the hybrid zone between *Chorthippus* grasshoppers in northern Spain, where female mate choice based on male calling songs generates strong premating isolation that seems independent from ecological differentiation, at least in some populations (Bridle *et al.*, 2001; Bridle *et al.*, 2002; Bridle *et al.*, 2006; but see Bailey *et al.*, 2012). Other examples come from situations where sexual isolation is reinforced by selection against hybridization. For instance, the hybrid zone between *Mus musculus musculus* and *M. m. domesticus* subspecies of the house mouse have been studied repeatedly in distant regions, confirming the role of sexual isolation regardless of geographic and ecological conditions (Smadja *et al.*, 2004; Smadja & Ganem, 2005; Bimova *et al.*, 2011).

When sexual isolation is found in conjunction with ecological isolation, it is interesting to understand the relative roles and interdependence between these two types of barriers. It informs us on the mechanisms that are currently shaping species boundaries, and in some cases on the origin and evolution of these mechanisms (Boughman, 2001; Jiggins *et al.*, 2001; Seehausen *et al.*, 2008).

Here we focus on isopods *Jaera albifrons* and *J. praehirsuta*, two closely related species that show strong sexual isolation and generally occupy distinct habitats but can also be found in a region where they coexist in the same habitat, therefore allowing us to ask whether sexual isolation persists in a situation where ecological isolation does not.

The two species *J. albifrons* and *J. praehirsuta* belong to the *Jaera albifrons* complex. This complex is composed by five species of small (2-5 mm) marine isopods that live on the shores of the temperate and cold waters on both sides of the North-Atlantic Ocean (Bocquet, 1953; Solignac, 1978). All species of the complex are phenotypically indistinguishable except for male secondary

sexual traits that are used for tactile courtship (Bocquet, 1953; Solignac, 1981). The males of each species have specific sets of setae and spines located at different places on their pereopods (Fig. 1) and they use these features to brush a particular region of the back of females to get them to engage in sexual intercourse. The males of both species share the same basic courtship behaviour, whereby they mount females in a head-to-tail position and exercise their brushes. Females accept or reject a male based on this tactile stimulus, and behavioural isolation is thought to ensure a nearly complete arrest of interspecific gene flow in nature (Solignac, 1978).

Sexual isolation is thus currently a very important barrier between these species, and it could have initiated speciation (Solignac, 1981). However, the five species of the complex also show some level of habitat segregation on the seashore. The marine intertidal zone is an interesting environment to study ecological isolation as it presents contrasted ecological conditions at a small spatial scale. The distribution of species composing the *Jaera albifrons* complex was found to vary with intertidal zonation, exposure, salinity, and substrate (Naylor & Haahtela, 1966; Jones, 1972). Most remarkably, while *J. albifrons* and *J. praehirsuta* occupy the same narrow belt of intertidal habitats along the American and European shores of the North-Atlantic Ocean, *J. albifrons* is primarily found under pebbles and stones while *J. praehirsuta* is primarily found on intertidal brown algae (at least along European coasts, Bocquet, 1953; Naylor & Haahtela, 1966; Naylor & Haahtela, 1967). These habitats are often in immediate proximity, and these preferences are not strict (Solignac, 1981; Ribardière *et al.*, 2017), but they still imply that ecological isolation is strong and thus its current relative contribution to total reproductive isolation must be important (because this is the first barrier to occur in the life-cycle, see Coyne & Orr, 2004 p. 58).

The *J. albifrons* / *J. praehirsuta* pair gives us an opportunity to examine the relative strength and interdependence of sexual isolation and ecological isolation because these two species that usually use distinct habitats were reported to exceptionally coexist in a population where they share the same habitat (under stones, that is, the primary *J. albifrons* habitat, Solignac, 1969b; a). This coexistence of the two species in a unique habitat was recently found to have persisted for decades

and to be more widespread than previously thought as it extends to several other sites at least in the
135 French region Normandy and in the United Kingdom (Ribardière *et al.*, 2017; see also Mifsud 2011).
Hybridization happens in these populations (Solignac, 1969a) and results in various levels of
introgression (Ribardière, 2017; Ribardière *et al.*, 2017), pointing toward reduced reproductive
isolation. Interestingly however, in these hybridizing populations most males bear sexual traits that
are clearly identified as belonging to one or the other species and intermediate phenotypes are
140 scarce, suggesting that reproductive isolation does not break down completely.

Ribardière *et al.* (2017) suggested that sexual isolation is one of the components allowing the
persistence of bimodality in spite of introgressive hybridization. If this hypothesis is correct and
sexual isolation does not disappear in absence of ecological isolation, then it would suggest that
sexual isolation has evolved without a direct dependence on ecological conditions and ecological
145 isolation.

Our main objective was to test whether sexual isolation persists in spite of introgressive
hybridization in populations showing no ecological isolation. To reach this objective we quantified
sexual isolation between *J. albifrons* and *J. praeheirsuta* using experimental "no-choice" crosses
between individuals that originated either from a region where ecological isolation is strong or a
150 region where ecological isolation is lacking and the two taxa hybridize. Using individuals from this
second region we also quantified sexual isolation in a multiple-choice experimental population where
females can escape males and there is competition between individuals, unlike in no-choice crosses
where mate rejection may be more constrained (e.g. Jiggins *et al.*, 2001).

In addition, no-choice crosses were also performed with individuals from across our two
155 regions in order to test whether sexual isolation could have evolved differently in different ecological
contexts. In particular, species *J. praeheirsuta* is found on markedly distinct shore substrates
(seaweeds vs pebbles) in our two study areas, giving us the opportunity to test for an effect of this
ecological difference on sexual isolation between populations within a unique species.

Finally, we took advantage of our experimental crosses to check for potential first-generation

160 intrinsic post-zygotic barrier effects. While such isolating barriers are more likely to operate at later generations, chromosomal differences have been reported in our two focal species (Staiger & Bocquet, 1956; Lécher & Prunus, 1971). Thus, we took the opportunity of our experiments to check for the possibility that such differences have an effect in first-generation hybrids.

Methods

165 *Species*

Contrary to males, females of the five species within the *Jaera albifrons* complex are morphologically indistinguishable (Bocquet, 1953; but see Khaitov *et al.*, 2007 for a discussion of potential phenotypic differences). Females follow the same reproductive cycle (total duration ca. 3 weeks) during which embryos develop in a marsupium (brood pouch) for about 12 days (Solignac, 170 1976). Development is direct, meaning that the dispersal capacity of *Jaera* species is certainly much more limited than that of other marine invertebrates with a pelagic larval phase. Offspring measure ca. 0.5 mm when they are released from the marsupium. Individuals become sexually mature and can be sexed within 4 to 5 weeks based on praeoperculum differentiation (e.g. Solignac, 1979).

175 *Experimental set-up*

Our study is based on the analysis of the reproductive output of virgin males and females used in intra- and inter-specific controlled mating experiments (set-up detailed in Fig. 2). In theory, whether or not juveniles are produced in these experiments could result not only from sexual barrier effects but also post-mating pre-zygotic or intrinsic post-zygotic barrier effects (e.g. inviability of 180 hybrid embryos). However, all past analyses of inter-specific crosses in the *Jaera albifrons* complex have shown that females either rejected hetero-specific males or produced a normal number of offspring. That is, females that produced no offspring did not mate (e.g. Solignac, 1978 p. 49), and females mated by a heterospecific male did not show any reduction in fecundity (e.g. Solignac, 1978 pp. 80-82). There is no postmating copulatory behavioural isolation or mechanical isolation (Bocquet, 185 1953 p. 297; Jones & Fordy, 1971; Veuille, 1978). The complete absence of offspring produced by a pair of individuals is thus a good indicator for sexual isolation (and most probably the behavioural component of sexual isolation, although gametic isolation has yet to be investigated in this group, see discussion).

To obtain virgin individuals of both sexes and both species, we first sampled (unidentified) females in natural populations where *Jaera albifrons* and *J. praehirsuta* coexist (Fig. 2). We chose populations where we knew from previous work (Solignac, 1978; Ribardière, 2017; Ribardière *et al.*, 2017) that the two species live on different substrates (pebbles vs seaweeds, populations from Brittany) or share the same substrate (pebbles, populations from Normandy). Then we individually reared in the lab the offspring produced by these females (which were fertilized by unknown males in nature prior to sampling) until they could be sexed and males could be identified. Females were sorted as *J. albifrons* or *J. praehirsuta* according to the sexual traits held by their brothers. At this stage we thus had a series of *J. albifrons* and *J. praehirsuta* virgin adults originating from populations with or without ecological isolation (region "Brittany" vs region "Normandy"). These individuals could then be used in the controlled experiments described below. Female sampling and experimental conditions are detailed in supplementary information.

No-choice crosses within each region

In order to understand reproductive isolation processes with or without ecological isolation, we first ran a series of crosses where one male and one female from the same region were paired and their reproductive output monitored. These crosses featured intraspecific and interspecific crosses using either a pair of individuals from Brittany (where ecological isolation is strong) or a pair from Normandy (where there is no ecological isolation).

We monitored 23 intraspecific and 17 interspecific crosses within each of these two conditions (that is, 40 crosses within each region of origin; details in Table 1). These numbers were somewhat constrained for three reasons. First, the number of individuals available for the experiment depended on the (unknown) species identity of the females sampled in the wild, their survival and fecundity in the lab, and the survival of their offspring (see preliminary steps in Fig. 2). Second, and more importantly, crosses were designed so that the male and the female that were paired never

shared the same mother. Third, each individual was used in a unique cross, so that each cross was an
215 independent replicate.

For each cross we recorded i) if it successfully produced offspring, ii) how long it took for the
first offspring to appear, iii) how many offspring were contained in each brood produced, and iv)
offspring survival at 35 days. These data were used to estimate reproductive isolation components as
described below. All analyses were performed in R v.3.3.3 (R Core Team, 2017).

220

Reproductive isolation estimated from no-choice crosses within each region

Ecological, sexual, and first-generation intrinsic post-zygotic components of reproductive
isolation were quantified following Sobel and Chen (2014) using estimators that vary between -1
(complete disassortative mating, probability of interspecific gene flow = 1) to 1 (complete
225 reproductive isolation, probability of interspecific gene flow = 0). To compare reproductive isolation
components in the presence vs absence of ecological isolation, all the computations described below
were performed independently using crosses featuring individuals "from Brittany" on one hand, and
"from Normandy" on the other hand (that is, two independent sets of analyses).

First, because *J. albifrons* and *J. praehirsuta* in Brittany do not have strictly non-overlapping
230 habitats, we used survey data from Ribardière *et al.* (2017) in the same study areas to quantify
ecological isolation as

$$RI_{eco} = 1 - (S/(S + U)) \quad (1)$$

where U was the proportion of individuals found on the primary habitat of their species and S
was the proportion of individuals found on the alternative habitat (i.e. S is the probability that an
235 individual is in a place where it will meet the other species, that is, "shared"). This equation gives an
estimate of the reduction in interspecific gene flow that would happen if individuals would mate
randomly within each habitat (i.e. ecological isolation only).

Second, we estimated three components of sexual and intrinsic post-zygotic isolation (listed in Table 2) from our experimental crosses. The strength of each reproductive isolation barrier i was

240 estimated as

$$RI_i = 1 - 2 \times (H_i / (H_i + C_i)) \quad (2)$$

where H_i and C_i refer to variables calculated for heterospecific and conspecific pairs (Sobel & Chen, 2014). For sexual isolation (RI_1), H and C were the proportions of inter- and intraspecific

245 crosses that successfully produced offspring. For components of post-zygotic isolation, H and C referred to brood size (number of offspring) or survival (proportion of offspring surviving at day 35)

observed from intra- and interspecific crosses (see column "parameter" in Table 2). Each of these components of reproductive isolation was thus estimated independently, giving the strength RI_i that each barrier would have if it were acting alone. Note that RI_1 , RI_2 , and RI_3 correspond here to different components of RI and should not be confused with similar notations in Sobel & Chen, 2014.

250 A 95% bootstrap confidence interval was calculated for each RI estimate by resampling 10000 times the observed data if sample sizes were not too small (i.e. S and U or H_i and $C_i \geq 14$, Table 2).

Total reproductive isolation was estimated using the product of H_i and C_i across all components of isolation (see details in supplementary information):

$$RI_{tot} = 1 - 2 \frac{S \times \prod_i H_i}{S \times \prod_i H_i + \prod_i C_i} \quad (3)$$

255 Finally, we estimated the absolute contribution of each individual barrier by subtracting the effect of previously acting barriers as (Sobel & Chen, 2014):

$$AC_i = RI_{[1,i]} - RI_{[1,i-1]} \quad (4)$$

With this definition, individual contributions AC_i can be seen as additive components of RI_{tot} .

260 *No-choice crosses across regions*

In addition to the 80 crosses described so far, we ran another series of 80 intraspecific and interspecific crosses (Table 1) pairing individuals from opposite regions (and thus opposite habitats in the case of *J. praeheirsuta*, which lives on algae in Brittany vs. under stones in Normandy). These

crosses were useful to test for an effect of habitat on sexual isolation within and between species.

265 They also provided a direct test that *J. albifrons* (or *J. praehirsuta*) from across our two separate regions actually belong to the same biological species, an implicit assumption of this study and most previous investigations with this system (Solignac 1969b; Ribardière et al. 2017). The reproductive output of these crosses was recorded as described above for no choice crosses within each region.

270 *Multiple-choice experiment*

The general aim of the multiple-choice experiment (Fig. 2) was to estimate sexual isolation based on the reproductive output of males and females *J. albifrons* and *J. praehirsuta* freely interacting in an experimental population (and thus experiencing intra-sexual competition and easier male avoidance by females, unlike in no-choice experiments). This experiment focused only on the situation where the two species occupy the same habitat in the wild and thus interact frequently.
275 Hence we used virgin adult individuals obtained from the no-choice crosses described above (region Normandy only), so that we could mix *J. albifrons*, *J. praehirsuta*, and F1 hybrids all obtained in the same controlled conditions and all originating from a region without ecological isolation (Fig. 2).

We used 15 females of each species, 17 males of each species, and 17 males produced by inter-specific crosses. We included F1 hybrid males (and more males than females altogether) in order to estimate sexual isolation in a situation where females would have free choice, by contrast with the no-choice design. These numbers were constrained by several parameters, including the fact that we avoided mixing related males and females (i.e. two males could be brothers, but we did not use males and females from within the same family). Moreover, 4 out of 17 “hybrid” males
285 turned out to have resulted from a cross between a brother and sister *J. praehirsuta* prior to the experiment and were removed from all analyses (see details in results and supplementary information). All remaining F1 hybrid males came from male *J. praehirsuta* x female *J. albifrons* crosses.

Here we outline the experimental set-up, which is presented in detail in supplementary
290 information. All adults were kept together in a small aquarium for 12 days. After that, all surviving
females were removed from the aquarium and kept individually until they produced offspring, which
were then reared individually. All adults were photographed before and after the experiment and
genotyped at 13 microsatellite loci (Ribardière *et al.*, 2015). All offspring were also genotyped at the
same loci. Photo-identification and genetic parentage assignment (using software Colony v2.0.6.1,
295 Jones & Wang, 2010) were used to identify adult females after the experiment (remember that
females of the two species cannot be distinguished otherwise) and identify the father of each
offspring.

In addition, the secondary sexual traits of all adult males (Fig. 1) were examined under a
microscope to determine their role in male mating success. This is useful in this experiment because
300 multiple-choice conditions give access to variance in male mating success with females of the two
species, and one can thus explore the link between male traits and sexual isolation. Male phenotypes
were summarized using principal component analyses (PCA) based upon 14 phenotypic variables
(see supplementary information). This approach produces linear combinations of traits that are more
efficient for investigating sexual isolation than multiple trait variables separately (Hohenlohe &
305 Arnold, 2010). We used coordinates on the first PCA axis to assess whether female mate choice
matched the distribution of male sexual traits within each species (building upon Ryan & Rand, 1993;
Arnold *et al.*, 1996).

Sexual isolation estimated from the multiple-choice experiment

310 We aimed to compare sexual isolation in these multiple-choice settings with that measured in
no-choice crosses. Hence in a first step we used the same theoretical framework (Sobel & Chen,
2014) to estimate sexual isolation RI_1 following equation (2) with H_1 and C_1 defined as the
proportions of inter-specific and intra-specific crosses that successfully produced offspring. These
proportions were calculated as the number of successful pairs divided by the number of potential

315 pairs that could possibly have formed. Note that because we initially introduced the same number of
males of each species in the experiment, using proportions (as above) or absolute numbers of
successful pairs (as in Sobel & Chen, 2014) would lead to the same result for RI_1 .

It turned out that sexual isolation was very strong: only one interspecific pair and two pairs
involving F1 hybrid males produced offspring, while all other successful pairs were conspecific (see
320 results). Hence downstream barriers involving brood size and survival were not quantified because
they would be based on too few samples.

In a second step, sexual isolation was estimated using a framework described by Rolan-Alvarez
and Caballero (2000) that applies to multiple-choice experiments. Rather than estimating the effect
of sexual isolation on interspecific gene flow (as in Sobel and Chen's method), this estimation
325 procedure aims to isolate the effect of mate choice as a cause of reproductive isolation. The
computation takes into account inequalities in mating frequencies rather than assuming that the two
species have the same propensity to mate. In addition, it can detect asymmetries in sexual isolation.
Note that this method can also be applied to no-choice data (Nosil *et al.*, 2002; Coyne *et al.*, 2005) if
they are obtained from equal numbers of no-choice trials of each type (unlike in this study, see Table
330 1).

To estimate sexual isolation we counted the number of male/female pairs of each type (e.g. *J.*
albifrons/J. albifrons, *J. albifrons/J. praehirsuta*, etc.) that successfully reproduced. Pair sexual
isolation (PSI) was estimated for every pair type following Rolan-Alvarez and Caballero (2000). This
method gives a conservative view of sexual isolation as it is defined for each pair type as the number
335 of observed pairs divided by the number of expected pairs given the actual mating success observed.
We then followed these authors' recommendation to estimate I_{PSI} , a modified joint isolation index
(Merrell, 1950) based on PSI statistics and that varies from 0 (no isolation) to 1 (complete isolation).
Details of the computation are described in Rolan-Alvarez and Caballero (2000) and Perez-Figueroa
et al. (2005). Values of PSI , I_{PSI} , and their statistical significance were computed using JMating v1.0.8
340 (Carvajal-Rodriguez & Rolan-Alvarez, 2006).

Results

Reproductive isolation within each region

Estimates of reproductive isolation are presented in Table 2. In region Brittany, Ribardière *et al.* (2017) reported 141 *J. albifrons* under pebbles vs 2 on algae, and 3 *J. praehirsuta* under pebbles vs 77 on algae. Ecological isolation can thus be estimated to $RI_{eco,Brittany} = 98\%$. Because this reproductive barrier is the first to occur in nature, all other barriers will have comparatively little effect in natural populations in this region. This is reflected in the values for absolute contributions (AC_i column in Table 2).

However, as shown in Table 2 and Figure 3, no-choice intraspecific crosses in Brittany were more successful (probability of success=0.9) than interspecific crosses (0.14, Fisher's exact test $p < 0.001$). These results indicate strong sexual isolation ($RI_{1,Brittany} = 0.73$). That is, sexual isolation would reduce interspecific gene flow by about 70% in situations where individuals of the two species would meet, as in the case for instance when bold *J. albifrons* individuals venture on algae, or *J. praehirsuta* on pebbles.

By contrast, there was no ecological isolation in mixed *J. albifrons* / *J. praehirsuta* populations from Normandy (Table 2, data from Ribardière *et al.*, 2017) and there we found sexual isolation to be half of that found in Brittany, although 95% confidence intervals were overlapping (probability of success = 0.73 vs. 0.33 for intra- and interspecific crosses, $RI_{1,Normandy} = 0.37$, Fig. 3). Yet in this region, sexual isolation was the first barrier to occur, and thus it effectively led to a strong reduction in interspecific gene flow ($AC_{1,Normandy} = RI_{1,Normandy} = 0.37$). This stands in contrast with the situation described above for Brittany where sexual isolation appeared stronger ($RI_{1,Brittany} = 0.73$) but had very little effect in nature ($AC_{1,Brittany} = 0.015$, Table 2).

The time that a pair of individuals took to produce offspring (Fig. S1) did not enter calculations of reproductive isolation following the framework of Sobel and Chen (2014), but it is interesting to note that intraspecific crosses produced offspring more quickly (41.2 days on average, $n=35$) than

interspecific crosses (58.9 days, $n=7$, generalized linear model GLM with quasi-Poisson family $p=0.042$). The time needed to produce offspring seemed also more variable among interspecific crosses (Fig. S1). These observations further support sexual isolation in the studied populations (see
370 discussion), but there were too few successful interspecific crosses to look at these data in each region separately.

Successful broods contained from 1 to 33 offspring (mean 7.9 ind.) and on average 79.8 % of the offspring were still alive at day 35. Detailed data for intra- and interspecific crosses within each region are presented in Table 2. These data did not indicate any first-generation post-zygotic barrier
375 effect due to reduced F1 hybrid inviability in either region (Table 2). Accordingly, pooling data from our two regions, brood size (Fig. S2) did not differ significantly between intraspecific ($n=35$ broods, mean 7.6 offspring per brood) and interspecific crosses ($n=7$ broods, mean 9.3 offspring per brood, GLM quasi-Poisson family, $p=0.5$). Although the few offspring produced by interspecific crosses gave little power for this test, we note that the point estimate for brood size was actually *higher* in
380 interspecific crosses. The same result was obtained for survival (Fig. S3, $n=265$ offspring from intraspecific crosses, 79% survival, $n=65$ offspring from interspecific crosses, 83% survival, GLM quasi-binomial family $p=0.6$).

No-choice crosses across regions

385 Experiments that crossed individuals from the same species but originating from distinct regions had a high success (28 out of 35 crosses produced offspring, Fig. 3) and this success was similar to that of intraspecific crosses within each region (Fisher's exact test $p=0.33$). Moreover, this high success was similar for *J. albifrons* pairs and *J. praeheirsuta* pairs (respectively 15 out of 19 and 13 out of 16 crosses produced offspring, $p=1$). That is, there was no difference in success when a male
390 and a female came from the same vs different regions, and this was true for each of the two species.

Focusing on the differences between intra- and interspecific crosses, all results obtained by crossing individuals from across two distinct regions were almost identical to the results presented

above for crosses within a region. Briefly, intra-specific crosses were more successful ($n=35$ crosses, probability of success = 0.8) than interspecific crosses ($n=35$, probability of success = 0.2, Fisher's exact test $p<0.001$, Fig 3), delay to offspring production was shorter in intraspecific ($n=28$ broods, 39.2 days on average) than interspecific crosses ($n=7$, 65.3 days, GLM quasi-Poisson $p=0.006$, Fig. S1), and there was no difference in brood size (intraspecific: $n=219$ offspring from 28 broods, 9.21 offspring per brood on average, interspecific: $n=68$ offspring from 7 broods, 11.7 offspring per brood, GLM quasi-Poisson $p=0.3$, Fig. S2) and survival at day 35, (intraspecific: 81% survival, interspecific: 79% survival, GLM quasi-binomial $p=0.8$, Fig. S3).

In summary, all differences in reproductive output from intra- versus inter-specific crosses were unaffected by the region of origin of the individuals.

Results from the multiple-choice experimental population

We could determine the reproduction patterns for 47 males (out of 51) and 22 females (out of 30), as detailed in the supplementary information. Out of 22 females, most (16) mated with a single male, while 5 had 2 mates and 1 had none (Fig. S4A). By contrast, the majority of males had no reproductive success (31 out of 47), while other males mated with 1 to 4 female partners. The distribution of reproductive success for males and females of each species is shown in Figs. S5 and S6.

A total of 26 male/female pairs produced offspring. As it turned out (Fig. 4), *J. albifrons* females reproduced only with *J. albifrons* males ($n=18$ pairs), while *J. praehirsuta* females reproduced with the three types of males: *J. praehirsuta* males ($n=5$), "hybrid" males, ($n=2$), and *J. albifrons* males ($n=1$). This distribution is not random (Fisher's exact test for independence between male and female species composing these pairs $p<0.001$) and leads to an estimate for sexual isolation between *J. albifrons* and *J. praehirsuta* equal to $RI_{1,exp.pop.}=0.92$ ($CI_{95} [0.75;1]$), a number that can be directly compared with $RI_{1,Normandy}=0.37$ obtained from no-choice crosses.

These results assume that all individuals had the same probability to mate. Rolan-Alvarez and Caballero's method based on the calculation of pair sexual isolation (*PSI*) is more conservative, since
420 it takes into account the effect of differences in mating success between individuals from each species (e.g. here *J. albifrons* individuals mated more frequently than others). Figure 4 shows the number of mating pairs of each possible type expected from random mating in absence of sexual isolation but taking into account the variation in mating frequencies. Estimates of *PSI* are given in Table S1. The global modified joint isolation index I_{PSI} (Rolan-Alvarez & Caballero, 2000) was equal to
425 0.46, while pair-specific values were $I_{PSI}^{albi/prae}=0.92$, $I_{PSI}^{albi/hybrid}=1$, and $I_{PSI}^{prae/hybrid}=0.38$, indicating strong isolation between *J. albifrons* and *J. praehirsuta*, and lower isolation between *J. praehirsuta* and hybrids. Bootstrap-based tests performed in JMating for all *PSI* and I_{PSI} estimates showed that only $I_{PSI}^{albi/prae}$ was statistically significant ($p=0.004$).

Most of the variation in parent phenotypes (male secondary sexual traits) could be reduced to
430 two PCA axes (61.8% and 18.3% of the variation explained, Fig. 5A). Using PCA axis 1 to summarize male sexual traits in a univariate space, we see in Figure 5B that the mates preferred by *J. albifrons* females appear to coincide with the distribution of male sexual traits of *J. albifrons*, while *J. praehirsuta* females mated with males showing a wider range of trait values.

435 Discussion

Sexual isolation was effective both with and without ecological isolation

We found strong sexual isolation both in a context where ecological isolation is nearly complete ($RI_{eco,Brittany}=98\%$, $RI_{1,Brittany}=73\%$) and in a context where there is no ecological isolation and individuals of the two species may hybridize ($RI_{eco,Normandy}=0$, $RI_{1,Normandy}=37\%$).
440 These RI values based on reproductive success in no-choice conditions suggest that sexual isolation is less effective when the two species co-occur in the same habitat (Normandy). However, the multiple-choice experiment showed that when the conditions allow females to escape males or choose amongst several males, then sexual isolation is in fact very efficient even in the hybridizing

populations from Normandy ($RI_{1,exp.pop.} = 92\%$). The exact same value was obtained using the joint
445 isolation index for *J. albifrons* and *J. prae-hirsuta* ($I_{PSI}^{albi/prae} = 0.92$), indicating that sexual isolation was
effectively due to mate choice rather than differences in mating propensity between species.

Moreover, most of our no-choice crosses were monitored for a long time (5 to 196 days,
median 22 days), meaning that females were confined with a given male in a tiny area for a long
period, and thus our estimates probably give us a lower bound on sexual isolation. We conclude that
450 sexual isolation remains strong in populations where the two species share the same habitat,
(despite hybridization and introgression, Ribardière, 2017; Ribardière *et al.*, 2017) and thus sexual
isolation in this system is largely independent of ecological isolation.

In addition, while males were identified as *J. albifrons* or *J. prae-hirsuta* based on phenotypes
that are directly relevant to reproductive isolation, this was not the case for females, which were
455 identified based on the phenotype of their brothers (see methods, Fig. 2, and supplementary
information). Hence in all our experiments we had no direct information on the sexual phenotype of
females (that is, preferences). This uncertainty on what really was a "*J. albifrons*" or a "*J. prae-hirsuta*"
female in our experiments makes it all the more remarkable that sexual isolation was found to be
strong, especially in the populations where the two species share the same habitat and hybridize.

460 Three potential caveats must be considered. First, we took offspring production in intra- vs
inter-specific crosses as an indicator of sexual isolation. This approach would be incorrect if intrinsic
post-zygotic barriers were involved. Here brood size did not differ between intraspecific and
interspecific crosses, and the abortion of an entire brood of embryos was never observed (oocytes
and developing embryos are visible in the marsupium of females). Hence, conforming to what had
465 been reported previously (Solignac, 1978), females in the presence of a heterospecific male either
produced no offspring because they did not mate, or produced a normal number of offspring. The
success of crosses (presence vs absence of juveniles produced) was thus a good indicator of sexual
isolation.

Second, the multiple-choice population was not replicated. Although clear-cut patterns were
470 observed, one cannot rule out that it was affected by an undetected experimental bias or that
individuals originating from other populations would behave differently. For instance, the 81
individuals mixed in the experiment were produced in the lab from 17 pairs of parents, and each
class of individuals (*J. albifrons* males, *J. albifrons* females, *J. praehirsuta* males, etc.) thus comprised
2 to 5 groups of siblings (1 to 9 siblings per family). Most notably, the fourteen *J. albifrons* females
475 that survived the experiment and reproduced came from only two families, which could have biased
the result if these 14 females were not representative of the range of female behaviour in natural
populations. No-choice crosses have shown that this is not the case (*J. albifrons* females were very
selective, both in intra-region and inter-region crosses), but repeating this experiment with more
individuals, and perhaps with individuals from other regions where the two species have been found
480 in the same habitat, would be particularly interesting (e.g. in the UK, Naylor & Haahtela, 1966;
Mifsud, 2011; Ribardière, 2017).

Did sexual isolation evolve independently of ecological contexts?

J. praehirsuta individuals in Brittany vs. Normandy dwell on different habitats (pebbles vs.
485 brown algae). However, intraspecific crosses had exactly the same success (and the same delay to
offspring production) whether or not males and females originated from the same region or different
regions (Figs. 3 and S1), and this success was the same as that of *J. albifrons* pairs (within or across
regions). Hence habitat differences did not generate any sign of sexual isolation between individuals
originating from algae vs. rock populations in species *J. praehirsuta*. Sexual isolation was unaffected
490 by the habitat of origin and thus we conclude that it did not evolve through sexual selection
mechanisms that are dependent of local ecological factors.

However, the ecological context could still have an effect on sexual isolation via two potential
mechanisms. First, to suit our purpose of estimating reproductive isolation in different populations,
all individuals were reared in identical, artificial lab conditions (see supplementary information)

495 where all five species of the *Jaera albifrons* complex had been shown to have high fitness (Bocquet
1953; Solignac 1978). This means in particular that all individuals used in cross experiments were
born and raised in the exact same conditions, regardless of their population of origin. Hence we
cannot exclude that contrasted substrates (algae vs. rocks) could have some proximate effect on
mate choice mechanisms, for instance through phenotypic plasticity in cuticular hydrocarbons or
500 other chemical cues involved in sexual signalling (see e.g. Breithaupt & Hardege, 2012, for a review
of chemical cues in aquatic organisms; and Zhu *et al.*, 2012 for an example involving sexual isolation).
Such chemical signalling mechanisms have yet to be investigated in the *Jaera albifrons* complex,
although we note that their effect on reproductive isolation would necessarily be somewhat limited
because sexual isolation is already strong in uniform lab conditions.

505 Second, as mentioned above we observed from no-choice trials that sexual isolation could be
twice as strong in populations where the two species are ecologically separated ($RI_{1,Brittany} = 73\%$,
 $RI_{1,Normandy} = 37\%$). One potential explanation is that mate choice is relaxed in introgressed
populations, but only in situations where mate options are constrained (in agreement with our
finding that sexual isolation was stronger in the multiple-choice experimental population
510 $RI_{1,exp.pop.} = 92\%$). Variations in the relative abundance and spatial distribution of individuals in
natural conditions may thus modulate the likelihood of interspecific mating. It is often argued that
this could happen when dynamic habitats modulate the intensity of sexual isolation by limiting mate
choice options, perhaps in some cases approaching no-choice conditions, if for example a female of
species A finds herself isolated for some time with males of species B only. This could happen in
515 some of our populations where the quantity of sand covering pebbles and stones varies with climatic
conditions across seasons and the density of individuals is low (e.g. Fig. S1 in supplementary material
from Ribardière *et al.*, 2017). Khaitov *et al.* (2007) also observed sharp variations in the relative
frequencies of *Jaera* species in mixed populations that were presumably attributable to the
destruction of nearby habitats.

520

Sexual isolation is not strict, and it is asymmetric

Sexual isolation was strong, but it was not 100%. Hence sexual isolation is not sufficient to prevent interspecific gene flow entirely on its own. As a result, when ecological isolation is lacking, hybridization is expected to happen occasionally.

525 Moreover, results from the multiple-choice experimental population showed sexual isolation to be asymmetric (Figs. 4 and 5). In this experiment, *J. albifrons* females mated exclusively with *J. albifrons* males while *J. praehirsuta* females mated with *J. praehirsuta* ($n=5$), *J. albifrons* ($n=1$) and F1 hybrid males ($n=2$). Accordingly, pair sexual isolation indices for heterospecific pairs were smaller (indicating stronger isolation) when the female involved was *J. albifrons* rather than *J. praehirsuta* 530 (Table S1). Summarizing male sexual phenotypes using the first axis of a principal component analysis (Fig. 5), we found that the probability that a male mated with a *J. albifrons* female matched relatively well the density distribution of *J. albifrons* male sexual traits. By contrast, the probability that a male mated with a *J. praehirsuta* female was clearly less concordant to the density distribution of *J. praehirsuta* male sexual traits (Fig. 5B). This shows that females *J. praehirsuta* mated with our three 535 categories of males (*J. praehirsuta*, F1 hybrids, and *J. albifrons*) because they are less selective than *J. albifrons* females with regards to male sexual traits (illustrative of "type d" preference function in Fig. 5 from Ryan & Rand, 1993).

This asymmetry is also confirmed by the no-choice experiments, where inter-specific crosses were systematically more successful with *J. praehirsuta* females. Over all interspecific crosses, 4 out 540 of 36 were successful when the female was *J. albifrons*, while 10 out of 28 were successful when the female was *J. praehirsuta* (Fisher's exact test $p=0.036$, Fig. 6). Interestingly, the same result was obtained by M. Solignac with no-choice experiments using individuals from a population from France (Brittany) crossed with individuals from Germany (0% success for German *J. albifrons* females crossed with French *J. praehirsuta* males vs 22 to 46% success for French *J. praehirsuta* females 545 crossed with German *J. albifrons* males, depending on the method of calculation, Solignac, 1978).

The asymmetry in sexual isolation is thus observed in all conditions: large-scale allopatry (between countries, Solignac, 1978), smaller-scale allopatry (between regions, this study), sympatry with ecological isolation and no hybridization (Brittany, this study), and sympatry without ecological isolation and with introgressive hybridization (Normandy, this study). This is interesting because it suggests that the asymmetry has a general cause of ancestral origin and has not evolved in response to local conditions, in agreement with our previous conclusion that sexual isolation has not evolved following mechanisms that depend on local habitat conditions.

Why do *J. albifrons* and *J. praehirsuta* coexist in hybridizing populations?

Individuals identified as *J. albifrons* in region Brittany (with ecological isolation) belong to the same biological species as individuals identified as *J. albifrons* in region Normandy (where there is no ecological isolation), and likewise for species *J. praehirsuta*. This was argued by taxonomists and evolutionary biologists based on the observation of secondary sexual traits (Bocquet, 1953; Prunus, 1968; Solignac, 1978) and confirmed in this study using cross experiments: conspecific crosses gave the same results within and across regions (no reproductive isolation between regions).

However, we also know that reproductive isolation is not complete in populations without ecological isolation, where hybridization leads to introgression (Bocquet & Solignac, 1969; Solignac, 1969a; 1978; Ribardière *et al.*, 2017). This situation raises questions about the conditions of coexistence of the two species in spite of hybridization and ecological equivalence (e.g. Coyne & Orr, 1998). Ribardière *et al.* (2017) discussed the peculiar nature of the hybridizing populations, emphasizing that they seem to receive no influx of individuals from pure parental populations, and that no fine-scale ecological differentiation was observed (the two species are repeatedly found to be intermingled in a number of different sites). The authors suggested sexual and post-zygotic isolation as two potential forces somehow acting to keep the species isolated. Here we found sexual isolation to be effectively strong in hybridizing populations, but no support for first-generation intrinsic post-zygotic mechanisms (AC_2 and AC_3 added to -0.04 and -0.06 in regions Brittany and

Normandy, Table 2). Moreover, this result was confirmed by between-regions crosses showing similar brood size and offspring survival for intra- and interspecific crosses. We could not quantify intrinsic post-zygotic isolation from the multiple-choice experimental population because only one
575 heterospecific mating pair and two backcrosses (F1-hybrid fathers) produced offspring. Yet these broods showed no sign of reduced fitness in any way (brood size and offspring survival, data not shown). Post-zygotic barrier effects need to be investigated further (especially in later generations), but so far, sexual processes (mate choice and/or intra-sexual interactions) constitute the only force identified that could maintain the two species separated in spite of extensive introgression. This
580 alone is unusual, and the fact that the two species are found to coexist in different places and on the long term despite ecological equivalence poses another conundrum that deserves further investigations.

Conclusion & open questions

585 In this study we looked at two closely related species that are reproductively isolated by ecological and sexual barriers, asking first if sexual isolation could maintain species integrity on its own or is only acting secondarily alongside ecological isolation, and second if sexual isolation may have evolved independently of local ecological context. We found that sexual isolation is a strong barrier that does not break down when ecological isolation is absent and most probably evolved
590 independently of local ecological conditions. The tactile courtship of *J. albifrons* and *J. praehirsuta* therefore probably exemplifies a situation where the set of genes and traits involved in male phenotypes and female preferences have no simultaneous role in local adaptation to different habitats and are not constrained by natural selection (no environmental effect on sexual traits, courtship behaviour, transmission of sexual signals, or choosiness). Under these conditions one can
595 expect sexual isolation to be largely independent of ecological isolation, as observed for instance in some *Chorthippus* grasshoppers (Bridle *et al.*, 2001; Bridle *et al.*, 2002) and *Laupala* crickets (Mendelson & Shaw, 2005) in which female mate choice is based on male calling songs. In some

Drosophila species, laboratory lines reared in artificial conditions provide another example where mate choice based on male courtship songs and pheromones (reviewed in Coyne & Orr, 1997) has no obvious ecological component (although this will not be the case of all *Drosophila* species, see e.g. Stennett & Etges, 1997; Etges, 2002). A few other potential examples have been reviewed by Svensson (2012). Will reproductive isolation be generally weaker in these conditions? Empirical examples reported in the literature show that reproductive isolation can be particularly strong when sexual isolation is inherently coupled with ecological isolation (Servedio *et al.*, 2011). Whether this is a general rule remains to be clarified.

Local ecological conditions could still affect the strength of sexual isolation if habitat dynamics modulate mate choice possibilities through stochastic effects on population densities. Whether such effects could constrain mate choice sufficiently to bypass sexual isolation and affect the likelihood of speciation completion remains an open question. More importantly, we found that reproductive isolation was not complete without ecological isolation. Both *J. albifrons* and *J. prae-hirsuta* are found in variable ecological contexts along the shores of the temperate and cold waters on both sides of the North-Atlantic Ocean and nearby seas. Whether or not there is niche differentiation may influence the local coexistence and hybridization of these species.

Finally, the conditions of coexistence of these two species in hybridizing populations are another open question. For that, further examination of post-zygotic barrier effects, fine-scale tests for cryptic habitat specialization, and interactions favouring density-dependence mechanisms (such as species-specific pathogen effects) may provide some answers.

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Author contributions

Conceptualization and methodology: AR and TB. Field sampling and species identification: AR,
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630 EP, JC, CH, SH, and TB. Genotyping and phenotyping: AR, EP, JC, CDT, CH and TB. Analyses and
writing: AR, EP, and TB. Supervision, project administration and funding acquisition: TB.

Conflict of interest disclosure

The authors of this preprint declare that they have no financial conflict of interest with the
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Data accessibility

The data associated with this study are available on the Dryad digital repository at
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Table 1 - Number of experimental "no-choice" crosses performed in order to estimate reproductive isolation between marine isopods *J. albifrons* and *J. praehirsuta* in two French regions where they are ecologically isolated (Brittany) or not (Normandy). Intra- and interspecific crosses were performed using males and females from the same region (intra-region) and different regions (inter-region). Inter-region crosses involved either females from Brittany (F. Britt) crossed to males from Normandy (M. Norm) or the opposite (F. Norm crossed to M. Britt).

Cross type	Female	Male	Intra-region		Inter-region	
			Brittany	Normandy	F. Britt. x M. Norm.	F. Norm. x M. Britt.
Intraspecific						
	<i>J. albifrons</i>	<i>J. albifrons</i>	17	6	10	10
	<i>J. praehirsuta</i>	<i>J. praehirsuta</i>	6	17	10	10
Interspecific						
	<i>J. albifrons</i>	<i>J. praehirsuta</i>	10	10	10	10
	<i>J. praehirsuta</i>	<i>J. albifrons</i>	7	7	10	10

Table 2 - Components of reproductive isolation between marine isopods *Jaera albifrons* and *J. praeheirsuta* in two regions with contrasting levels of isolation. Reproductive isolation (RI) was calculated following Sobel & Chen (2014). Bootstrap confidence intervals (CI₉₅) based on 10000 resampling of observed data are given whenever sample sizes were not too small (e.g. sample sizes for survival of interspecific offspring in Brittany were particularly small since interspecific crosses were rarely successful). RI gives the reduction of interspecific gene flow that would be caused by each specific barrier acting alone. AC gives the actual absolute contribution of each barrier given that other barriers are acting earlier in life cycle. The sum of AC over all barriers is equal to the total strength of reproductive isolation. Sexual isolation contains a strong behavioural component, but could also include a (so far untested) gametic component. RI due to F1 hybrid inviability is based on brood size (number of offspring produced by a mother after intra-marsupial development) and survival of these offspring after 35 days.

Barrier	Notation	Parameter	Brittany			Normandy				
			Data		RI [CI ₉₅]	AC	Data		RI [CI ₉₅]	AC
Ecological isolation	RI_{eco}	Probability of heterospecific encounter	5/222 ind. in shared habitat	217/222 ind. in unshared habitat	0.98 [0.955 ; 0.995]	0.977	80/80 ind. in shared habitat	0/80 ind. in unshared habitat	0.00	0.00
Sexual isolation	RI_1	Proportion of successful crosses	19/21 successful intrasp. crosses	2/14 successful intersp. crosses	0.73 [0.45 ; 1]	0.015	16/22 successful intrasp. crosses	5/15 successful intersp. crosses	0.37 [0.06 ; 0.73]	0.37
F1 hybrid inviability	RI_2	Number of offspring released per brood	6.2 ind. ($n=19$ intrasp. broods)	5.5 ind. ($n=2$ intersp. broods)	0.06	0.001	9.3 ind. ($n=16$ intrasp. broods)	10.8 ind. ($n=5$ intersp. broods)	-0.08	-0.07
	RI_3	Survival probability at 35 days	0.74 ($n=117$ intrasp. offsp.)	0.91 ($n=11$ intersp. offsp.)	-0.10	-0.001	0.83 ($n=148$ intrasp. offsp.)	0.81 ($n=54$ intersp. offsp.)	0.01 [-0.06 ; 0.09]	0.01
Total reproductive isolation	RI_{tot}				0.99	0.99			0.31	0.31

Figure 1

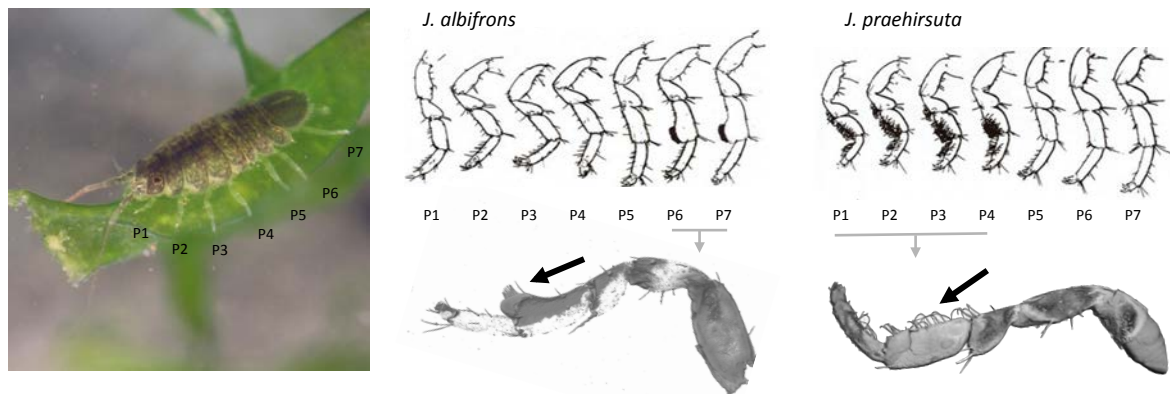


Figure 1

Sexual traits used for tactile courtship by male *Jaera albifrons* and *J. praehirsuta*. In male *J. albifrons*, the second segment (carpus) of peraeopods 6 and 7 extends as a lobe bearing a patch of setae (indicated by the black arrow on the left). Male *J. praehirsuta* instead have curved setae distributed on the first three segments (propus, carpus, merus) of peraeopods 1-4 (right black arrow), and one or two spines on the carpus of peraeopods 6 and 7. The photo on the left shows a 4mm-long adult female (photo credit to Guillaume Evanno & Thomas Broquet). The drawings are reproduced from Solignac (1981) with authorization. After fluorescent labelling of dissected appendices, close-up pictures were obtained with a confocal laser scanning microscope and processed using software Fiji and IMARIS (photo credit to Sébastien Colin).

Figure 2

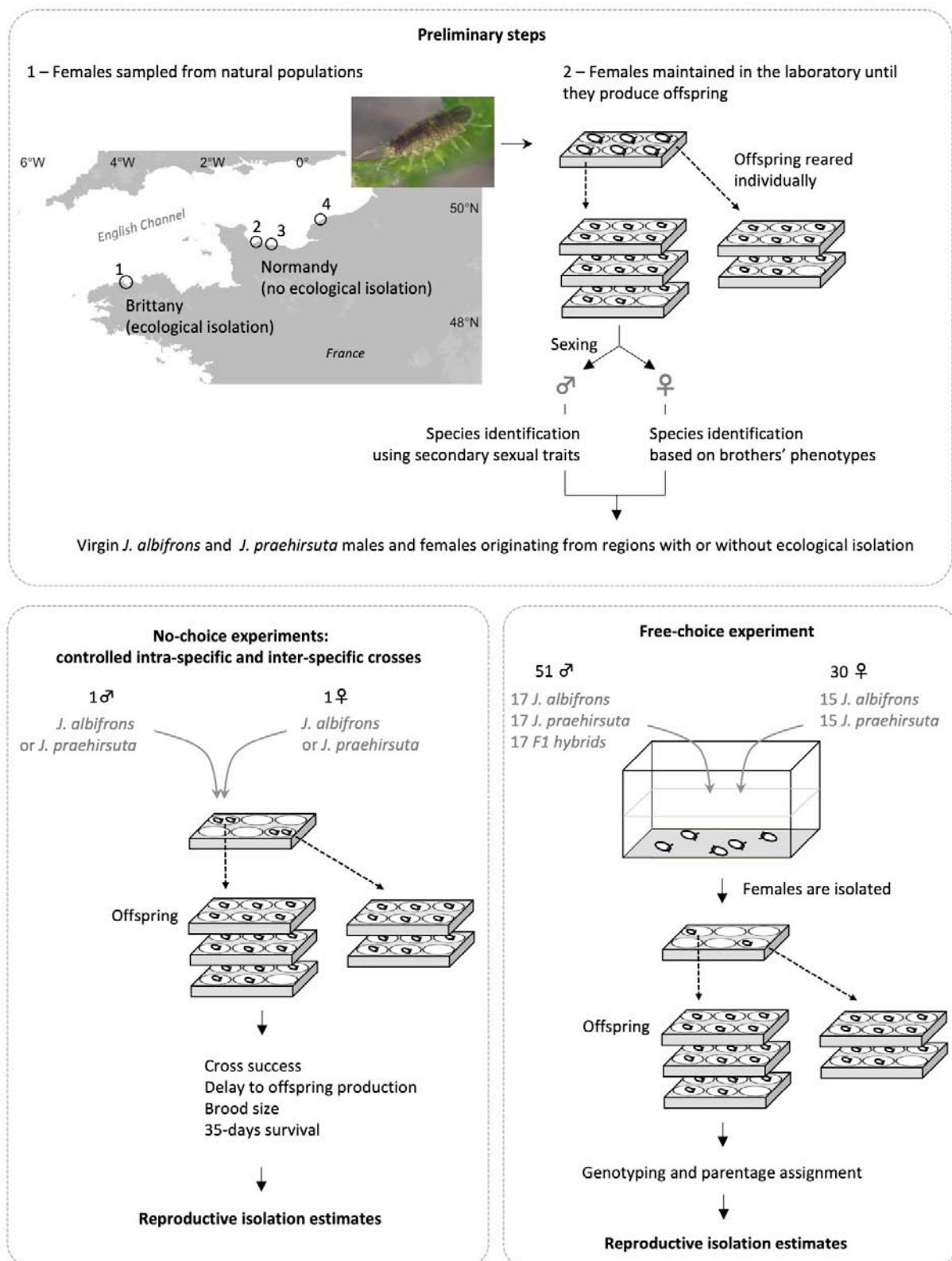


Figure 2 – Outline of experimental protocols. The upper panel presents the preliminary steps that were taken to obtain virgin males and females of each species, which could then be used in

controlled experiments. Adult females were sampled from natural populations as shown on the map. We knew from previous genetic analyses that local sympatric populations of *J. albifrons* and *J. prae-hirsuta* were reproductively isolated (region Brittany) or not (introgressive hybridization, region Normandy). Adult females were kept in the laboratory until they produced offspring that were then raised until the male offspring could be identified. Virgin individuals born in the lab were then chosen to be used in no-choice cross experiments. Individuals born and reared in the lab following these no-choice experiments were then used in a multiple-choice experimental population.

Figure 3

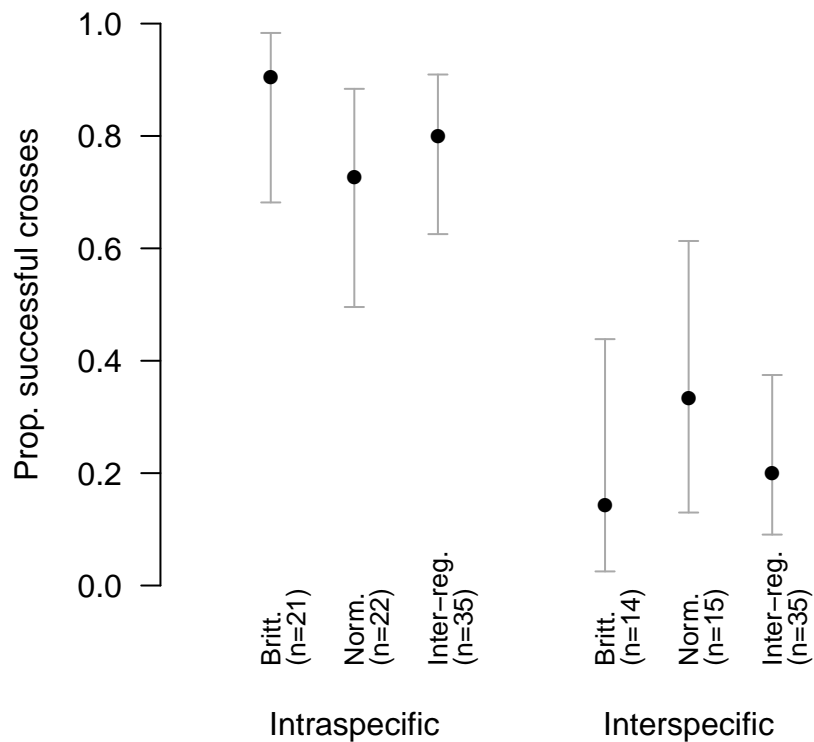


Figure 3 - Proportion of successful crosses in no-choice experiments involving intraspecific crosses (either *Jaera albifrons* or *J. praehirsuta*) and inter-specific crosses. The male and female of a given cross could come from the same region (Brittany or Normandy, see text) or from a different region (inter-reg. crosses). The sample sizes (number of experimental crosses) are given within brackets, and the bars give 95% confidence intervals around each observed proportion. A cross was successful if it produced at least one offspring. Interspecific crosses were consistently less successful than intraspecific ones, pointing towards sexual isolation between *J. albifrons* and *J. praehirsuta*.

Figure 4

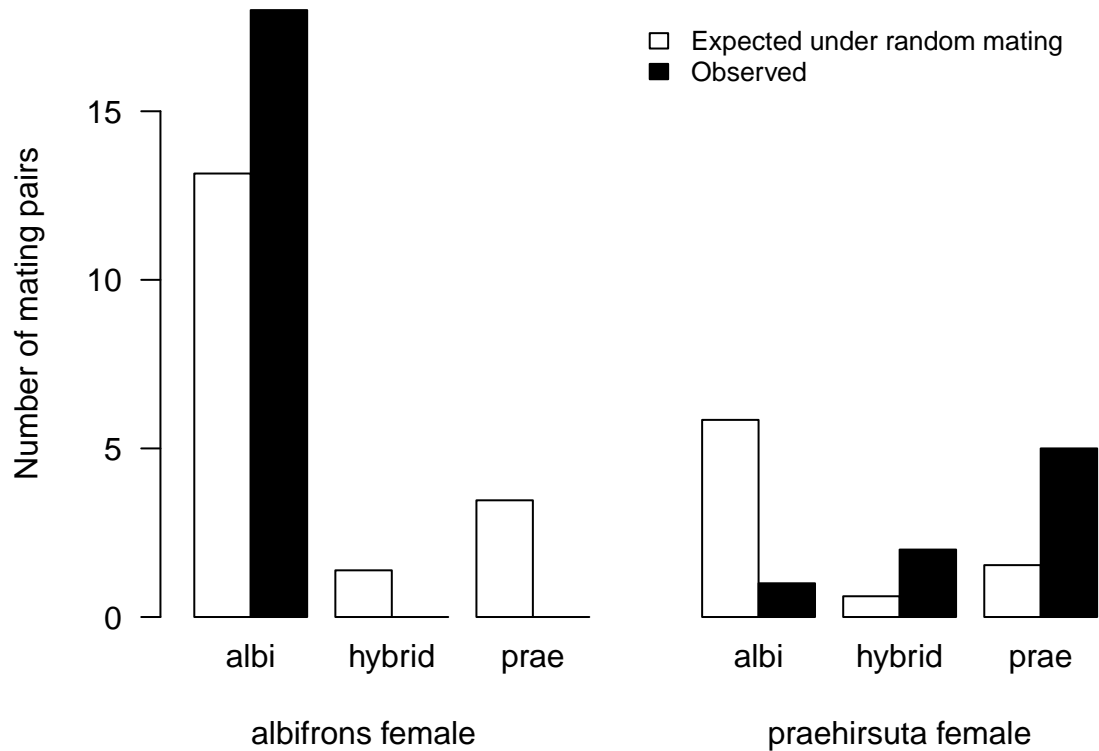


Figure 4

Number of mating pairs of each possible type expected from random mating in absence of sexual isolation (white bars) and observed from an experimental population (black bars). The expected numbers take into account not only the number of males and females of each type that entered the experiment but also their propensity to mate (see text). We see that *J. albifrons* females mated successfully only with *J. albifrons* males, while *J. praehirsuta* females produced offspring with males of the three different types, although not in equal proportions. These results point to strong but imperfect, asymmetric sexual isolation.

Figure 5

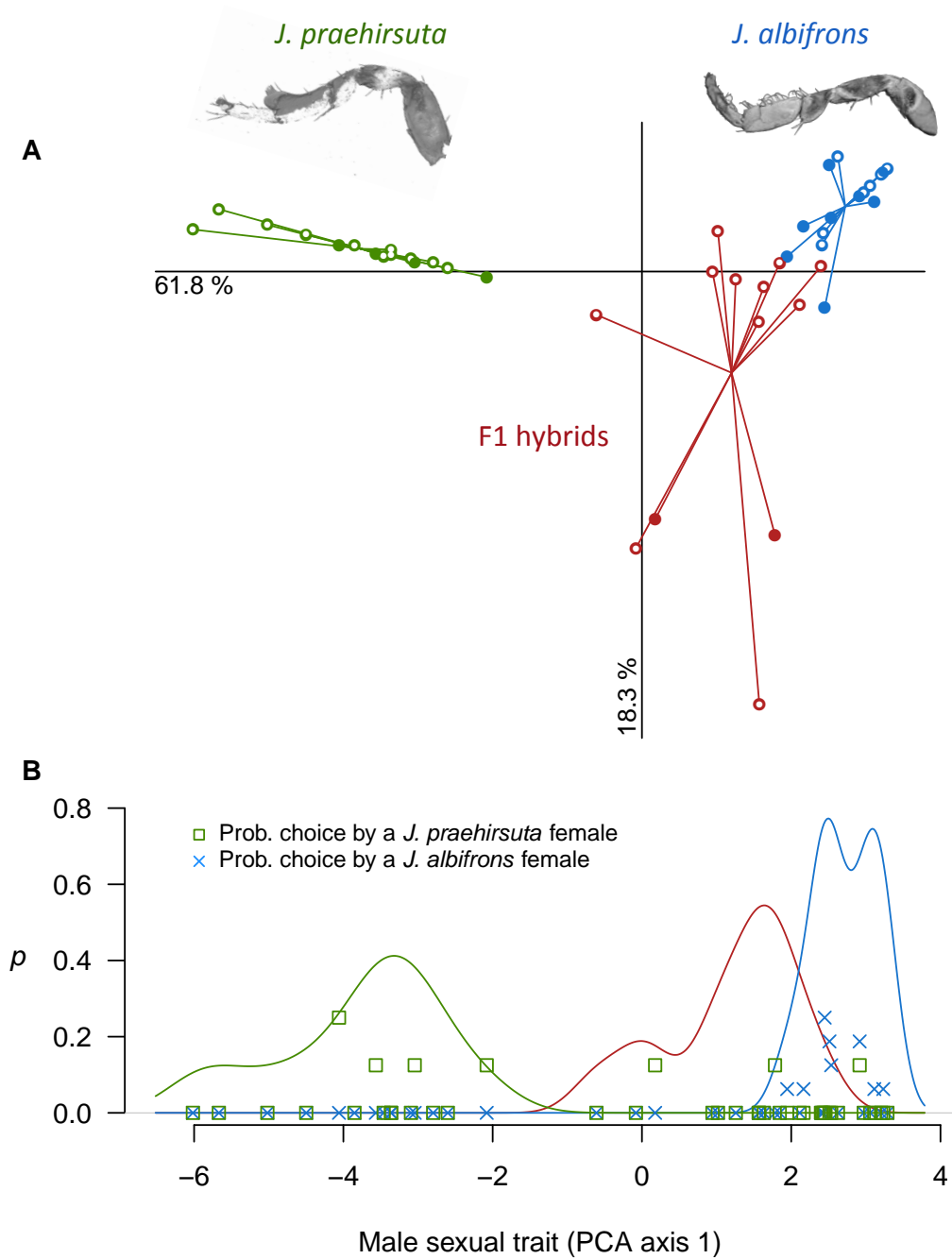


Figure 5

Asymmetric sexual isolation between *Jaera albifrons* and *J. praeheirsuta*. Panel A) shows the first two components of a principal component analysis of male phenotypes (potential parents in the multiple-choice experimental population) grouped by types. We see that individuals of the *albifrons* or *praeheirsuta* types are phenotypically differentiated, while individuals of the hybrid type (i.e.

produced by an "interspecific" cross) show more phenotypic variability, including phenotypes indistinguishable from the parental morphs. In this PCA plot, empty circles represent the males that did not sire any offspring, while solid dots show the males that successfully reproduced. In panel B), the solid curves show the density distribution of male sexual trait values in univariate space (PCA axis 1 only). The probability that a female mated with a male showing a particular sexual trait value is shown by green squares (*J. praeheirsuta* females) and blue crosses (*J. albifrons* females). The preferred mates of *J. albifrons* females coincide with the distribution of *J. albifrons* male sexual trait. By contrast, this concordance is much relaxed for *J. praeheirsuta* females, which mated with males showing a wide range of trait values.

Figure 6

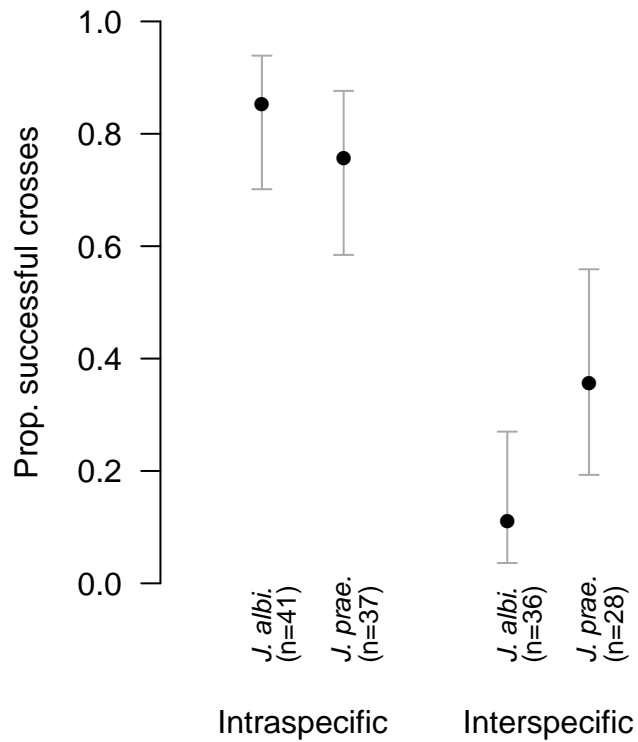


Figure 6 - Proportion of successful intra- and interspecific crosses in no-choice experiments involving either *J. albifrons* or *J. praeahirsuta* females. The sample sizes (number of experimental crosses) are given within brackets, and the bars give 95% confidence intervals around each observed proportion. A cross was successful if it produced at least one offspring. Sexual isolation between species is stronger in one direction (*J. albifrons* female).