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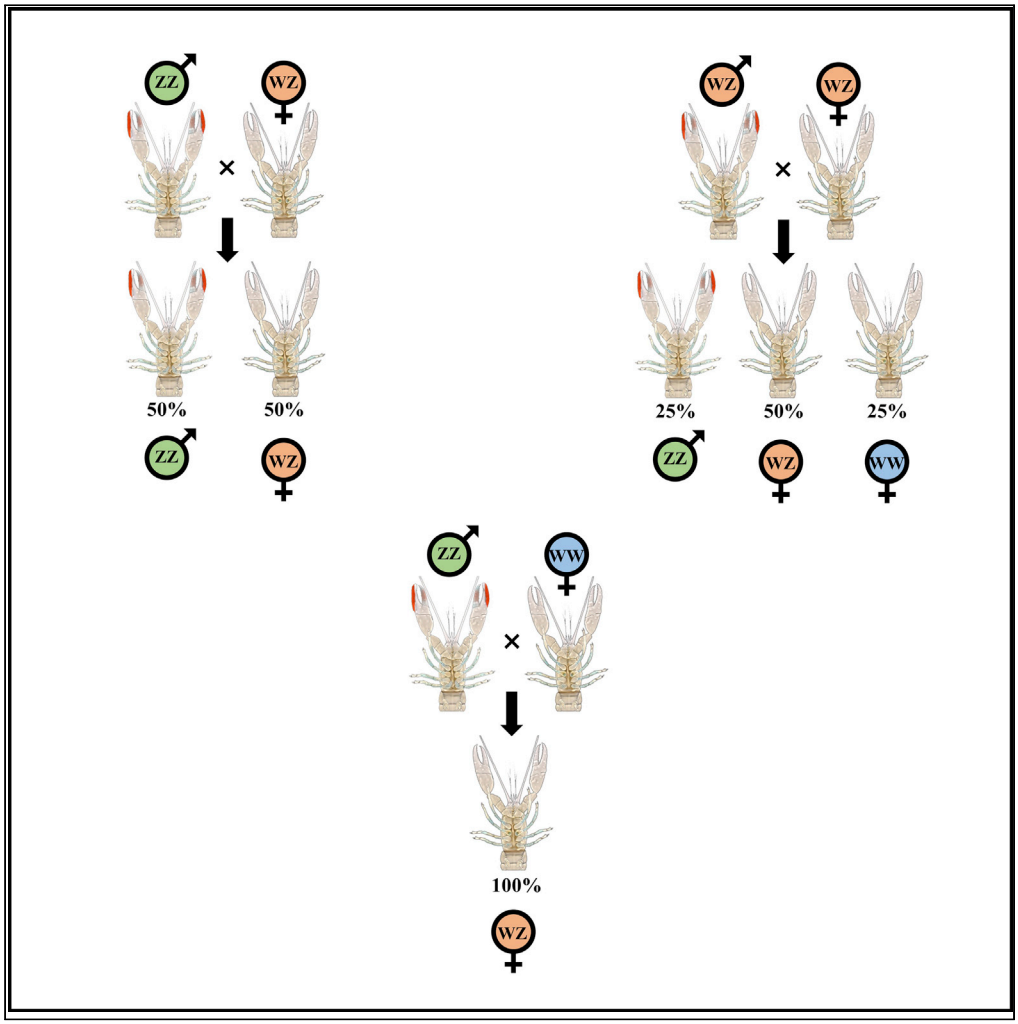

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HIGHLIGHTS

Two sexes and four sexual genotypes are consequences of crayfish intersexuality

W/Z genomic sex markers were developed for the Australian redclaw crayfish

Homogametic WW females were found for the first time in crayfish populations

Intersexuals may contribute to fitness by increasing population growth rate

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Two Homogametic Genotypes – One Crayfish: On the Consequences of Intersexuality

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SUMMARY

In the Australian redclaw crayfish, *Cherax quadricarinatus* (WZ/ZZ system), intersexuals, although exhibiting both male and female gonopores, are functional males bearing a female genotype (WZ males). Therefore, the occurrence of the unusual homogametic WW females in nature is plausible. We developed W/Z genomic sex markers and used them to investigate the genotypic structure of experimental and native *C. quadricarinatus* populations in Australia. We discovered, for the first time, the natural occurrence of WW females in crustacean populations. By modeling population dynamics, we found that intersexuals contribute to the growth rate of crayfish populations in the short term. Given the vastly fragmented *C. quadricarinatus* habitat, which is characterized by drought-flood cycles, we speculate that intersexuals contribute to the fitness of this species since they lead to occasional increment in the population growth rate which potentially supports crayfish population restoration and establishment under extinction threats or colonization events.

INTRODUCTION

Intersexuality, a term first coined by Richard Goldschmidt (Goldschmidt, 1938; Reinboth, 1975), is not a deviated type of reproduction but is rather used to describe an individual of a gonochoristic species failing to fit into the typical sex definition of a male or a female. Instead, intersexuals may bear an unusual combination of male and female features such as sex chromosomes, genital opening, and gonads (Abdel-Moneim et al., 2015; Reinboth, 1975).

The occurrence of intersexuality in the animal kingdom is sometimes attributed to environmental effects or to external interventions, such as temperature (Olmstead and Leblanc, 2002; Olmstead and LeBlanc, 2007; Souissi et al., 2010), photoperiod (Dunn et al., 1993), pollutants (Barnhoorn et al., 2004; Ford et al., 2006), parasitism (Ford et al., 2007; Ianora et al., 1987), and bacterial infections (Rigaud and Juchault, 1998), and even to designated crossbreeding and hybridization of two species (Gomot, 1975). The natural occurrence of intersexuality is well established in some species—vertebrates and invertebrates alike (Adolfi et al., 2019; Armstrong and Marshall, 1964; Grilo and Rosa, 2017; Reinboth, 1975). In terms of fitness, previous studies have shown that intersexuality results in low fecundity, suggesting lower reproductive fitness for populations with higher occurrences of intersexuality (Ford et al., 2003, 2004; Jobling et al., 2002). It has also been suggested that morphological abnormalities in certain intersexuals impair mating success (Martins et al., 2009). However, although it is plausible that during evolution there is negative selection for intersexuals, the common occurrence of natural intersexuality could also suggest a possible fitness advantage.

For animals bearing the WZ/ZZ chromosomal system (WZ females and ZZ males), such as the fowl *Gallus gallus*, it has been suggested that the occurrence of the WW sex chromosome combination is lethal since most W-bearing primordial germ cells fail to differentiate into spermatozoa (Tagami et al., 1997). Similarly, in the fowl *Meleagris gallopavo*, WW blastoderms produced by parthenogenesis did not survive beyond two days of incubation (Harada and Buss, 1981). In contrast, laboratory experiments in crustaceans and amphibians involving artificial sex manipulations of a WZ female to a WZ male and crossing the WZ male with a normal WZ female resulted in a progeny that contains 25% of viable WW females. The sex manipulation was

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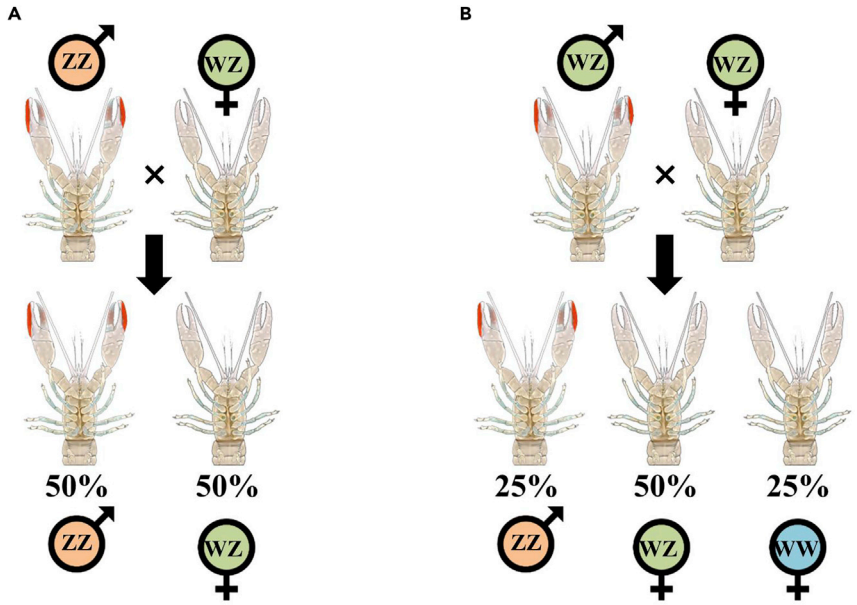

Figure 1. Crosses of (A) male and female and (B) intersex and female crayfish. The resulting genotype and phenotype ratios in each progeny are shown in the figure.

performed in isopod crustaceans using hormonal treatment (Juchault and Rigaud, 1995) and in the decapod *Macrobrachium rosenbergii* using implantation of the unique masculine crustacean androgenic gland (AG) or injection of AG cell suspension into females (Levy et al., 2016; Malecha et al., 1992), while testis implantation was used to masculinize females in frogs and salamanders (Humphrey, 1948; Mikamo and Witschi, 1964).

Natural occurrence of WW animals was suggested to exist in the branchiopod clam shrimp *Eulimnadia texana* (Weeks et al., 2010). However, this branchiopod is not gonochoristic but androdioecious hermaphrodite in which males and hermaphrodites coexist (Weeks et al., 2006). Additionally, it is noteworthy that unlike gonochoristic species bearing the W/Z system, the sex chromosomes in this androdioecious species are considered as sex chromosomes at a very early developmental stage (i.e. proto-sex chromosomes) (Weeks et al., 2010).

In the Australian redclaw crayfish, *Cherax quadricarinatus*, a gonochoristic species in which, according to progeny testing, sex is determined by the WZ/ZZ mode of inheritance (Parnes et al., 2003), a natural occurrence of 1–8% of intersexuals (i.e., bearing a combination of both male and female gonopores) within a given population has been reported (Brummett and Alon, 1994; Curtis and Jones, 1995; Sagi et al., 1996; Thorne and Fielder, 1991). In contrast to protandric hermaphrodite species in which male and female phases are well defined (except during episodic transitional phases between sexes [Benvenuto and Weeks, 2020]), intersexuals of the redclaw crayfish, although bearing both male and female gonopores, do not shift between sexes but are actually males with a female genotype (WZ males) (Parnes et al., 2003; Sagi et al., 1996). Anatomically, they have a constantly arrested ovary in a pre-vitellogenic state (Sagi et al., 1996, 2002). Morphologically, they exhibit male secondary characteristics, such as a red patch on the chela (Rosen et al., 2010; Sagi et al., 2002) and, like normal ZZ males, they present masculine behavior in terms of courtship and fighting (Barki et al., 2006). In a previous work that included progeny testing under laboratory conditions, it was shown that *C. quadricarinatus* intersexuals (WZ males) crossed with females (WZ) could skew the female:male ratio to 3:1 (Figure 1; [Parnes et al., 2003]). Also, when females from the progeny of such crosses were further crossed with normal males (ZZ), a third of them were viable WW females as indicated by their production of all-female progenies. In addition, seven types of *C. quadricarinatus* intersexuals, exhibiting seven different combinations of male and female gonopores, have been reported (Figure 2 and Sagi et al. 1996).

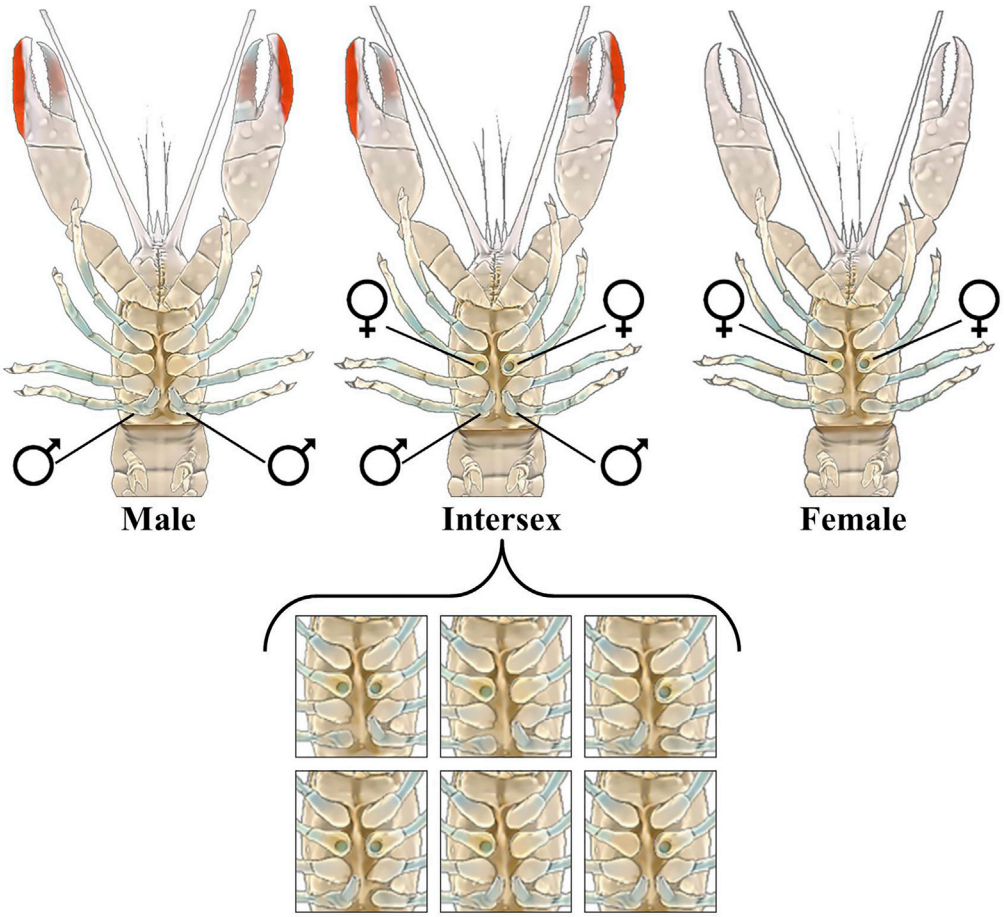

Figure 2. Genital Openings (Gonopores) in *C. quadricarinatus*

Male – two gonopores at the base of the fifth pereiopod (left). Female – two gonopores at the base of the third pereiopod (right). Intersex – seven combinations of male and female gonopores observed and reported by Parnes et al., in 2003 (middle).

The above mentioned *C. quadricarinatus* WW females, similarly to other species in which WW females were achieved only through external intervention, have been reported only under laboratory conditions (Parnes et al., 2003). Although possible, even if existed in nature, according to the above reports, WW females in the laboratory are phenotypically indistinguishable from WZ females. To the best of our knowledge, the occurrence of WW females within gonochoristic species in nature has never been reported for any wild population in the animal kingdom. However, since intersexuals are found in wild populations of *C. quadricarinatus* (Curtis and Jones, 1995), the natural occurrence of Z-lacking WW females could be hypothesized. In such a case, questions could be asked regarding the essentiality of the Z chromosome in a WZ/ZZ sex determination system. Moreover, WW females, which should comprise third of the progeny of an intersexual crossed with a female (WZ × WZ), could cause a female bias in native populations.

In the current study, using genomic sex markers, we screened the genotypic composition of both cultured and native crayfish populations. This screening has enabled us to report for the first time Z-lacking WW females in native populations of *C. quadricarinatus*. We also modeled the population dynamics structure and tested the propensity of intersexuals and WW females to skew sex ratios in crayfish populations. Finally, in light of the potential contribution of intersexuals and WW females to the fitness of the species, we examined possible evolutionary advantages for female-biased populations, even though the notion is seemingly contradictory to the theory of sex allocation (Charnov, 1982).

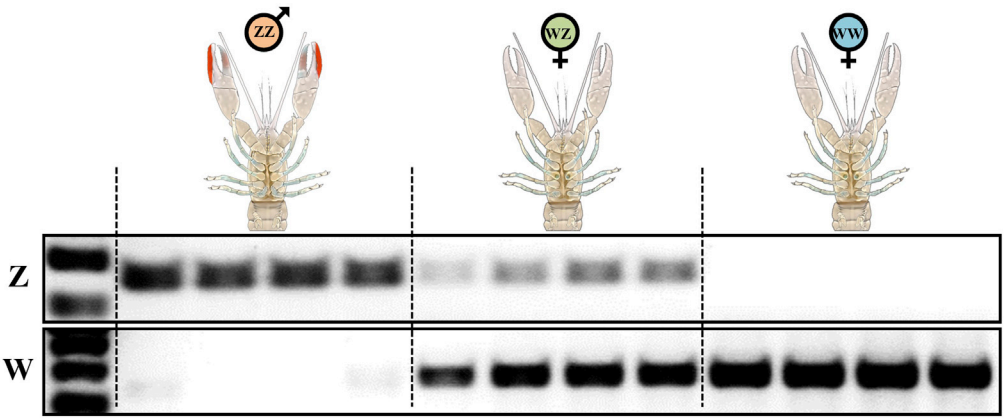

Figure 3. Identification of the Genotype of *C. quadricarinatus* Using Genomic Sex Markers

DNA that was extracted from 4 animals of each genotype (ZZ, WZ, WW) was used as a template to amplify sex chromosome–specific markers (Z–top, W–bottom). A 100 bp DNA ladder is given in the left part of the gels.

RESULTS

Sex-Specific Markers in *C. quadricarinatus*

Obtaining sex-specific markers in *C. quadricarinatus* was a crucial prerequisite for performing the different analyses in this study. To this end, restriction-site associated DNA sequencing (RADSeq; Davey and Blaxter, 2010) was applied to 12 male (ZZ) and 12 female (8 WZ + 4 WW) samples, resulting in a total of 50,052,191 single-end 90-bp sequence reads. Clustering of the reads yielded 147,952 unique RAD tags. To identify sex-specific tags, the reads of each sample were aligned to the RAD tags and quantified. RAD tags present in most (>60%) of the female samples but absent in all the male samples were considered as possible W-associated sex markers. Of the eight identified candidates (Table S1), 'Tag 906' seemed most precise based on a preliminary polymerase chain reaction (PCR) screening. Therefore, this tag was extensively validated resulting with a W-specific marker, as attested by genotyping more than 1,500 animals in different populations coming from different continents (see details in the experiments below). Interestingly, the primers that amplified the W-band (120 bp) also amplified a Z-band (200 bp), as shown in Figure S1. Sequencing both W and Z products enabled us to design more specific primers, which amplified bands of higher intensity associated with either the W chromosome or the Z chromosome, separately, in thousands of animals that were different from those which were sampled for the RADSeq analysis (Figure 3).

Genotyping the Various Intersexual Types

In addition to the intersexual types previously reported (Sagi et al., 1996), during the process of sampling the animals, we found a type of intersex individual bearing one male gonopore and one female gonopore on the same side of the animal. According to the sex-specific markers used in this study, intersexuals with 2 female and 1 or 2 male gonopores (n = 8) as well as intersexuals with 1 female gonopore and 1 male gonopore on opposite sides (n = 1) were found to bear a female WZ genotype. Intersexuals with 1 female and 2 male gonopores (n = 6) as well as intersexuals with 1 female gonopore and 1 male gonopore on the same side (n = 1) were found to bear the masculine ZZ genotype. Genotyping intersexuals bearing every possible male-female gonopore combination is shown in Figure 4.

Progeny Testing of Females Crossed with Intersexuals

An examination of the progeny of females crossed with intersexuals (WZ × WZ) under laboratory conditions revealed that the observed ratios of WZ/ZZ:WW in the three progenies tested (3.8:1, 4.6:1, and 2.2:1) were not statistically different from the expected 3:1 ratio, according to the chi-square goodness of fit test (p *value* ≥ 0.16). Detailed results are presented in Table 1.

Intersex Progeny in Cultured Populations

Sex ratios were determined during the field experiments performed in earthen ponds in Australia and Israel. A clear 1:1 sex ratio was observed in control ponds stocked only with females and males, whereas ponds stocked only with females and intersexuals showed a clear female bias of a 5:1 ratio in Israel and

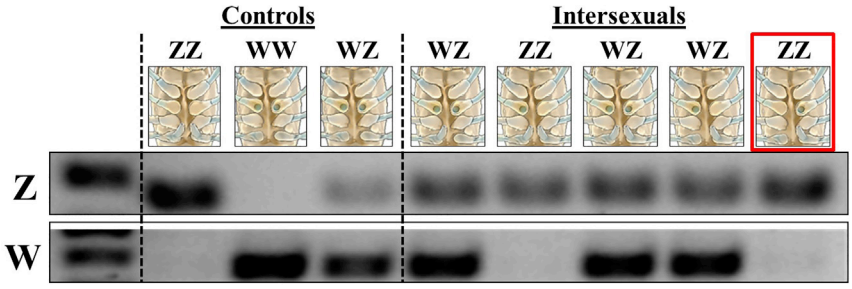

Figure 4. The Genotype of *C. quadricarinatus* Intersexuals

Genomic sex markers were used to test the genotype of each intersex with every possible combination of female and male gonopores (right). The new type of intersexual that we found in this study, with one male and one female gonopore on the same side, is marked out with a red frame. ZZ male, WZ female, and WW female are given as controls. A 100 bp DNA ladder is given in the left part of the gels.

ratios ranging from 2:1 to 3:1 in the Australian ponds. The sex-specific markers revealed that WW females were found in all treatment ponds, and the ratio of WZ to WW females was 4:1 in Israel and ranged from 2:1 to 4:1 in Australia (Table 2).

The Sexual Composition in Australian Crayfish Populations

An investigation of the sex ratio in native Australian crayfish populations at two different locations (A and B, shown in Figure 5) revealed that the female:male ratio was ~1:1, with a very low proportion of intersexuals in the population (1.1 and 1.2%, respectively). Testing the sexual genotype of the females sampled at those two locations and at two more revealed that the samples from A, B, and C (but not from D) did indeed contain WW females, but only rarely, namely, 0.65, 1.05, and 0.7% of all the females sampled at locations A, B, and C, respectively. A detailed summary of the results for the native populations is presented in Table 3.

Population Dynamics of *C. quadricarinatus*

A discrete-time and density-independent Malthusian growth model was developed to track the dynamics of the four phenotypes and genotypes in *C. quadricarinatus* populations, namely, ZZ males, WZ intersexuals (IS), WZ females, and WW females (for further details, see the Transparent Methods section in the supplemental information).

Simulations of *C. quadricarinatus* population dynamics (see Excel file: Data S1 in the supplemental information) showed that, regardless of the initial conditions (as long as the number of males [♂] = $ZZ_0+IS_0 > 0$ and the number of females [♀] = $WZ_0+WW_0 > 0$), the population reached a stable distribution (constant fraction of individuals for each phenotype/genotype) in about 30 generations (i.e., 15 years), with the fraction of individuals in the long run being $ZZ_\infty = 47.96\%$, $WZ_\infty = 48.96\%$, $IS_\infty = 2.04\%$, and $WW_\infty = 1.04\%$, respectively (Figure S2A). Simulations also showed that in the long term, the fraction of intersexual (IS) individuals will be about $^1/_2\alpha$, i.e., half the fraction of WZ progeny will emerge as intersexuals ($\alpha = 4\%$, while $IS_\infty = 2.04\%$), while the overall sex ratio (being the total number of functional males [ZZ + IS]: the total number of functional females [WZ + WW]) will be balanced at 1:1, as in a binary sex population, i.e., populations in which ZZ = 50%, WZ = 50%, and $\alpha = 0\%$ (no WZ progeny emerging as intersexuals) (Figure S2B). The long-term annual growth rate of a population with a fraction $\alpha > 0$ of WZ progeny emerging as intersexuals will be the same as that for a population with a binary sex structure (i.e., $\alpha = 0$, only ZZ and WZ individuals, Figure 6A, blue line). In the short term, the growth rate of a population with $\alpha > 0$ will be lower than the growth rate of a binary sex population ($\alpha = 0$), if the initial fraction of intersexuals in the founder population (i.e., at time t = 0) is lower than that in the long-term stable distribution (LTSD), i.e., $ZZ_0 = 50\%$ and $WZ_0 = 50\%$, with $IS_0 = WW_0 = 0 < IS_\infty$ and $\alpha > 0$ (Figure 6A, red line). In contrast, in the short term, the annual growth rate for a population with $\alpha > 0$ will be higher than that in the case of $\alpha = 0$, if the fraction of intersexuals in the founder population (i.e., at time t = 0) is larger than that in the LTSD; for instance, for $IS_0 = 50\% > IS_\infty$ and $WZ_0 = 50\%$, with $ZZ_0 = WW_0 = 0$ (Figure 6A, green line). As a consequence, in the short term, the size of a population with $\alpha > 0$ and initial conditions of intersexuals instead of ZZ males will grow much faster and the population will reach 1,000 individuals after as little as 15 years, in contrast to the 26 years required for the same

Progeny	Total N	Genotypic Composition Observed		Genotypic Composition Expected		p *value*
		WZ/ZZ	WW	WZ/ZZ	WW	
1	83	66 (80%)	17 (20%)	62 (75%)	21 (25%)	0.34
2	68	56 (82%)	12 (17%)	51 (75%)	17 (25%)	0.16
3	61	42 (69%)	19 (31%)	46 (75%)	15 (25%)	0.27

Table 1. Observed and Expected Genotypes in WZ × WZ (Intersex × Female) Crayfish Progenies
Values in the table are the number (percentage) of sampled animals from each progeny. p *values* represent the level of significance between the observed and expected results according to chi-square goodness of fit test.

population with ZZ males instead of intersexuals (Figures 6B and 6C). We note that the growth rate pattern of the two Australian native populations shown in Figure 6A (AU-A, black line and AU-B, gray line) is similar to the pattern of both the population with a binary sex structure (blue line) and the population with $\alpha > 0$ and initial conditions of $ZZ_0 = 50\%$ and $WZ_0 = 50\%$, with $IS_0 = WW_0 = 0 < IS_\infty$ (red line).

The actual fraction of individuals in each class in the short and long run depends on the specific values of the model parameters. However, the general properties listed above are invariant with respect to the actual values of the model parameters, i.e., finite growth rate (λ), survival of newborns (σ_0), adult life expectancy ($1/-\ln(\sigma_a)$), per capita fecundity (ϕ), and the length of a generation (i.e., whether the time step represents ~6 months, as assumed here). In particular, the LTSD, i.e., the relative size of each of the four classes, is uniquely a function of α and does not rely on the other model parameters. Further simulations showed that, in the long term, the population will tend to converge into a stable structure (i.e., fraction of individuals in each class) also under the assumption that the total number of individuals in the population remains constant (either because $\lambda = 1$ or because the population has reached the maximum carrying capacity and assuming that newborn individuals replace only the adults that die at each time step).

Evolutionary Insight into Sexual Reproductive Strategies

According to our phylogenetic analysis (Figure 7), *C. quadricarinatus*, which exhibits a viable intersexual phenotype, is found within the Astacidea infraorder clade. To the best of our knowledge, among the species that have been analyzed, the only other astacidean species with a reproduction form that deviates from pure gonochorism (other than *C. quadricarinatus*) is the parthenogenetic marbled crayfish, *Procambarus virginalis*, in which a virginal form of reproduction occurs, resulting in an all-female clone (Martin et al., 2007). Out of the 38 decapod species used in our phylogenetic analysis, the two protandric hermaphrodite species, *Pandalus platyceros* (family Pandaloidea) and *Hippolyte inermis* (family Alpheoidea), were found to be phylogenetically closely related. However, both are within the Caridea infraorder clade, which is the furthest from the Astacidea clade, according to our phylogenetic tree. To summarize, species exhibiting hermaphroditism and the intersexual *C. quadricarinatus* are evolutionary distinct from each other.

DISCUSSION

To date, homogametic WW females in the WZ/ZZ sex determination system have been reported only following laboratory-induced manipulations (Levy et al., 2016; Malecha et al., 1992). The present study is the first to report the presence of naturally occurring WW females in native wild populations. The genomic sex markers established in this study – based on an experimental laboratory population of the redclaw crayfish in Israel – were found to be universal on the basis of validation in five geographically distinct native populations in Australia. Therefore, the markers facilitated, first, the screening of a large sample of animals from different locations, from both experimental and native populations, and, second, a comprehensive study eventually proved, for the first time, the existence of homogametic WW females in native *C. quadricarinatus* populations.

At first glance, the occurrence of WW females in native populations does not seem to be feasible because – extrapolating from avian species – the Z chromosome is believed to be vital in the WZ/ZZ sex determination scheme since WW animals were not developed (Harada and Buss, 1981; Tagami et al., 1997). However, previous studies of decapod crustaceans showed that artificial sex manipulations have yielded not only viable

Pond	Stocked Phenotype			Phenotype of Sampled Progeny				Genotype of Sampled Females			
	Females	Males	Intersexuals	N	Females	Males	F:M Ratio	n	WZ	WW	WZ/WW Ratio
IL	20	0	5	52	43	9	5:1	43	35	8	4:1
AU 1	130	0	30	121	80	41	2:1	48	33	15	2:1
AU 2	130	0	30	144	109	35	3:1	48	32	16	2:1
AU 3	130	0	30	218	158	60	3:1	48	39	9	4:1
AU 4	130	30	0	160	80	80	1:1	20	20	0	–
AU 5	130	30	0	131	53	78	1:1	20	20	0	–

Table 2. Populations Cultured in Earthen Ponds in Israel and Australia

Four ponds were stocked with females and intersexuals (one as a preliminary experiment at Dor station, Israel [IL], and three at Cherax Park Aquaculture, Australia [AU 1–3]). Two control ponds were stocked with males and females at Cherax Park Aquaculture (AU 4–5). The number of sampled animals from each sex and the ratio between the two sexes are given.

WW females (Levy et al., 2016, 2017; Malecha et al., 1992; Molcho et al., 2020) but also viable WW males lacking the masculine Z chromosome (Levy et al., 2019). Also, in the androdioecious hermaphrodite clam shrimp, WW hermaphrodites have male traits but lacking the Z proto-sex chromosome (Weeks et al., 2010). Therefore, in crustaceans, it is possible not only that the Z chromosome is not essential for life but also that it is not essential for masculine development. It is hence also possible that part of the sex determination and differentiation "tool kit" could instead reside in autosomal chromosomes. This notion has similarly been posited for the male-determining factors in some *Musca* flies (Sanchez, 2008) and for the autosomal *dpy-21* locus regulating the X chromosome in *Caenorhabditis elegans* (Meneely and Wood, 1984). In light of this this theory, it now becomes conceivable that in crustaceans viable WW females may occur in nature, as we did indeed find in *C. quadricarinatus* in the course of this study.

On the assumption that intersexuals and WW females do indeed exist in nature, one of the major questions raised in this study was whether they contribute to the fitness of the population. To address this question, we first confirmed that intersexuals were able to alter the common 1:1 female:male ratio in pond experiments in both Australia and Israel stocked with high fractions of intersexuals. The finding that this ratio ranged from 2:1 to 4:1 served as the first evidence that the presence of high fraction of intersexuals instead of males may alter the population sexual structure within a single generation. It is thus puzzling that while WW females produce all-female progenies and intersexuals produce female-biased progenies, native populations always showed a ~1:1 female:male ratio, as described in the present study and by Bortolini et al. (2007). This conundrum could suggest that at low frequencies, intersexuals and WW females are not able to alter the sex ratio of *C. quadricarinatus* populations in the long term, as confirmed by our population dynamics model. Analysis of the population dynamics structure in terms of sex ratios of *C. quadricarinatus* populations with different initial conditions confirmed that irrespective of the initial ratios of ZZ males, WZ intersexuals, WZ females, and WW females, the long-term sex ratio was not biased, and the annual growth rate of the entire population converged to a constant value with a stable distribution of ZZ males, WZ females, WZ intersexuals, and WW females (approximately 48, 49, 2, and 1%, respectively). The prediction of our model, which showed that every *C. quadricarinatus* population, even if initiated with biased sex ratios, converged to a 1:1 female:male ratio, is in line with Fisher's principle claiming that this convergence is a result of natural selection because parental expenditure should be equal for both sexes (Fisher, 1930). In contrast, even if all crayfish populations converge in the long term to a 1:1 female:male ratio, our model suggests that occasionally an initial condition of high fractions of intersexuals contributes significantly to the annual increment of a given population in the short term. This result suggests that intersexuality and WW females might contribute to the fitness of this species under conditions of small founder populations, in which occurrences of intersexuals, and consequently of females, may result in a higher short-term population growth rate, contributing to colonization or restoration. Moreover, in a near extinction event, the higher the intersex ratio, the better the chances of the population to recover and reach steady state of equal sex ratios, according to Fisher's principle (Fisher, 1930).

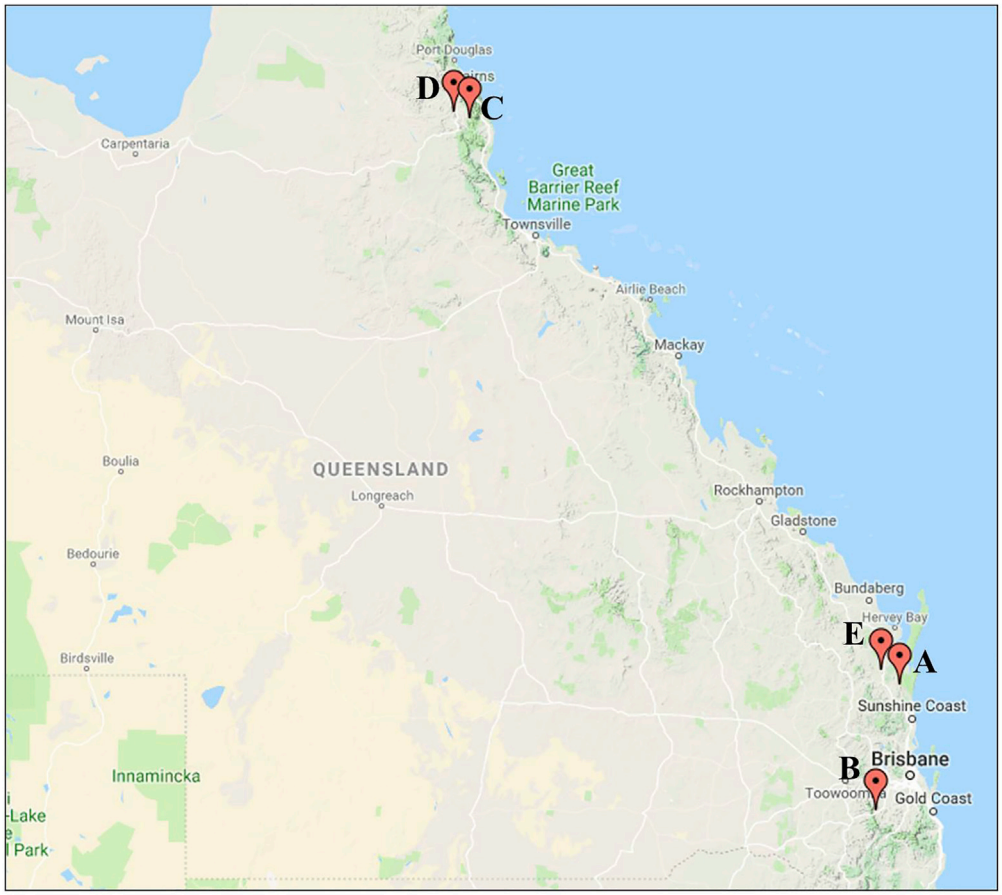

Figure 5. Sampling Locations in QLD, Australia

(A–E) (A) Ironbark Redclaw Crayfish Farm, (B) Freshwater Australian Crayfish Traders Pty Ltd., (C) Klaus Cazzonelli Redclaw Farm, (D) AquaVerde Redclaw Hatchery & Farm, (E) Cherax Park Aquaculture. The map was created online through http://www.pinmaps.net.

All freshwater crayfish from Australia, New Zealand, New Guinea, and Madagascar belong to the same Parastacidae family, dating back hundreds of millions years to the Neoproterozoic era when Australia was part of the Gondwana supercontinent (McCormack, 2012). When Australia became dryer and warmer, crayfish populations experienced colonization events, became isolated, and evolved into the many crayfish species, including *C. quadricarinatus*, that are currently found across Australia (McCormack, 2012). In view of the vastly fragmented landscape with multiple drought-flood cycles where this species originated (Jones, 1990), it could be speculated that not only a fast growth rate and high fecundity (McCormack, 2012) but also intersexuality provided a competitive advantage for the settlement of *C. quadricarinatus* populations in new niches vs other crayfish species.

Although not related to intersexuality but rather to rearrangement of the sex chromosomes, some species of mammalian rodents (XX/XY system), including the African pygmy mouse (*Mus minutoides*) and several lemming species, exhibit frequent occurrence of females with a male genotype (XY females) (Bull and Bulmer, 1981; Fredga, 1983; Veyrunes et al., 2010). The contribution of mammalian XY females (the equivalent of intersexuals [WZ males] in *C. quadricarinatus*) to the fitness of the species is even more questionable than that of the WZ intersex crayfish since quarter of their progeny (YY genotyped) is lethal. However, since the progeny of such XY females is female biased, it was suggested that producing an excess of females could be an adaptive strategy for rapid recovery from low densities (Veyrunes et al., 2010). The latter is in line with our theory regarding the short-term advantage of female-biased crayfish populations.

Location (See Figure 5)	Phenotype of Sampled Progeny				Genotype of Sampled Females			
	n	Female	Male	Male Intersexual	N	WZ	WW	WW [%]
A	852	419	423	10	307	305	2	0.65
B	570	287	276	7	287	284	3	1.05
C	-	-	-	-	286	284	2	0.70
D	-	-	-	-	294	294	0	0.00

Table 3. Native Populations of the Australian Redclaw Crayfish

Four locations in QLD, Australia, (see map, Figure 5) were examined. At two locations (A and B), animals were sampled at random and sorted phenotypically. Of the sampled animals, all or most of the females were genotypically tested. At the other two locations (C and D), the entire population was not examined, but only females were randomly sampled and genotypically tested. The number of females bearing each genotype and the percentage of WW females in the sampled population are given.

Selection for a biased sex ratio could be also attributed to species whose sex is determined by environmental factors in which environmental conditions during embryogenesis affect the sex of the offspring. Under this assumption, an embryo will develop into a male phenotype under conditions where males have a higher fitness than females or vice versa under conditions in which females have a higher fitness than males (Uller et al., 2007). However, frequent fluctuations in the sex ratio in a given population, as a result of rapid changes in the environmental conditions, may also reduce the optimal matching of the offspring sex to the changed conditions (Uller et al., 2007) and, potentially, affect the fitness of the species. However, to the best of our knowledge, the occurrence of the intersexual phenomenon in *C. quadricarinatus* is not dependent on environmental factors.

In terms of morphology, in addition to the seven types of intersexuals reported before (Sagi et al., 1996), the present study revealed a type of intersex animal, which bears one female gonopore and one male gonopore on the same side. To date, only the genotype of the two most common intersexual types of *C. quadricarinatus*, those with two female and one or two male gonopores, proved to bear the WZ genotype, according to progeny testing (Parnes et al., 2003). However, genotype determination of each of the eight possible intersexual types is now possible as a result of genomic sex markers obtained in this study.

Unlike protandric hermaphrodite species, which episodically transform from one sex to the other (Bauer and Holt, 1998; Benvenuto and Weeks, 2020; Heath, 1977; Levy et al., 2020; Mutalipassi et al., 2018), the intersexual form in *C. quadricarinatus* uniquely represents a natural case of permanent co-occurrence of male and female characteristics within a gonochoristic scheme. The intersex animal is a functional male with a female genotype (WZ), bearing a combination of male and female gonopores and a mix of male and female reproductive systems (Khalaila et al., 1999; Parnes et al., 2003; Sagi et al., 1996). This intersexuality phenomenon has some similarities to gynandromorphism (i.e., bilateral manifestation of male and female phenotypes; [Farmer, 1972; Johnson and Otto, 1981; Micheli, 1991]) in other decapod crustaceans. However, gynandromorphs are the outcome of random and rare events of abnormalities produced through several possible mechanisms, including improper migration of chromosomes or cytogenetic complications in early embryonic development (Gjershaug et al., 2016), while *C. quadricarinatus* intersexuals are not rare and their fraction in the population is not random but relatively constant (Sagi et al., 1996; Webster et al., 2004). While intersexuality in *C. quadricarinatus* could be attributed to a simply random phenomenon, it could also potentially represent "evolution in the making" in which intersexuality is the current form in an evolutionary process from a gonochoristic mode of reproduction toward hermaphroditism or vice versa, as has been suggested to have occurred repeatedly in crustaceans along evolution (Benvenuto and Weeks, 2020). However, discussions of "evolution in the making" with respect to intersexuality are fraught with controversial theories regarding the direction of the transition process, suggesting either that the first crustacean lineage, the "ur-crustacean" (Hessler and Newman, 1975; Richter and Wirkner, 2020), had a gonochoristic mode of reproduction that changed to hermaphroditism at low population densities (Cisne, 1982) or vice versa (Hessler and Newman, 1975; Juchault, 1999).

The following line of thought should throw some light on the above argument: Reproductive strategies in the animal kingdom are highly diverse, ranging from gonochorism (Subramoniam, 2013) to different types

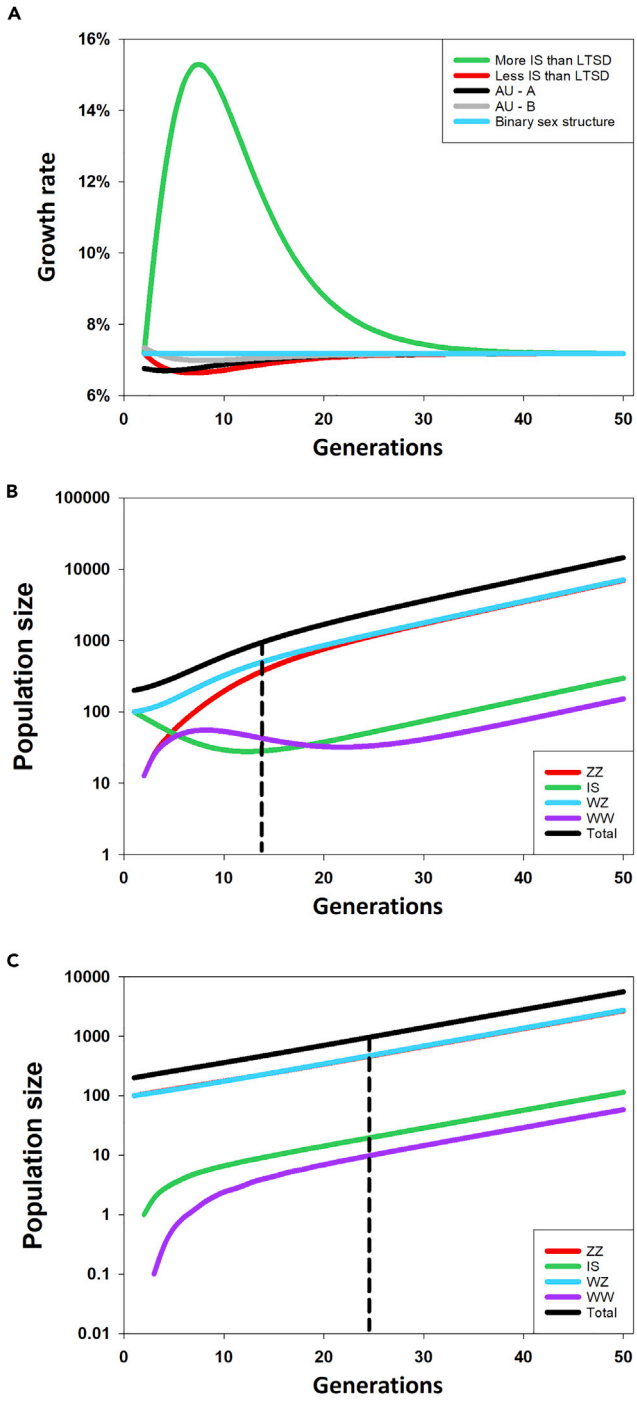

Figure 6. Simulated Dynamics of *C. quadricarinatus* Population Structure

(A) Finite growth rate (rate of change in a generation) of crayfish populations as a function of time. Green line: a population with α = 4% (fraction of WZ females emerging as intersex IS) and the initial fraction of intersexuals in the founder population (i.e., at time t = 0) larger than that at the long-term stable distribution (LTSD) (IS_0 = 50% > IS_∞ and WZ_0 = 50%, with ZZ_0 = WW_0 = 0); red line: same α as above but with the initial fraction of intersexuals in the founder population lower than that at LTSD (ZZ_0 = 50% and WZ_0 = 50%, with IS_0 = WW_0 = 0 < IS_∞); black and gray lines: finite growth rate for two Australian native crayfish populations as described in Table 3 with an initial structure of N_0 = [ZZ_0 = 423, IS_0 = 10, WZ_0 = 417, WW_0 = 2] and N_0 = [ZZ_0 = 276, IS_0 = 7, WZ_0 = 284, WW_0 = 3] for AU-A and AU-B, respectively, and α = 4%; blue line: a population with a binary sex structure (no WZ progeny emerging as intersexuals, i.e., α = 0, only ZZ and WZ individuals).

Figure 6. *Continued*

(B) Trajectories of population size in time for $\alpha = 4\%$ and an initial fraction of intersexuals that is larger than that at the LTSD [$IS_0 = 100\ (50\%) > IS_\infty$ and $WZ_0 = 100\ (50\%)$, with $ZZ_0 = WW_0 = 0$]. After an initial transitory period, depending upon the initial conditions, the population reaches a stable structure (i.e., a constant fraction of individuals in each class) while growing exponentially (long-term Malthusian growth in the semi-logarithmic diagram is represented by a straight line). (C) As in (B) but with an initial fraction of intersexuals that is lower than that at the LTSD [$ZZ_0 = 100\ (50\%)$ and $WZ_0 = 100\ (50\%)$, with $IS_0 = WW_0 = 0 < IS_\infty$]. The vertical dashed lines in B and C represent the generation in which the population size reaches or exceeds 1,000 individuals.

of hermaphroditism (Bauer and Holt, 1998; Brook et al., 1994; de Almeida and Buckup, 2000; Levy et al., 2020) and even to parthenogenesis (Benvenuto and Weeks, 2020; Scholtz et al., 2003). Our phylogenetic analysis of available transcriptomes from different decapod species with respect to reproduction strategies indicates that *C. quadricarinatus* is found within the Astacidea clade, closely related to another *Cherax* species that also exhibits intersexual forms, *C. destructor* (Austin and Meewan, 1999) [*C. destructor* is missing from the phylogenetic analysis in the present study due to lack of transcriptional data in the National Center for Biotechnology Information (NCBI) server]. This clade is evolutionarily remote from the Caridea clade, which comprises a cluster of many decapod crustaceans exhibiting hermaphroditism (Baeza, 2007; Bauer and Holt, 1998; Benvenuto and Weeks, 2020; Subramoniam, 1981; Yaldwyn, 1966) but has a common ancestor with gonochoristic species. This finding is in agreement with a recent comprehensive phylogenomic analysis indicating that the Astacidea and Caridea infraorders diverged from a common ancestor ~450 mya (Wolfe et al., 2019). Thus, if the intersexual phenomenon in *C. quadricarinatus* represents a case of "evolution in the making," it most probably reflects a process of transition from gonochorism toward hermaphroditism rather than the opposite direction.

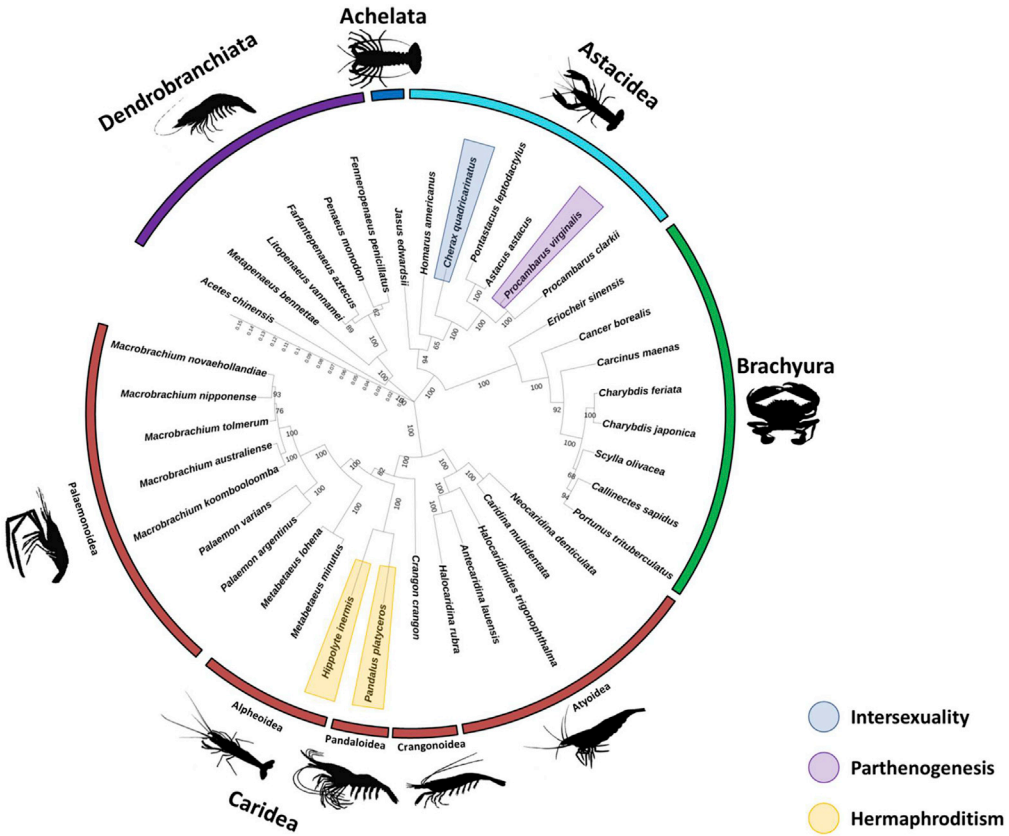

Figure 7. Phylogenetic Analysis of 38 Crustacean Species

The evolutionary history was inferred by using the maximum likelihood method and Jones-Taylor-Thornton (JTT) matrix-based model (Jones et al., 1992). Supporting values (bootstrapping 1000 tests) and branch length scale are given. The infraorder of each species is indicated. Species with reproductive strategies other than gonochorism are colored.

In summary, intersexuality in *C. quadricarinatus* represents a unique reproductive strategy, providing a window to peek into the evolutionary background of reproduction in crustaceans. To the best of our knowledge, this is the first gonochoristic species in the animal kingdom reported to contain both naturally occurring homogametic males and females (ZZ and WW, respectively). We speculate that the propensity of intersexuals and WW females to alter the sexual composition of *C. quadricarinatus* populations, and hence to increase the short-term population growth rate, might contribute to the fitness of this species and might assist it in colonization or in overcoming events of mass extinction. However, our findings do not resolve the open questions of whether this intersexual form contributes to fitness or just presents a random case of unusual reproduction.

Limitations of the Study

Unlike animals within the X/Y mode of inheritance, in which the X chromosome is essential for life and YY animals are most likely inviable, and unlike avian species (W/Z system), in which the Z chromosome is vital, our study shows that in crustaceans bearing the W/Z mode of inheritance, viable WW females indeed exist. The latter raises questions regarding the genomic content of the sex chromosomes in crustaceans and calls for further investigation of sex determination within this W/Z system.

Resource Availability

Lead Contact

Further information and requests should be directed to and will be fulfilled by the Lead Contact, Amir Sagi (sagia@bgu.ac.il).

Material Availability

This study did not generate new materials.

Data and Code Availability

All the data are available within the article.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101652.

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AUTHOR CONTRIBUTIONS

T.L., T.V., N.G., F.A.A, R.M., and A.S. conducted the experiments. G.D.L. developed the population dynamics model. M.Y.S. and V.C.C. performed the bioinformatics analyses. T.L., T.V., and A.S. conceived the study. T.L., M.Y.S., V.C.C., and D.M. performed the phylogenetic analysis. The paper was written by T.L. and reviewed and approved by all co-authors.

DECLARATION OF INTERESTS

A patent regarding sex-specific genomic markers in the Australian redclaw crayfish is pending (International application number: PCT/IL2018/051046, International publication number: WO/2019/058371).

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Supplemental Information

Two Homogametic Genotypes – One Crayfish: On the Consequences of Intersexuality

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Table S1. W-associated candidate tags from RADSeq, Related to Figure 3.

Rad tag	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	Males	Females
	ZZ	ZZ	ZZ	ZZ	ZZ	ZZ	ZZ	ZZ	ZZ	ZZ	ZZ	ZZ	WZ	WZ	WZ	WZ	WZ	WZ	WZ	WZ	WW	WW	WW	WW		
Tag 906	0	0	0	0	0	0	0	0	0	0	0	0	18	106	0	39	45	81	136	13	1856	245	391	190	0	11
Tag 473	0	0	0	0	0	0	0	0	0	0	0	0	0	31	16	7	72	55	43	13	708	84	80	111	0	10
Tag 803	0	0	0	0	0	0	0	0	0	0	0	0	0	104	0	38	71	47	146	0	1045	235	115	105	0	9
Tag 935	0	0	0	0	0	0	0	0	0	0	0	0	7	67	0	13	24	47	114	0	1014	260	170	129	0	9
Tag 881	0	0	0	0	0	0	0	0	0	0	0	0	0	37	0	48	23	48	79	0	957	133	78	77	0	9
Tag 358	0	0	0	0	0	0	0	0	0	0	0	0	0	24	3	24	36	14	29	8	381	38	73	28	0	9
Tag 368	0	0	0	0	0	0	0	0	0	0	0	0	0	76	0	50	18	27	27	0	385	28	74	30	0	9
Tag 546	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	38	25	80	72	0	614	92	63	76	0	8
Total NGS reads	1,859,296	15,153,807	2,281,606	2,206,581	2,630,175	2,306,972	1,466,234	463,264	2,036,338	2,141,013	2,337,766	2,311,612	477,119	2,429,117	586,004	1,992,287	2,215,997	2,307,454	2,493,313	356,236	28,535,646	5,462,686	2,790,830	2,334,642		

NOTE.—The number of reads from each male (M) and female (F) that aligned to each RAD tag candidate is shown along with the total animals (males and females) in which the RAD tag appeared (i.e., >10 aligned reads). The total NGS reads that were sequenced per sample are given in the bottom row. The genotype of each sampled animal is denoted.

Table S2. Relationship between Mating Parents and their Progeny for the Australian Redclaw Crayfish, Related to Figure 6.

		Mating type			
		ZZ(♂)×WZ(♀)	IS(♂)×WZ(♀)	ZZ(♂)×WW(♀)	IS×WW(♀)
Mating frequency for ZZ and IS males	*p*	$\frac{ZZ}{ZZ + IS}$	$\frac{IS}{ZZ + IS}$	$\frac{ZZ}{ZZ + IS}$	$\frac{IS}{ZZ + IS}$
Reproductive output	*RO*	$\varphi \cdot P_{ZZ\times WZ} \cdot$ WZ	$\varphi \cdot P_{IS\times WZ} \cdot$ WZ	$\varphi \cdot P_{ZZ\times WW} \cdot$ WW	$\varphi \cdot P_{IS\times WW} \cdot$ WW
Progeny					
Males	ZZ	$\frac{1}{2} RO_{ZZ\times WZ}$	$\frac{1}{4} RO_{IS\times WZ}$		
Intersexual	IS	$\alpha \frac{1}{2} RO_{ZZ\times WZ}$	$\alpha \frac{1}{2} RO_{IS\times WZ}$	$\alpha RO_{ZZ\times WW}$	$\alpha \frac{1}{2} RO_{IS\times WW}$
Females	WZ	$(1-\alpha) \frac{1}{2} RO_{ZZ\times WZ}$	$(1-\alpha) \frac{1}{2} RO_{IS\times WZ}$	$(1-\alpha) RO_{ZZ\times WW}$	$(1-\alpha) \frac{1}{2} RO_{IS\times WW}$
WW females	WW		$\frac{1}{4} RO_{IS\times WZ}$		$\frac{1}{2} RO_{IS\times WW}$

NOTE.—φ is the per capita fecundity (egg per WZ and WW female per reproductive event) and α the fraction of WZ progeny emerging as an intersexual (IS).

Table S3. List of the Genes used for the Phylogenetic Analysis, Related to Figure 7.

#	Gene
1	Serine tRNA ligase
2	Splicing factor 3B subunit 2
3	40S ribosomal protein S3a
4	60S ribosomal protein L8
5	4-hydroxybenzoate polyprenyltransferase, mitochondrial
6	Stomatin-like protein 2, mitochondrial
7	V-type proton ATPase subunit H
8	40S ribosomal protein S24
9	Coatomer subunit beta
10	Dihydrolipoyl dehydrogenase, mitochondrial
11	2-oxoglutarate dehydrogenase complex component E2

NOTE.—Out of 87 BUSCOs shared by all 38 crustacean transcriptomes, the 11 proteins indicated above appeared only once per transcriptome. These proteins were further concatenated and used for phylogenetic analysis.

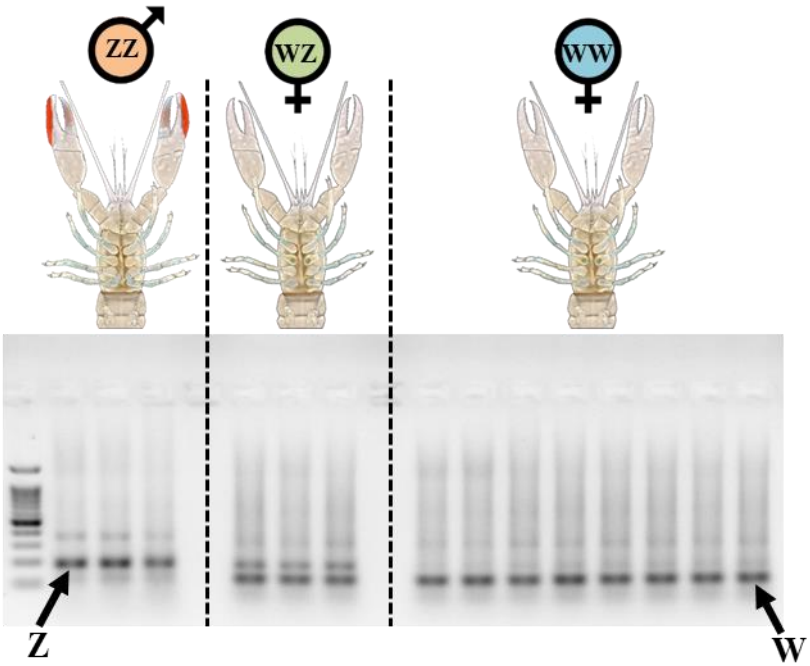

Figure S1. Tag 906 in *C. Quadricarinatus*, Related to Figure 3. DNA that was extracted from 3 ZZ males, 3 WZ females and 8 WW females was used as a template to amplify Tag 906 which resulted in a lower 120 bp W-band and a higher 200 bp Z-band. A 100 bp DNA ladder is given in the left part of the gel.

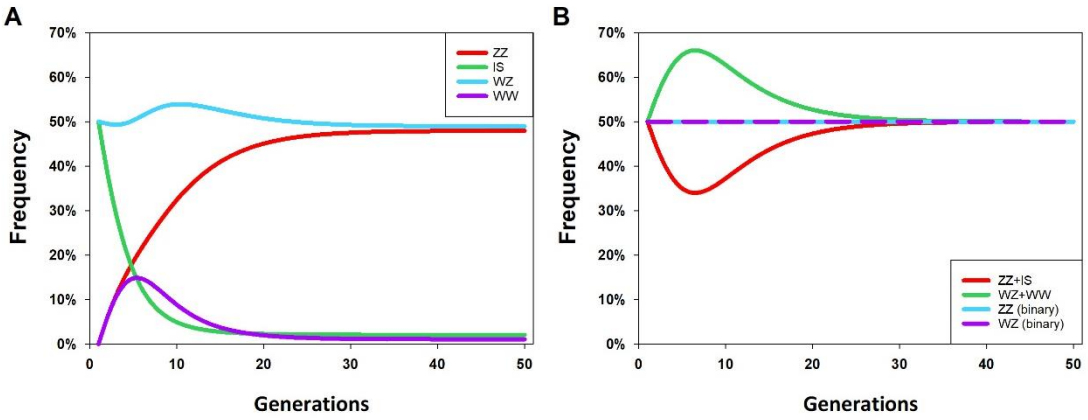

Figure S2. Simulated dynamics of *C. quadricarinatus* population structure, Related to Figure 6. (A) Population structure [ZZ males (red), IS male-intersexuals (green), WZ females (blue) and WW females (purple)] as a function of time for α = 4% (fraction of WZ females emerging as IS) with initial fraction of male-intersexuals in the founder population (i.e., at time t = 0) larger than the long term stable distribution (LTSD) (IS_0 = 50% > IS_∞ and WZ_0 = 50%, with ZZ_0 = WW_0 = 0). (B) Fraction of ZZ + IS males (red) and WZ + WW females (green) in a population with α = 4% with initial fraction of male-intersexuals larger than the LTSD (IS_0 = 50% > IS_∞ and WZ_0 = 50%, with ZZ_0 = WW_0 = 0). In a binary population (i.e., α = 0, ZZ and WZ individuals only), the sex ratio is 1:1 (blue and dashed purple lines).

Transparent Methods

Animals

Cherax quadricarinatus animals (used for DNA extraction, analysis of sex-specific markers and progeny testing) were reared in 600-L tanks at 26±2 °C with constant aeration, a light regime of 14:10 (L:D) and food (shrimp pellets comprising 30% protein) *ad libitum* at Ben-Gurion University of the Negev (BGU), Israel.

Identification of genomic sex markers in *C. quadricarinatus*

C. quadricarinatus DNA was extracted from 12 males (ZZ) and 12 females (8 WZ and 4 WW, confirmed by progeny testing). Briefly, muscle tissue was dissected from each animal and frozen using liquid nitrogen. Then, the tissue was ground with a mortar and pestle, and the DNA was extracted with a DNeasy Blood & Tissue DNA isolation kit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions. The DNA samples were sent to Floragenex (Portland, OR, USA) for restriction site-associated DNA sequencing (RADSeq) using Illumina technology. Subsequent bioinformatics analysis was carried out at the Bioinformatics Core Facility of BGU using NeatSeq-Flow (Sklarz et al., 2018) and additional R scripts. Since the quality of the reads, determined with FastQC version 0.11.2, was satisfactory, no further trimming was performed. Reads from female samples were clustered at 98% similarity using vsearch (Rognes et al., 2016). All reads were aligned against the resulting RAD tags with bowtie2 using the "--very-sensitive preset" option. Reads with mapping quality below 10 were discarded. The number of reads per sample that mapped to each RAD tag was obtained with the idxstats tool included in samtools 1.3 (Li et al., 2009). A tag was considered as a W-associated candidate if it had more than 10 mapped reads in 60% or more (i.e., > 7 animals) of the female samples but did not appear in any of the male samples.

To verify the sex-specific markers identified by RADSeq, 4 μL of gDNA of male and female *C. quadricarinatus* animals (differed from those which were sampled for the RADSeq

analysis) as a template were amplified by PCR (94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and then a final elongation step of 72 °C for 10 min) with 1 μL of forward primer, 1 μL of reverse primer (10 μM each), 12.5 μL of Ready Mix REDTaq (Sigma) and water to a final volume of 25 μL. PCR products were separated on 2% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA). Bands were excised from the gel, isolated using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), and sent for Sanger sequencing. The tag validated as a sex-specific marker was used to test the genotypes of the animals in the different experiments described in this study and also to reveal the genotypes of the different types of intersexuals.

Progeny testing

Adult intersexuals with two female gonopores and one male gonopore (the mostly common gonopore combination that was available in our experimental population) were bred with females in a communal 600-L tank. Upon fertilization, three berried females were transferred to individual 100-L tanks. After hatching, a sample of animals from each progeny ($n_1 = 83$, $n_2 = 68$, $n_3 = 61$) was taken, and the genotype of each sampled animal was determined using the sex-specific markers described above. The observed ratio of WZ/ZZ to WW animals was compared to the expected ratio (3:1) using the chi-square test for goodness of fit.

Field experiments in earthen ponds

As a preliminary experiment, 20 females and 5 intersexuals (each bearing two female gonopores and one male gonopore) were stocked in a 350-m^2 earthen pond at the Aquaculture Research Station, Dor, Israel. All animals weighed between 60 to 100 g. During the five months of grow-out, from May to November, the animals were fed *ad libitum* and water temperature was maintained at 25 ± 4 ºC. In addition, a comparative study of females stocked with males vs females stocked with intersexuals was conducted at the Cherax Park Aquaculture farm, Queensland, Australia. In this experiment, three 375-m^2 earthen ponds were stocked with 130

females and 30 intersexuals (treatment), while two ponds were stocked with 130 females and 30 males (control). Females were in the weight range of 65 to 100 g, and males/intersexuals, 120 to 150 g. During the 6 months of grow-out, from July to December, the animals were fed *ad libitum* and water temperature was maintained at 25 ± 4 ºC. At the end of the grow-out period, both in Israel and Australia, the animals harvested, and the progeny (animal weight ranging from 9 to 60 g) were phenotypically sex sorted by assessment of male and female gonopores. To test whether the females were WZ or WW, the sex markers described above were used. In Israel, all females (n = 43) were genetically tested, while in Australia, 48 females from each treatment pond and 20 females from each control pond were tested.

WW females in native Australian crayfish populations

During this study, we did not have access to or permission to sample wild populations in Australian rivers. Therefore, to reliably assess the native crayfish population, we sampled populations of natively farmed crayfish that had been introduced from the wild eight years prior to sampling and believed not to have artifacts of inbred lines. To determine whether there were WW females in those populations, animals were sampled from four locations. In two locations (A and B; Figure 3) we had access to the entire population. For those populations, 852 animals from A and 570 from B were sorted for sex, and of those 307 and 287 females, respectively, were genetically tested using our sex markers. In the other two locations (C and D; Figure 3), males and females were separated in advance, so we could not examine the sex ratio of the entire population, but we did sample 286 and 294 females to test their sexual genotype (WW or WZ).

Modelling population dynamics of *C. quadricarinatus*

We developed a simple demographic model to simulate discrete-time, density-independent Malthusian growth of *C. quadricarinatus* populations and to track the dynamics of the four

phenotypes-genotypes, namely, ZZ males, WZ intersexuals (designated IS), and WZ and WW females.

We assumed that the mean generation time, i.e., the mean age at which females give birth, to be equal to 6 months, and the length of the time step *t* was set accordingly. We also assumed that the fraction σ_o of the progeny generated at time *t* and surviving to the next time step would be reproductive 6 months later, namely, at time *t+1*. The species-specific maximum life span of *C. quadricarinatus* depends on environmental (temperature, salinity, pH, water quality, etc.) and ecological (productivity, presence of predators, etc.) conditions. Field studies have provided evidence of *C. quadricarinatus* populations with a life span of about 5 years (Jones, 1990). To account for the higher mortality of younger/smaller individuals, we assumed that the mean life expectancy *LE*, i.e., the mean number of years that a 1-year old crayfish could be expected to live, was about half the life span, i.e., 2.5 years; we then computed the natural mortality rate μ [year^{-1}] as 1/*LE* and derived the 6-month survival, i.e., the fraction σ_A of adult crayfish surviving between time *t* and time *t+1*, as $\sigma_A = \sqrt{e^{-\mu}} = 81.9\%$. We assumed a rapidly growing population capable of doubling its abundance in a 10-generation time span, i.e., about 5 years, which corresponds to a 6-month finite growth rate (λ) of $\lambda = 2^{\frac{1}{10}} = 1.0718$, i.e., a 7.18% of population increment in 6 months or, equivalently, a 14.88% increment per year. We assumed a per-capita fecundity of φ=300 eggs per reproductive event for both WZ and WW females [which is within the range of previous reports (Curtis and Jones, 1995)], and set the fraction of eggs hatching and surviving to reproductive maturity 6 months later as σ_o = $(\lambda - \sigma_A)/(½\ \varphi) = 0.169\%$, which is consistent with a 7.18% population growth rate in 6 months. We assumed that at each time step adult WZ and WW females in the population are fertilized by males and intersexuals in proportion to the males' relative abundance in the population, namely ZZ/(ZZ+ IS) and IS/(ZZ+ IS), respectively. A small fraction α of the WZ progeny will emerge as intersexuals (IS). While according to the literature IS proportion can range from 1% to 8%; (Brummett and Alon, 1994; Curtis and Jones, 1995; Sagi et al., 1996; Thorne and Fielder, 1991), most of the published data report on 4% of WZ progeny that emerges as intersexuals.

Therefore, in most scenarios described below we have used α=4%. However, since it is unknown whether this value is fixed within given populations and the genetic basis behind the intersexuality phenomenon is not clear, we allowed the option of changing the α in the model's Excel file provided with this article (Data S1). The formulas for deriving the reproductive output in each mating type, given the abundance of males and females in the brood stock, are given in Table S2. Accordingly, the equation describing the dynamics of the population may be expressed as follows:

$$\mathbf{N}_{t+1} = \mathbf{M} \times \mathbf{N}_t + \sigma_o \cdot \mathbf{R}_t \quad (1)$$

where:

- $\mathbf{N}_t = [ZZ_t, IS_t, WZ_t, WW_t]$ is the vector with the number of individuals of type ZZ, IS, WZ and WW, respectively, at time *t*, just before reproduction*;*
- **M** is a 4 × 4 square matrix with sub-diagonal elements $m_{ij} = \sigma_A = 0.819$ ($i = 2..4$, $j = 1..3$), zero otherwise, "×" indicates matrix multiplication;
- $\sigma_o = 0.169\%$ is the fraction of eggs that hatch and survive to the next generation;
- $\mathbf{R}_t$ is the vector for egg reproductive output at time *t* for each type computed by using the reproduction formula reported in Table S2, namely:

$$R_t^{ZZ} = \frac{1}{2}\varphi \frac{ZZ_t}{ZZ_t + IS_t} WZ_t + \frac{1}{4}\varphi \frac{IS_t}{ZZ_t + IS_t} WZ_t = \frac{1}{2}\varphi WZ_t \left(\frac{ZZ_t}{ZZ_t + u \cdot IS_t} + \frac{1}{2} \frac{IS_t}{ZZ_t + IS_t} \right) \quad (2a)$$

$$R_t^{IS} = \alpha \frac{1}{2}\varphi \frac{ZZ_t}{ZZ_t + IS_t} WZ_t + \alpha \frac{1}{2} f \frac{IS_t}{ZZ_t + IS_t} WZ_t + \alpha \varphi \frac{ZZ_t}{ZZ_t + IS_t} WW_t + \alpha \frac{1}{2}\varphi \frac{IS_t}{ZZ_t + IS_t} WW_t$$

$$= \alpha \frac{1}{2}\varphi \left(WZ_t + \frac{2 \cdot ZZ_t + IS_t}{ZZ_t + IS_t} WW_t \right) \quad (2b)$$

$$R_t^{WZ} = (1 - \alpha) \frac{1}{2}\varphi \left(WZ_t + \frac{2 \cdot ZZ_t + IS_t}{ZZ_t + IS_t} WW_t \right) \quad (2c)$$

$$R_t^{WW} = \frac{1}{4} f \frac{IS_t}{ZZ_t + IS_t} WZ_t + \frac{1}{2} f \frac{IS_t}{ZZ_t + IS_t} WW_t = \frac{1}{2} f \frac{IS_t}{ZZ_t + IS_t} \left(\frac{1}{2} WZ_t + WW_t \right) \quad (2d)$$

where φ = 300 eggs is the per-capita female fecundity, ½ accounts for the sex ratio, and $\alpha = 4\%$.

Population dynamics were simulated for 50 generations (25 years) with α, the fraction of WZ animals emerging as IS, set either to 4% or 0% (the 0% is representing a population generated by only males and females but no intersexuals). For initial conditions (i.e., number of individuals in each class at time t = 0), we considered four cases, two hypothetical cases, with the fraction of intersexuals in the population being either larger or smaller than the fraction of intersexuals at the long term stable distribution (LTSD), and two cases representing the estimated population structure for two of the native Australian populations sampled in this study (populations A and B described in Table 3), as follows:

- 0% intersexuals (all males are ZZ); $\mathbf{N}_0 = [ZZ_0 = 100, IS_0 = 0, WZ_0 = 100, WW_0 = 0]$.
- 50% intersexuals (all males are WZ); $\mathbf{N}_0 = [ZZ_0 = 0, IS_0 = 100, WZ_0 = 100, WW_0 = 0]$.
- AU – A ; $\mathbf{N}_0 = [ZZ_0 = 423, IS_0 = 10, WZ_0 = 417, WW_0 = 2]$.
- AU – B ; $\mathbf{N}_0 = [ZZ_0 = 276, IS_0 = 7, WZ_0 = 284, WW_0 = 3]$.

For the four scenarios above, the fraction α of WZ animals emerging as IS was set to 4%. We also ran an additional hypothetical simulation for α = 0% (intersexuals cannot occur) to represent the case of 1:1 males:females sex ratio. The Excel file used to run the model is available in the online supplementary information (Data S1).

Phylogenetic analysis of decapod crustaceans

The identification of orthologs from diverse crustaceans and their multiple sequence alignment were carried out at the Bioinformatics Core Facility of BGU using the NeatSeq-Flow platform (Sklarz et al., 2018). The transcriptomes of all decapod crustacean species that were available in NCBI (http://www.ncbi.nlm.nih.gov) were downloaded (36 decapod species) ("NCBI,") ("NCBI,"). Two more transcriptomes, one from *Pandalus platyceros* and the other from *Hippolyte inermis*, had been assembled in currently performed studies in our laboratory. All 38 transcriptomes were translated with Transdecoder version 5.5.0 (Haas and Papanicolaou, 2016), and the protein sequences of the longest open reading frames (ORFs) for each transcript were used for further analysis. BUSCO analysis (Simão et al., 2015) was performed on all

transcriptomes, using "--mode transcriptome" against the Metazoa database (metazoa_odb9). An R script was used to select BUSCO accessions that appeared at least once in all transcriptomes, resulting in 87 (of 978) "shared BUSCOs." The protein sequences of the 87 shared BUSCOs were extracted from the "ancestral" file of the metazoa_odb9 database. A blast database was constructed for each of the transcriptome predicted protein sets, and blastp version 2.7.1+ (Altschul et al., 1997) was used to find the predicted proteins matching the shared BUSCO protein sequences. The blastp result tables were processed with parse_blast (https://github.com/bioinfo-core-BGU/parse_blast) with the following arguments: --max_evalue 1e-10 --min_align_len 60 --min_coverage 70 --min_pident 50. For each shared BUSCO, matched protein sequences from all transcriptomes were extracted using samtools faidx version 1.3 (Li et al., 2009). Out of the 87 protein sequences, 11 (Table S3) appeared only once in each of the 38 transcriptomes. We have used only proteins that were represented once to avoid the complications of analyzing the presence of isoforms. The sequences of these 11 proteins were concatenated and submitted to multiple sequence alignment with MAFFT version 7.427 (Katoh and Standley, 2013). To find the best phylogenetic model to select with likelihood-based criteria, we used the Smart Model Selection (SMS) function with Bayesian information criterion (BIC) in PhyML (Lefort et al., 2017). Finally, the evolutionary history was inferred by the maximum likelihood method and the Jones-Taylor-Thornton (JTT) matrix-based model (Jones et al., 1992) with bootstrapping 1000 replicates. The evolutionary phylogenetic analysis was conducted and visualized using MEGA X (Kumar et al., 2018).

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