



# All-female crayfish populations for biocontrol and sustainable aquaculture

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## ABSTRACT

Snail control in aquaculture and control of snail vectors implicated in animal and human diseases are global challenges. Potential biocontrol agents could be species of crayfish, which are known to be voracious predators of snails. However, at present, the use of crayfish as biocontrol agents is limited due to their invasive nature. A promising candidate biocontrol agent is the Australian redclaw crayfish, *Cherax quadricarinatus*, a key species in the aquaculture industry. However, the downside of using this species for biocontrol is that *C. quadricarinatus* aquaculture escapees – being highly robust, fast growing, and ferocious omnivores – have become invasive, establishing feral populations around the world. Here we suggest the production of monosex *C. quadricarinatus* populations that open two exciting biotechnology applications—as a possible sustainable solution for snail control and as a means to improve yields and management in crayfish aquaculture, without the inherent danger of establishing invasive populations of aquaculture escapees. In the first part of this study, *C. quadricarinatus* was tested as a snail eradicator. Both males and females were highly efficient in predation experiments, eliminating 89% and 98% of *Thiara scabra* snails, respectively. The superior efficiency of the females in the predation experiments and the possible advantage for aquaculture of all-female crayfish populations provided the rationale for the second part of the study—the development of an economically feasible and non-labor-intensive biotechnology for the production of all-female *C. quadricarinatus* populations. To this end, we leveraged the naturally occurring intersexuality in *C. quadricarinatus* populations to produce female-biased populations. Females bearing only the W sex chromosome isolated from such populations using W and Z-specific genomic markers were found to be reproductive when bred with WZ intersexuals. Resulting progenies from the above crosses were 100% female populations including 50% WW females, thus paving the way to the establishment of an all-female producing brood stock. We present a quantitative scheme that demonstrates how our approach, which does not include any genomic intervention, can be implemented both for sustainable aquaculture and to fight harmful snails damaging aquaculture, agriculture, and human health.

## 1. Introduction

### 1.1. Biocontrol of snail pests in aquaculture, agriculture, and human health

Snail pests are responsible for crop damage in both agriculture and aquaculture and also for damage to human and animal health (Chai et al., 2013; Pinto and De Melo, 2011; Stenseth et al., 2003). For example, in agriculture, apple snails inflict significant direct damage on

rice wet-land fields world-wide (Cowie, 2002; Horgan et al., 2014). In the aquaculture industry, snails act as secondary hosts to disease-causing parasites (Edgerton et al., 2002; Pinto et al., 2018). These snails inflict major damage both in the wild and in fish aquaculture ponds, where severe pathologies in major cultured species could reduce fish yields and survival by approximately 25% (Fleming et al., 2011; Gjursevic et al., 2007; Mitchell et al., 2002). At present, there is no sustainable treatment for fish parasitic diseases, and thus control efforts are focused on disease prevention through snail control. Finally, snail-

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borne diseases, such as schistosomiasis (infecting 36 out of 100,000 people every year in Africa alone), constitute a major threat to human health (De Boni et al., 2021). The damage inflicted by trematode-carrying snails both in natural habitats and in agricultural and aquacultural systems thus calls for the control of snails in a sustainable manner. The solution to this problem may lie in the exploitation of decapod crustaceans as biocontrol agents (Hoover et al., 2019), as is proposed herein.

### 1.2. Crustaceans as natural predators of snails

Decapod crustaceans are ferocious predators, known to feast on organisms of various trophic levels in their natural habitats and in captivity (Mao et al., 2016; Nelson, 1981). As long ago as the early 1970s, it was therefore suggested that crustaceans could be used as predators of snail pests (Sohn and Kornicker, 1972). Subsequently, evidence for the predation of disease-carrying snails has been well documented (Arostegui et al., 2019; Wood et al., 2019), with several crustacean species being reported to be highly effective predators of snails (Hamilton, 1976; Lorda et al., 2016; Pechenik et al., 2010; Savaya-Alkalay et al., 2018a; Savaya et al., 2020). Pechenik et al., for example, demonstrated the predation efficiency of the invasive crab species, *Hemigrapsus sanguineus*, on the slippershell snail *Crepidula furcata* (Pechenik et al., 2010). Among decapod crustaceans, crayfish in particular were found to be highly efficient in snail predation. In both laboratory and field studies, the crayfish *Procambarus clarkii* voraciously devoured native snail species (Ibrahim et al., 1995; Sulieman et al., 2013). However, *P. clarkii* has, itself, become an invasive species in many areas around the globe (Gherardi, 2006; Service, U.F.A.W, 2015; Souty-Grosset et al., 2016). To address the problem of the potential of crustacean species to become invasive, it has become necessary to find innovative – and sustainable – biocontrol systems that are nonetheless based on crustacean predation on snail hosts for breaking parasite life cycles (Hoover et al., 2019; Savaya et al., 2020). Here, we propose to obviate the potential for invasiveness by using an all-female population of the Australian redclaw crayfish *Cherax quadricarinatus* (Crustacea) as a predator of *Thiara scabra*, a fish-disease carrying pest snail, which is an invasive species in many parts of the world (Heller et al., 2014; Moustafa and Hussien, 2019; Thompson et al., 2009).

### 1.3. The invasive nature and global invasive incidence of *C. quadricarinatus*

*C. quadricarinatus*, which is a freshwater species that is native to Australia and southern Papua New Guinea, has become a successful invasive species (Akmal et al., 2021), facilitated by successful competition for shelter (Chivambo et al., 2020) and predation on macro-invertebrates (De Moor, 2002; Marufu et al., 2018) and the eggs of nesting fish (Marufu et al., 2018). In addition, aggressive behavior, either intraspecific between *C. quadricarinatus* individuals or interspecific toward other aquatic vertebrates, has been reported (Austin and Ryan, 2002; Bradsell et al., 2002). Invasions of *C. quadricarinatus* worldwide have led to the establishment of populations in Indonesia, Singapore, Martinique, Zambia, Zimbabwe, Mexico and more (Ahyong and Yeo, 2007; Baudry et al., 2020; Marufu et al., 2014; Patoka et al., 2016), where any introduction of *C. quadricarinatus* to new habitats resulted in the establishment of feral populations from aquaculture escapees, due to their robustness and resistance to relatively low temperatures (Ahyong and Yeo, 2007; Rigg et al., 2020). Attempts to eradicate invasive populations of *C. quadricarinatus* have been unsuccessful, and there is currently no established protocol for eradicating such populations in the wild (Haubrock et al., 2021).

### 1.4. *Cherax quadricarinatus* aquaculture, sexual heritability and intersex individuals

In recent years, there has been a growing demand for the production of unisex populations in crayfish husbandry and aquaculture alike (Tacon et al., 2011) as a means to improve yields and ensure sustainability (Bardhan et al., 2021; Levy et al., 2017). In the past decade, a number of studies have indeed been devoted to the advantages and cost effectiveness of all-female or all-male aquaculture, including those for the aquaculture species *C. quadricarinatus* (Naranjo-Paramo et al., 2018; Naranjo-Paramo et al., 2021; Rigg et al., 2020). This species is an high demand globally due to its fast growth rate and good survival compared to shrimps or crabs (Ankesheva et al., 2021). However, *C. quadricarinatus* is a ferocious omnivore (Marufu et al., 2018) that is considered a classical invasive species: since it is a robust species without natural competitors or predators outside of its native habitat, it quickly establishes invasive feral populations in new habitats (Bortolini et al., 2007; Doupe et al., 2004; Madzivanzira et al., 2021; Snovsky and Galil, 2011). Nonetheless, in monosex populations, these characteristics could be leveraged for the dual purpose of enhancing aquacultural yields (Cortes-Jacinto et al., 2004; Curtis and Jones, 1995; Rodgers et al., 2006) and providing sustainable biocontrol of disease-carrying snails, with no threat of invasion. However, the production of monosex populations of *C. quadricarinatus* by manual sorting of juveniles is not practical for breeders and growers, since the process is labor intensive and prone to human error. Furthermore, distinguishing between the sexes in *C. quadricarinatus* is possible only after an initial growth period of the juveniles; thus, manually sorted populations could not be grown as monosex from day zero and their pure monosex nature would be both questionable and prone to human error. To increase reliability and to save time and effort, a possible solution would be the use of temporal RNA-interference (RNAi) to perform sex reversal, as has been done in other crustacean species (Molcho et al., 2020; Ventura et al., 2012). However, growing interest in non-manipulated food sources is providing the rationale for developing alternative biotechnologies not based on RNAi for the production of monosex populations of a number of crustacean species, which are characterized by the WZ/ZZ system of inheritability. In particular, our group has focused on exploiting newly devised W and Z molecular sex markers along with the use of naturally occurring intersex individuals (Jin et al., 2022; Levy et al., 2020), where relevant, for the production of monosex populations. In *C. quadricarinatus*, intersexuality (Levy et al., 2020; Parnes et al., 2003) is manifested as the presence of different combinations of male and female gonopores in the same individual that bears the WZ genotype (Parnes et al., 2003). While the main obstacle to generating monosex female populations in crustacean species is the production of neomales bearing the WZ genotype, intersex *C. quadricarinatus* individuals represent naturally born equivalents of WZ males (Parnes et al., 2003). These intersex crayfish can be used to generate the WW females needed for the production of all-female progenies.

### 1.5. *Cherax quadricarinatus* all-female production for the dual purpose of snail biocontrol and aquaculture

Use of all-female *C. quadricarinatus* populations could provide a means for biocontrol of disease-carrying snails and also to improve aquaculture yields without the risk of establishing feral populations from aquaculture escapees. In the present study, we first tested the utility of exploiting *C. quadricarinatus* crayfish as predators of pest snails. Since our experiments showed that the female crayfish were superior predators and since the sustainable use of crayfish as biocontrol agents will require populations with no invasive capabilities, we investigated the possibility of producing all-female (rather than all-male) populations without using methods based on molecular manipulations, thus producing all-female populations of non-genetically modified organism (GMO) *C. quadricarinatus*. Such populations would also be suitable for

use in aquaculture as a means of enhancing crayfish yields.

## 2. Materials and methods

### 2.1. Crayfish and snails

*C. quadricarinatus* crayfish were grown in a dedicated facility at Ben-Gurion University of the Negev in 600-L tanks at  $26 \pm 2$  °C with light regime of 14:10 (L:D) and constant aeration. Feed [shrimp pellets comprising 30% protein and Frozen Single Ingredients food (i.e., bloodworms, brine shrimp, and cyclops; Ocean Nutrition, Essen, Belgium)] was provided ad libitum.

*T. scabra* snails were collected from Kibbutz Tirat Tzvi (N 32.420907, E 35.536143) and Kibbutz Nir David (N 32.501062, E 35.469657) and from a water canal near Kibbutz Shluhot (N 32.462482, E 35.474086), Northern Israel.

### 2.2. Predation of *Cherax quadricarinatus* crayfish on *Thiara scabra* snails

Twenty male and twenty female *C. quadricarinatus* crayfish, each weighing  $20 \pm 7$  g, were used for the predation experiments. Before starting the experiments, all 40 crayfish were X-rayed (Shechter et al., 2008) to make sure they were at the intermolt molt-stage. The reason for this step was to reduce the chance that the crayfish would molt during the predation experiment, since freshly molted crayfish do not resume eating immediately after molt. Each individual crayfish (whether male or female) was placed in a 130-L tank with constant aeration and water filtration. Water temperature was kept at  $27 \pm 3$  °C. After *C. quadricarinatus* acclimation in the tank and feeding, each tank was supplemented with 10 small (2–4 mm) and 10 large (4–6 mm) *T. scabra* snails. The predation rate was monitored – three times a day for the first three days and then once a day for the next seven days – by siphoning off shell fragments from the bottom of each tank and then counting the number of live snails. The crayfish were fed with Frozen Single Ingredients food on the first day of the experiment and with dry pellets daily thereafter. GoPro cameras (GoPro Inc., San Mateo, CA, USA) were placed on constant video mode to monitor the predation in two randomly selected predation tanks for the duration of the experiment. Videos were screened for predation events, and selected scenes are attached to this article as proof of concept (POC) videos.

Statistical analyses of predation efficiency for the male and female experiments were performed with the Cox proportional hazards model from the *Survminer* and *Survival* packages (Fox and Weisberg, 2002) in R software, as previously described (Savaya-Alkalay et al., 2018b), with treatment (control = 0, predator = 1), predator sex (F, M) and snail size (2–4 mm, 4–6 mm) as covariates, using the time-to-event data;  $p < 0.05$  was considered statistically significant. This kind of data enables evaluation of the different predictors (such as predator sex) on the rate of the occurrence of predation events by the crayfish. Kaplan-Mayer curves were plotted to depict snail survival in the presence of *C. quadricarinatus* predators and tested with the Gehan-Breslow test.

### 2.3. Progeny testing of female-biased *Cherax quadricarinatus* populations

To test whether progenies from WZ intersexuals crossed with wild-type females are female biased, adult intersex crayfish were crossed with adult females ( $N = 3$ ) and adult males were crossed with adult females ( $N = 3$ ). *C. quadricarinatus* intersex individuals were genotyped as WZ using sex-specific markers reported before: For chromosome Z, the sex marker reported by Levy et al. (Levy et al., 2020) was used. For chromosome W, one specific marker pair (designated W1-SM) from the work of Jin et al. (Jin et al., 2022) was utilized. Every female found with eggs was moved to a separate tank until the juveniles hatched. After hatching, samples of crayfish from the progeny of intersex  $\times$  females ( $n_1 = 58$ ,  $n_2 = 69$ ,  $n_3 = 82$ ) and from the progeny of males  $\times$  females ( $n_1 = 63$ ,  $n_2 =$

67,  $n_3 = 70$ ) were taken, and the phenotype of each sampled crayfish was determined based on the observed gonopores. The observed ratio of females + intersex to males was compared to the expected ratio (3:1 in intersex  $\times$  female progenies and 1:1 in male  $\times$  female progenies) using the chi-square test for goodness of fit;  $p < 0.05$  considered statistically significant. Females from the progenies of intersex  $\times$  adult WZ females were separated from males and intersex crayfish and grown separately in 600-L tanks. Sex chromosome composition of these females was determined.

### 2.4. Isolation of *Cherax quadricarinatus* WW females from female-biased populations

Females from test crosses were genotyped using the same sex-specific markers described above. Determination of female bias was performed for each test cross by sampling and calculating M:F:intersex ratios. To isolate WW 'super-females', all the females from each test cross were isolated and genotyped using the above sex markers. DNA extraction and PCR detection of the Z sex marker was performed as described previously (Levy et al., 2020). For the W sex chromosome and the 18S rRNA gene (housekeeping gene that served as a positive control), the PCR program was as follows: 94 °C for 3 min, followed by 40 cycles of 94 °C for 30s, 55 °C for 30 s and 72 °C for 45 s, and then a final elongation step of 72 °C for 10 min. The PCR reaction mix included 1  $\mu$ l of forward primer, 1  $\mu$ l of reverse primer, 12.5  $\mu$ l of Ready Mix REDTaq (Sigma-Aldrich, Burlington, MA, USA) and water to a final volume of 25  $\mu$ l. PCR products were separated on 2% agarose gel stained with SYBR Safe DNA gel stain (ApexBio, Houston, TX, USA).

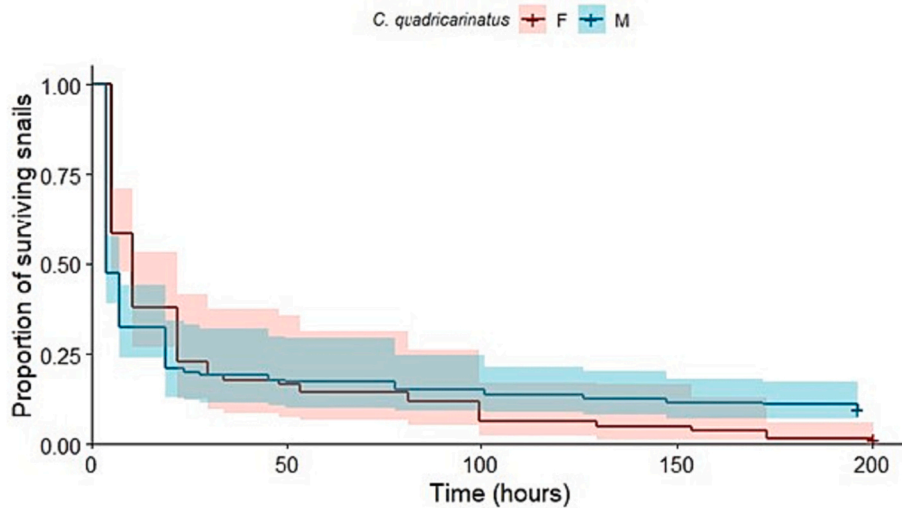
### 2.5. Progeny testing of *Cherax quadricarinatus* all-female populations

Four adult WW females were isolated from the female-biased populations described above. Each female was crossed with an intersex crayfish bearing the WZ chromosome. Each female with eggs was moved into a separate tank until the juveniles hatched. Two weeks after hatching, a sample of 30 crayfish from each progeny was taken, DNA was extracted, and the sex chromosomes were determined using the same sex markers as described above. All samples were analyzed statistically using the chi-square test for goodness of fit to test for deviations from the 1:1 expected ratio for WZ:WW genotypes;  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. *Cherax quadricarinatus* predation on *Thiara scabra* snails

Predation experiments were performed to assess the ability of *C. quadricarinatus* to eradicate snails. To test for differences in predation efficiency between the sexes, two separate experiments were performed, one with female crayfish and the other with male crayfish. The Cox proportional hazard model (whole model statistics: Wald test = 266.9, DF = 2,  $p < 0.001$ , Table S1) indicated that snail mortality rate was 40-fold higher in the presence of a crayfish, be it a male or a female ( $e^\beta = 40.20$ ,  $z = 14.192$ ,  $p < 0.001$ ). Mortality rate of the larger snails (4–6 mm) was nearly 50% lower than that of the small snails (2–4 mm) ( $e^\beta = 0.507$ ,  $z = -7.323$ ,  $p < 0.001$ ). The survival rate of the snails in the control tanks was 96% (Fig. S1), proving that predation, and no other factor (such as water quality), was responsible for snail mortality. Fig. 1 shows the proportion of surviving snails in the presence of a *C. quadricarinatus* predator according to predator sex. Both males and females were successful in significantly reducing the snails in the tank by 89% and 98%, respectively, within a 200-h period. At the beginning of the predation experiments, the predation efficiency of the males was superior, with the crayfish preying on 79% of the snails in the first 10 h and reducing the snails to 17% of the original population within the first 50 h of the experiment. Thereafter, the predation rate slowed down,



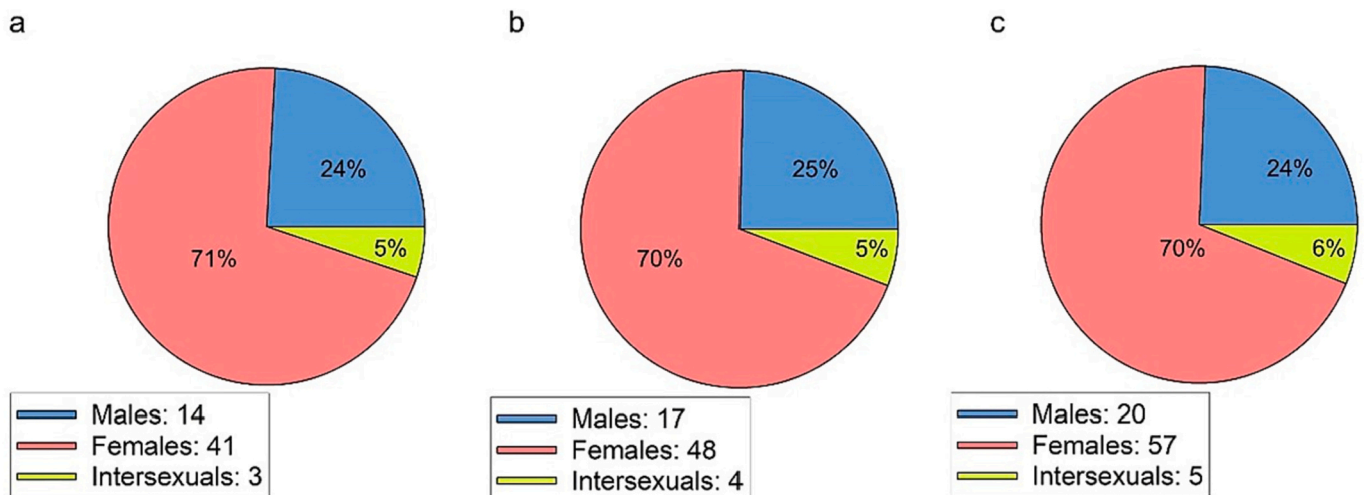
**Fig. 1.** Kaplan-Meier plot of snail survival in the presence of a *C. quadricarinatus* predator. A total of 50 water tanks were used for the experiment: the tanks were divided into five blocks, and in each block one tank served as a control tank without a predator, and the other four tanks each contained one crayfish (either male or female) weighing  $20 \pm 7$  g. Each of the 50 tanks also contained a total of 20 *T. scabra* snails. The X axis represents time in hours, and the Y axis, the proportion of surviving snails. The results for the female *C. quadricarinatus* crayfish are indicated in red and those for the males in teal. The corresponding shadows are the 95% confidence intervals. The ending + sign is the censor ending. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nearly reaching a plateau and reducing the snail population slowly from 17% to 15% between 50 and 100 h, and from 13% to 11% between 100 h and the end of the experiment. The predation efficiency of the females was also good the first 10 h, reducing the snails in the tank to 58%, but thereafter it improved considerably to reduce the snails to 16% in the first 10 to 50 h of the experiment and to 2% by the end of the experiment. Increments of 10 h showing the number of snails remaining as prey in each experiment is presented in Table S2, showing predation efficiency trends for both male and female *C. quadricarinatus*. Nonetheless, both male and female *C. quadricarinatus* were very efficient in preying on the *T. scabra* snails, with no overall significant difference in predation between the sexes ( $z = 1.371, p = 0.17$ , Table S3). Videos from the GoPro camera showing predation of *T. scabra* snails by a male and a female crayfish are appended to this article as Supplemental Videos S1 and S2, respectively. From the videos and observations during the male experiment (Supplemental Video S3), the males seemed less consistent in their preying activity and at a certain point they did not continue to attempt to crack open the larger snails, while the females continued to

prey on the larger ‘more-difficult-to-handle’ snails until they had eliminated all the snails in the tank, as may be seen in Fig. S1. With regard to the association between predation efficiency and prey size, both male and female crayfish exhibited a marked preference for the smaller snails (Fig. S1, Tables S1, S3). In particular, *C. quadricarinatus* males were less efficient in preying on the larger snails vs. the smaller snails and vs. the females’ ability to prey on the larger snails: While the males successfully eliminated all the small (2–4 mm) snails from their tanks (Fig. S1, light blue line), at the end of the experiment 23% of the larger snails (4–6 mm) in their tanks survived (Fig. S1, dark blue line).

**3.2. Progeny compositions toward all-female production of *Cherax quadricarinatus***

An examination of the progeny of intersex crayfish crossed with females (WZ × WZ) under laboratory conditions revealed that the observed ratios of females+intersexuals to males in the three progenies tested were female biased. The female bias of WZ × WZ test crosses is



**Fig. 2.** Pie charts of progenies from three WZ × WZ test crosses showing the proportions of sexes in each progeny. Percentages of each sex (female, male or intersex) are given on the pie charts, and numbers of crayfish of each sex is given below each pie chart.

clearly evident in Fig. 2 (3.1:1 in all three progenies) and was not statistically different from the expected 3:1 ratio ( $p > 0.05$ ). In contrast, the observed ratios of females+intersexuals to males in the three progenies tested of males crossed with females (ZZ  $\times$  WZ) resulted in almost equal proportions of males and females (1.25:1, 1.2:1, and 1:1; Fig. 3) and did not differ significantly from the expected 1:1 ratio ( $p > 0.05$ ). Detailed chi-square goodness of fit test results are presented in Table 1. Interestingly, WZ  $\times$  WZ test crosses did not significantly alter the percentage of intersexual individuals compared to control test crosses.

### 3.3. Isolation and growth of viable *Cherax quadricarinatus* WW females

Females from test crosses were isolated, and their sex chromosomes were genotyped using molecular Z and W markers. Out of all the females genotyped, six females lacked the Z sex chromosome (Fig. 4). A ZZ genotyped male, a WZ genotyped female and a WW genotyped female served as controls for the presence of W and Z sex chromosomes. The ZZ male had a single representative Z chromosome band. The WZ female had both the Z and W representative bands, and the WW female had only the W band; 18S served as a positive control to ensure genomic DNA integrity. Negative controls in all three PCR reactions were performed without the addition of any genomic DNA to the reaction tube and all resulted in null PCR products. For all six females tested, there was no evidence of the Z sex chromosome PCR product, and thus confirming that these females were validated to be carrying only the W sex chromosome.

### 3.4. Crossing WW females with WZ intersex crayfish

Four of the WW females isolated were crossed with WZ intersex crayfish. All WW females tested were found to be reproductively viable. Sex chromosomes were genotyped for samples of 30 crayfish from each progeny, and all crayfish were found to be females, either WZ or WW (see Fig. 5 for representative sample). No statistical difference from the expected 1:1 ratio ( $p > 0.05$ ) was found according to chi-square goodness of fit test (Table 2).

## 4. Discussion

In this study, male and female *C. quadricarinatus* crayfish were investigated as potential biocontrol agents against fish-disease-carrying *T. scabra* snails (Savaya et al., 2020). Although both sexes were very efficient as snail eradicators, the male crayfish were less persistent in snail predation than the female crayfish, pointing to possible advantage of using an all-female population as a biocontrol agent against pest snails. This difference between males and females in snail predation behavior should be explored further in the future, as to date there is no evidence of differences in prey handling between female and male

**Table 1**

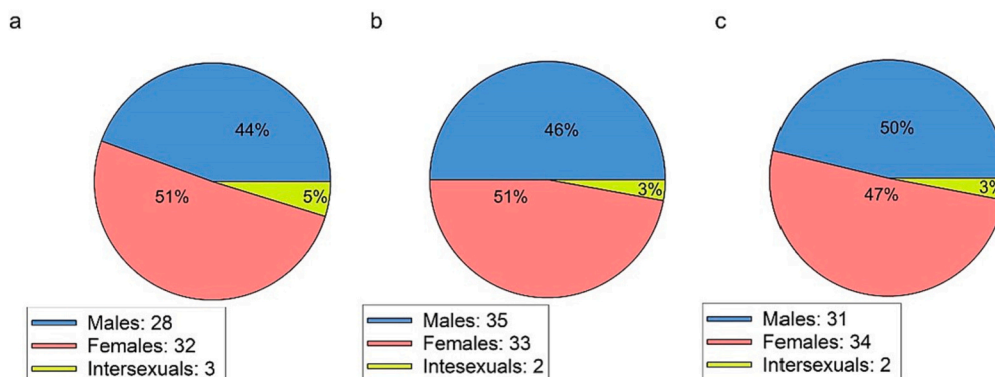
Observed and expected phenotypes in crayfish progenies of crosses of males (M)  $\times$  females (F) (ZZ  $\times$  WZ) and intersex crayfish (I)  $\times$  females (F) (WZ  $\times$  WZ).

Progeny	Total N	Observed phenotype		Expected phenotype		p value
		M	F + I	M	F + I	
M $\times$ F (1)	63	28 (44%)	35 (56%)	31.5 (50%)	31.5 (50%)	0.38
M $\times$ F (2)	67	31 (46%)	36 (54%)	33.5 (50%)	33.5 (50%)	0.54
M $\times$ F (3)	70	35 (50%)	35 (50%)	35 (50%)	35 (50%)	1
I $\times$ F (1)	58	14 (24%)	44 (76%)	14.5 (25%)	43.5 (75%)	0.88
I $\times$ F (2)	69	17 (25%)	52 (75%)	17.25 (25%)	51.75 (75%)	0.94
I $\times$ F (3)	82	20 (24%)	62 (76%)	20.5 (25%)	61.5 (75%)	0.9

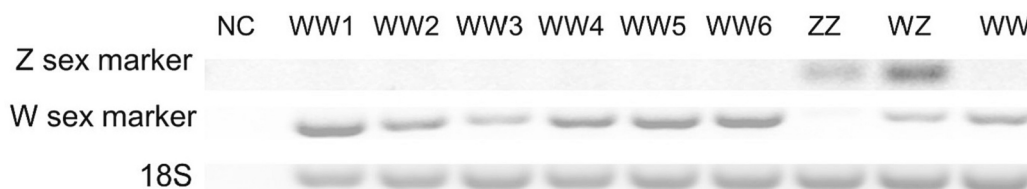
Values in the table are the numbers and percentages of sampled crayfish from each progeny.  $p$  values represent the level of significance between the observed and expected results according to the chi-square goodness of fit test.

crustaceans. Nonetheless, Fig. 1 clearly shows the superior predation abilities of *C. quadricarinatus* females in the laboratory under conditions of ad libitum food availability. Thus, this research adds a potential agent to the biocontrol toolbox, thereby addressing some of the pressing challenges of snail control in aquaculture [e.g., preventing gill-infecting fish diseases transported by snails (Gjurcevic et al., 2007; Mitchell et al., 2002)], in agriculture (e.g., eradicating apple snails in rice paddies) and in human health [fighting the transmission of schistosomiasis (De Boni et al., 2021; Wood et al., 2019)]. Including all-female *C. quadricarinatus* populations in integrative pest control (IPC) without concern that the species could become invasive will thus pave the way for many exciting applications, from poverty alleviation and the control of schistosomiasis in African villages (Hoover et al., 2019) – through fighting agricultural damage and crop loss due to apple snails in rice paddies (Cowie, 2002; Horgan et al., 2014; Savaya-Alkalay et al., 2018b) – up to the sustainable aquacultural production of crayfish without any molecular manipulations. Given that monosex crayfish populations will be available in high quantities and at an affordable price as a result of this research, the voracious predation ability of *C. quadricarinatus* on snails presented in this study for the first time suggests that monosex populations of this species could serve as excellent biocontrol agents. The idea of utilizing crustaceans to control pests such as disease-carrying snails has been presented before (Savaya et al., 2020; Sulieman et al., 2013), making the trials described in this work a proof of concept for *C. quadricarinatus*.

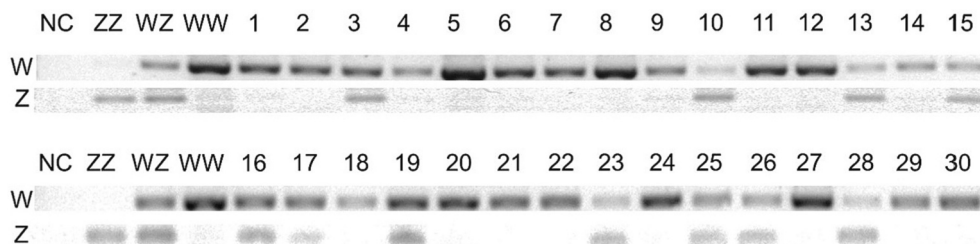
From an aquaculture perspective, *C. quadricarinatus* is a popular aquaculture species due to its simple life cycle (no larval stages), fast growth and robustness (Rigg et al., 2020). However, it was shown some



**Fig. 3.** Pie charts of progenies from three ZZ  $\times$  WZ test crosses showing the proportions of sexes in each progeny. Percentages of each sex (female, male or intersex) are given on the pie charts, and numbers of crayfish of each sex is given below each pie chart.



**Fig. 4.** In vitro validation of sex chromosomes for WW *C. quadricarinatus* females. PCR of the genomic DNA of six WW females for the W and Z sex markers. Top, middle and bottom rows are the bands of Z, W and 18S genomic PCR products, respectively. NC – negative control. WW1 to WW6 – six WW females tested. ZZ, WZ, WW – known controls of the three known sexual genotypes of *C. quadricarinatus*.



**Fig. 5.** Results of progeny genotyping (30 crayfish) from a WZ genotyped intersexual × genotyped female WW test cross. PCR products of the specific W and Z sex markers are displayed in the top and bottom rows, respectively. NC – negative control. ZZ, WZ, WW – crayfish with known genotypes serving as positive controls.

**Table 2**  
Observed and expected phenotypes in progenies of intersex × female (WZ × WW) crayfish.

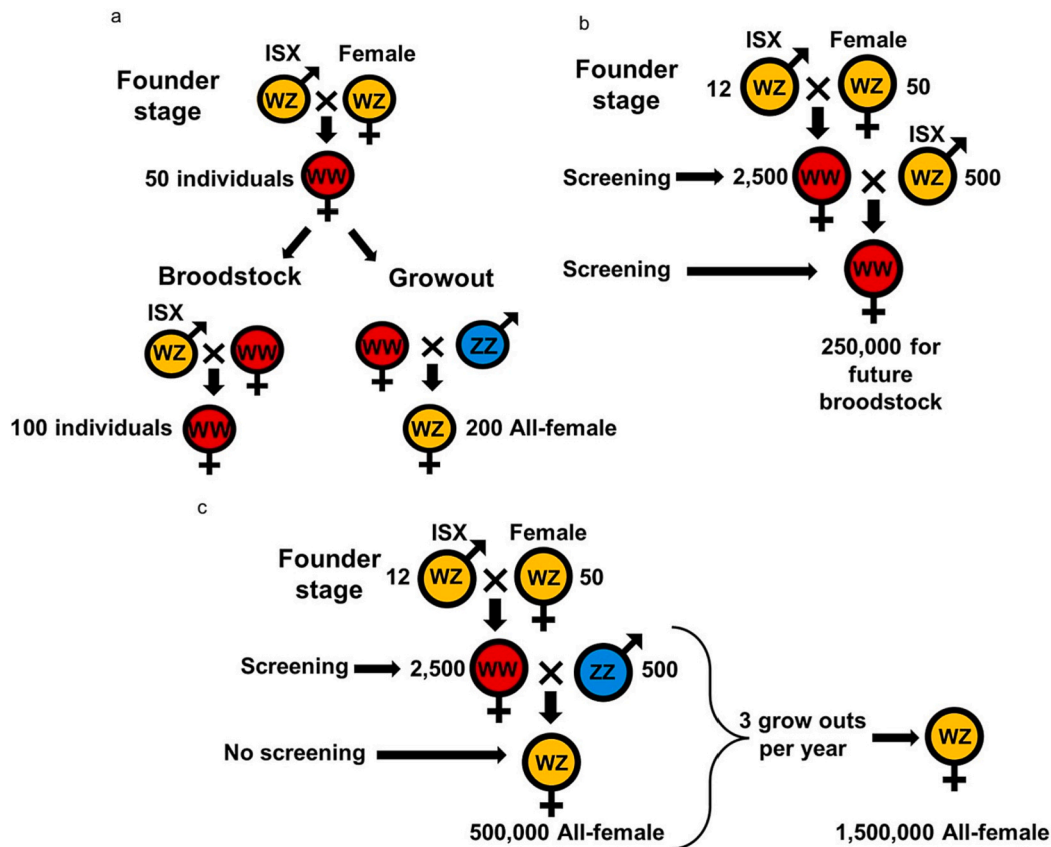
Progeny	Total N	Observed phenotype			Expected phenotype			p value
		ZZ	WZ	WW	ZZ	WZ	WW	
WZ X WW (1)	30	0 (0%)	11 (36.67%)	19 (63.33%)	0 (0%)	15 (50%)	15 (50%)	0.34
WZ X WW (2)	30	0 (0%)	12 (40%)	18 (60%)	0 (0%)	15 (50%)	15 (50%)	0.54
WZ X WW (3)	30	0 (0%)	13 (43.33%)	17 (56.67%)	0 (0%)	15 (50%)	15 (50%)	0.76
WZ X WW (4)	30	0 (0%)	11 (36.67%)	19 (63.33%)	0 (0%)	15 (50%)	15 (50%)	0.34

Values in the table are the numbers and percentages of sampled crayfish from each progeny. P-values represent the level of significance between the observed and expected results according to the chi-square goodness of fit test.

time ago that *C. quadricarinatus* grown in mixed-sex populations weigh less than those growing in monosex populations (Curtis and Jones, 1995; Rodgers et al., 2006). With respect to aquaculture yields, several reports from different crustacean species have demonstrated monosex populations, either all-male or all female, to be more cost beneficial than mixed populations (Duan et al., 2021; Khatun et al., 2009). For *C. quadricarinatus*, Naranjo-Paramo and his colleagues (Naranjo-Paramo et al., 2018; Naranjo-Paramo et al., 2021) evaluated the benefits of monosex all-female and all-male populations grown at different densities and suggested the latter to be more cost beneficial. However, although average final weight and biomass of the males were higher than the values for females, the females seemed to be less sensitive to crowding, with an all-female body weight of approximately 80 g being reached, regardless of the stocking density (Naranjo-Paramo et al., 2018). This lack of sensitivity of females to stocking density indicates that higher stocking densities could be used to improve total biomass. Similar results were obtained in a large-scale field study showing higher prawn yields and survival rates of all-female populations compared to mixed-sex populations (Levy et al., 2017), leading to the conclusion that all-female populations can result in higher total biomass at higher stocking densities compared to all-male aquaculture (Karplus and Barki, 2004; Karplus et al., 1998; Parnes and Sagi, 2002; Pinto and Rouse, 1996). For *C. quadricarinatus*, the above notion is supported by studies showing that females are less aggressive and less territorial than males (Karplus and Barki, 2004; Karplus et al., 1998; Parnes and Sagi, 2002; Pinto and Rouse, 1996).

As monosex technologies are used in aquaculture to enable higher stocking density and increase crop yields (Bardhan et al., 2021;

Harlioglu and Farhadi, 2017; Ventura, 2018), it has been suggested that they would also be suitable for *C. quadricarinatus* aquaculture (Levy et al., 2020). In particular, they would be especially suitable for ensuring that escapees from ponds would not result in the establishment of new feral populations, as has happened previously in different parts of the world (Ahyong and Yeo, 2007; Baudry et al., 2020; Marufu et al., 2014). Previous studies in crustacean aquaculture showed that WW populations are viable as demonstrated in prawns (Levy et al., 2017; Molcho et al., 2020). Moreover, also previous results in the crayfish show that WW females are reproductively viable (Parnes et al., 2003). When WW females are crossed with normal males, it results with an all-female population. With the aim of improving the process of all-female crayfish production, in the present study we developed a high precision non-GMO method to identify potential brooders for large-scale production. The identification of brooders for all-female production was done by applying our validated genomic sex marker to efficiently and accurately isolate WW females (produced as described above) without the need to perform prolonged and human-error-prone progeny tests. We thus elaborated a quantitative scheme for the large-scale production of all-female *C. quadricarinatus* populations (Fig. 6) in which isolated WW ‘super females’ are used as brooders for the mass production of all-female monosex populations without the need for any molecular, genomic, or endocrine interventions. The devised scheme starts by crossing WZ intersex crayfish and WZ females to produce WW females (Fig. 6a). These females are identified by using the genomic markers described above. The second-generation crosses of WW females with WZ intersexuals yields 50% WW females (Fig. 6b), which are again validated using the above genomic markers. This step increases the quantities of



**Fig. 6.** Proposed models for all female *Cherax quadricarinatus* production. (a) Production of WW super females from a founder generation of intersexuals and wild-type WZ females. Utilization of WW females to create all female WZ populations. (b) Screening and utilization of WW females as seen in (a) to produce a substantial number of WW females for future broodstock. (c) Further screening for WW females and intersexuals and their utilization for mass production 100% WZ female populations. \*The models assume 200 individuals from each progeny that reach puberty.

WW females obtained and the efficiency of WW female production: According to our model, starting from 12 intersex crayfish and 50 WZ females, we can produce a large population of WW females in only two generations to be used as breeding stocks. For the mass production phase, even a brood stock composed of only 2500 small-sized WW females can be crossed with regular ZZ males to produce an all-female WZ population without the need for further offspring screening (Fig. 6c), resulting in an all-female population of 500,000 crayfish for Growout. Some of the WW females obtained in the first and second steps should be periodically crossed with WZ intersexuals to maintain WW female brooders, which serve as founders for the subsequent monosex all-female populations (see Fig. 5 for proof of concept). Furthermore, eliminating the need to screen and manually sort the offspring from such large progenies saves time and investment, eliminates human errors, and thus contributes to the economic feasibility of the process. Affordable production of such all-female populations in large quantities will have major implications for biocontrol and aquaculture sustainability and profitability, all of which call for further research and development. Potential avenues for R&D studies could be based on our recently constructed transcriptome of insulin androgenic gland (IAG) knocked-down *C. quadricarinatus* intersexuals, which revealed new potential genes involved in the sexual plasticity mechanism (Smith et al., 2023). In addition, since there are still many open questions regarding sexual plasticity in crustaceans, the production of all-female populations could also serve as an incentive for generating new knowledge regarding sexual determination and differentiation, thereby revealing the upstream genetic toolkit behind such processes. Additional investigations leveraging this transcriptome could shed further light on the sexual functionality of intersexual individuals upon IAG knock down. Such experimentation, however, should be done carefully due to the unlikely

possibility of all-female driven population to result in parthenogenetic reproduction, as manifested in *Procambarus virginialis* (Scholtz et al., 2003). The probability of such phenomenon is low since it will depend on occurrence of reproduction in the all-female population that might arise from the spontaneous emergence of WZ intersexuals. Such occurrence in the all-female *C. quadricarinatus* populations is yet unknown, thus more research should be carried out before all-female population could be employed as biocontrol agents in the field. Such research could permit the utilization of *C. quadricarinatus* all-female populations for biocontrol, as shown before with *Macrobrachium rosenbergii* (Savaya-Alkalay et al., 2018a).

In conclusion, the non-GMO production of an all-female *C. quadricarinatus* population suggested in this study opens the path for future studies and applications on more productive and sustainable aquaculture of crayfish with major implications for biocontrol and basic research.

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#### Authors contributions

S.A.S - Conceptualization, Data curation, Methodology, Validation, Writing - original draft, Writing - review and editing. T.L.- Methodology, Validation, Writing - review and editing. S.M. - Data curation, Methodology, Validation, Writing - original draft. H.W. and R.M. - Methodology and Validation. O.O - Conceptualization, Data curation, Methodology, Validation, Writing - review and editing and Supervision. E.D.A. - Conceptualization, Data curation, Methodology, Validation, Writing - original draft, Writing - review and editing and Supervision. A. S. - Conceptualization, Data curation, Methodology, Validation, Writing

- original draft, Writing - review and editing, Supervision and Funding acquisition.

## Declaration of Competing Interest

A patent regarding sex-specific genomic markers in the Australian redclaw crayfish has been issued (International application number: PCT/IL2018/051046, International publication number: WO/2019/058371). The patent's link is attached: [https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2019058371&\\_cid=P21-K2OIO0-43037-1](https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2019058371&_cid=P21-K2OIO0-43037-1)

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Amir Sagi reports financial support was provided by Emek Hamaayanot Regional R&D. Tom Levy reports financial support was provided by Gruss Lipper Postdoctoral Fellowship. Tom Levy and Amir Sagi has patent #International application number: PCT/IL2018/051046, International publication number: WO/2019/058371 issued to World Intellectual Property Organization.

## Data availability

Data will be made available on request.

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