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Three generations of prawns without the Z chromosome: Viable WW *Macrobrachium rosenbergii* all-female populations in polyculture with *Oreochromis niloticus*

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1. Introduction

Tilapia has become an increasingly popular aquaculture species, with a crop of 4.2 million tons produced in more than 78 countries at a value of ~11 billion USD in 2016 (FAO, 2018). Paralleling this increasing popularity of tilapia in recent years is that of crustacean species: The Food and Agriculture Organization of the United Nations (FAO) reported a production of about 7.8 million tons in 2016 (FAO, 2018), of which over 230 thousand tons comprised freshwater prawns (~3% of total crustacean culture) valued at ~1.7 billion USD. In light of the high demand for aquaculture products and projections for increases in demand in the coming decades (Godfray, 2010), attempts are underway to increase global production by different means.

Alongside agro-technological efforts to increase efficiency and to enlarge culture areas, polyculture – using the same water and land resources for more than one crop during the same growing season – may be an effective methodology to increase yields. Such a methodology may be particularly relevant for the large tilapia aquaculture industry, which has an estimated global pond area of 110,830 km^2 (Boyd et al., 2010), but which provides only a moderate income to the grower from the relatively inexpensive tilapia. A number of studies have demonstrated successful tilapia–prawn polyculture (Martínez-Porchas et al., 2010; Wang and Lu, 2016), with the tilapia occupying the water column and feeding on floating feed and zooplankton, and the prawns mostly occupying the bottom area of the pond, where they feed on benthic flora and tilapia waste (Cruz et al., 2008). For farmers, the attractiveness of the higher yields of such polyculture systems is further enhanced by the added economic value provided by the high-value crustacean species: The incorporation into tilapia ponds of a high-value prawn crop could make a significant contribution both to the growers' income and to the productivity of the global aquaculture industry. Furthermore, if monosex populations of both tilapia and prawns were to be integrated into such a polyculture approach, profitability could be increased even further.

Recent studies have shown that the sexual dimorphism in many fish and decapod species can be exploited by growers to farm monosex populations, which may confer an economic advantage (Beaumont et al., 2011; Poissant et al., 2010; Rogers, 2016). In fish, due to the large variation in sex hereditary mechanisms, which include both XY and WZ modes of heredity and high sexual plasticity (Kikuchi and Hamaguchi, 2013; Volff, 2004), a variety of approaches have been used to develop monosex cultures (Beardmore et al., 2001; Fuentes-Silva et al., 2013). In tilapia, all-male culture offers the dual advantages of faster growing fish (Mei and Gui, 2015) and the prevention of wild spawning in the aquaculture ponds (Beardmore et al., 2001; Guerrero, 1975). Several technologies have been suggested for producing tilapia all-male cultures, including hormonal/chemical treatments or genetic manipulations, and some have already been instituted in aquaculture (Arai, 2001; Beardmore et al., 2001; Fuentes-Silva et al., 2013; Guerrero, 1975). For prawns, two monosex culture options have been suggested—all-male culture at relatively low densities (Aflalo et al., 2006) or all-female culture of the naturally more uniform sized females under more intensified conditions (Levy et al., 2017; Malecha, 2012). For the prawn species used in this study, the giant freshwater prawn *Macrobrachium rosenbergii*, the productivity of all-female aquaculture was found to be superior to that of mixed populations due to the higher survival rates of the females, which may be attributed, at least in part, to the lack of dominant males that repress the growth of smaller males and females alike (Levy et al., 2017).

The novel technology that has been developed for producing all-female populations of *M. rosenbergii* (Levy et al., 2016) exploits the WZ mode of heredity of most decapods (Benzie et al., 2001; Defaye and Noel, 1995; Parnes et al., 2003), which has been studied far less than the XY mode of inheritance. Subsequently, the contents of the sex

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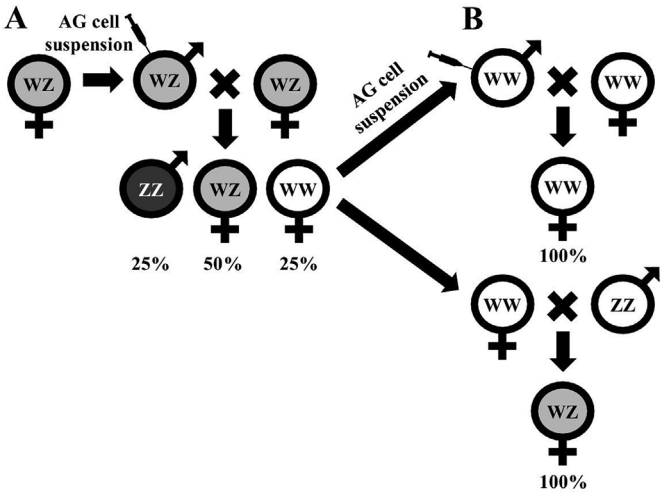

Fig. 1. Biotechnology to produce all-female prawn populations. (A) Sex reversal of WZ females through injection of androgenic gland (AG) cells to obtain WW females. (B) Sex reversal of WW females through injection of an AG cell suspension and crossing with WW females to obtain all-WW female progeny (top). Crossing WW females with normal males (ZZ) to obtain all-WZ female progeny (bottom).

chromosomes in decapods is not yet known. The technology is based on the injection of an androgenic gland (AG) cell suspension into WZ genotype females, leading to their sex reversal to WZ 'neo-males,' with populations of these neo-males exhibiting the typical male morphotypes (Levy et al., 2016). When such neo-males are crossed with WZ females, the progeny will include 25% WW females (Fig. 1A), which have been found to be reproductively viable. Crossing of such female progenies, designated 'super-females,' with normal males (ZZ genotype) gives rise to all-female WZ progenies. Alternatively, WW females can be turned into fully functional neo-males (by AG cell suspension injection), which are then crossed with WW females to produce an all-female WW population (Levy et al., 2019). This latter approach led, for the first time, to the recent production of all-female WW populations that could be grown for consecutive generations without the Z chromosome. However, at that time, the viability and performance of such all-female WW *M. rosenbergii* populations remained to be demonstrated—a shortcoming that we address in the current study.

In the present study, we thus demonstrated the viability and performance of the first *M. rosenbergii* all-WW female population produced using the above novel biotechnology, and we also demonstrated the production of a third generation without the Z chromosome. The study set out to compare the productivity of two polyculture systems, one consisting of all-male Nile tilapia *Oreochromis niloticus* grown together with all-female WW *M. rosenbergii* prawns, and the other comprising the same all-male tilapia population but all-female WZ prawns (under identical polyculture conditions).

2. Materials and methods

2.1. Animals

All-female WW and WZ populations of the *M. rosenbergii* BGU line were produced by previously developed novel biotechnologies (Levy et al., 2016; Levy et al., 2017). In brief, WZ progenies were produced by crossing WW females with ZZ males, while WW progenies were produced by crossing WW females with WW neo-males, as described above (Fig. 1). The WW and WZ females were supplied by Enzootic Ltd. and Northern Prawns Ltd., Israel, respectively. The all-male tilapia population was obtained by cross spawning between selected hybrid populations that provides 95–100% male population. Both parental and maternal populations are Chitrelada mixed with Aureus (Pruginin et al., 1975; Shirak et al., 2019). All tilapia crosses were performed at the Aquaculture Research Station, Dor, Israel.

All experimental protocols were approved by the Institutional Animal Care and Use Committee and the Animal Experimentation Ethics Committee of the Ministry of Agriculture and Rural Development. https://www.moag.gov.il/en/Pages/default.aspx (accessed 16 may 2019).

2.2. Experimental design and management

In early May 2018, three ponds of WW and three of WZ all-female prawns were stocked at the post-larvae stage PL_{20} (20 days post-metamorphosis) in 350-m^2 earthen ponds at the Aquaculture Research Station, Dor, Israel, under an extensive stocking density of 4 prawns/m^2 (total of 1400 prawns per pond). At stocking, average PL weight was 0.03 g. Fish were stocked 35 days later to prevent early predation on prawn PLs. Fish with an average fish weight of 116 g were stocked at a density of 2 fish/m^2, a total of 729 per pond. these densities were chosen to resemble common polyculture conditions. Ponds were supplied twice daily with floating, fish-meal-free feed containing 35% protein and 4% fat (Zemach Feed Mill Ltd.). This feed was supplied according to fish growth (% of biomass), with no added feed for the prawns. Fish were sampled and weighed every two weeks to enable the amount of feed to be adjusted according to average fish weight. Pedal wheels were used to aerate the ponds for 13 h each day (1800 to 0700). Ammonia concentration in the ponds ranged from 0.5 to 1 mg/l; nitrite from 0.05 to 0.1 mg/l; oxygen from 6.4 to 8.9 mg/l; and temperatures from 16.1 to 33.1 °C, with an average of 28.4 ± 0.3 °C.

The grow-out period was 160 days for the prawns and 125 days for the fish, i.e., the aquaculture crops were harvested in late October. At harvest, all the fish were collected first, a sample of at least 80 fish from each pond was individually weighed, the rest were individually counted and the proportion survival calculated. The ponds were then drained and examined for wild spawning of fish, and the prawns were collected and transferred to aerated holding tanks. A representative sample of at least 300 prawns was taken from each pond. All the prawns in each sample were individually weighed and the reproductive state of each individual prawn was recorded as one of the following: virgin (had not yet undergone a reproductive cycle according to brood chamber conditions and the absence of external signs of ovarian development), virgin with developed ovaries, spent (having laid and discarded eggs during the growth period), spent with developed ovaries, or gravid (holding eggs) (Levy et al., 2017). The remainder of the prawns from each pond were individually counted and weighed in bulk to determine the proportion of survival, the mean final body weight and the total crop from each pond.

The survival rate and the specific growth rate (SGR) were calculated for the fish and prawns according to the data at harvest:

$$\text{Survival rate}(\%) = \frac{no.\ \ of\ animals\ collected\ at\ harvest}{no.\ \ of\ animals\ stocked} x100,$$

$$\text{SGR}\ (\%/\text{day}) = \frac{(\ln Wf - \ln Wi)}{t} \times 100,$$

where *Wf* - final wet weight, *Wi* - initial wet weight and t – time from stocking until harvest in days.

2.3. Genomic validation of all-WW female populations

WW populations were repeatedly produced to achieve three generations without the Z chromosome. For each generation, the absence of a Z chromosome and the appearance of the W chromosome alone in the population was genomically validated. To validate the genotype of the two populations of *M. rosenbergii* females, DNA was extracted from pleopod tissue by incubating pleopods in NaOH (0.2 M) for 20 min at 70 °C, followed by the addition of Tris-HCl (0.04 M). The specific

markers were evaluated by high-resolution melt curve analysis of real-time quantitative PCR (qPCR) products. Briefly, fluorescent dye was used to detect and quantify the different PCR products according to the manufacturer's specifications (Hylabs, Israel), and the results were analyzed with micPCR v2.2.0 software (Bio Molecular Systems, Australia). TCTGTTATCTGGTCAACTTGAAATATCGAGA served as the universal reverse primer. W and Z chromosome markers were based on Ventura et al. (2011) and Levy et al. (2016), respectively. Representative samples of each generation of the WW populations were collected and validated at two distinct stages of development, namely, five days post hatching (n = 24) and at PL_1, immediately after metamorphosis (~20 days post hatching, n = 46).

2.4. Statistical analysis

The weight of the animals in the pond is dependent on many factors specific to each pond that could not be treated as independent samples. Furthermore, ponds were stocked with a population of a single genotype (WW or WZ). We thus analyzed the data using a Nested design ANOVA with the genotype as the whole plot treatments and pond as the plot nested within the genotype. Genotype was included in the statistical model as a fixed factor, while the ponds were treated as a random factor. The data was not normally distributed, and the variances were not homogenous. To correct for variance heterogeneity, the data were log10 transformed. The data still did not normally distribute but due to the robustness of the ANOVA test we decided to preside. The differences between the distinct genotype all-female populations in all other documented aquaculture parameters were tested for each parameter by a two-tailed *t*-test ($P < 0.05$). The weight distributions were tested for normality by the Shapiro-Wilk test. $P < 0.05$ was defined as a statistically significant difference. All statistical analyses were performed using Statistica v9.0 software (StatSoft Ltd., Tulsa, OK).

3. Results

There were no significant differences in fish yields ($P = 0.77$), average weights ($P = 0.71$) and SGR ($P = 0.17$) between the two polyculture conditions. Fish survival rates ranged from 44.6% (polyculture with WZ prawns) to 89.3% (polyculture with WW prawns), with no significant difference between conditions ($P = 0.76$) (Table 1). Uncontrolled spawning of tilapia was observed in all six ponds, indicating that a percentage of the fish population collected was female (Usually up to 5% as observed in these strains), however uncontrolled spawning scale was relatively low in a manner that didn't result in lower growth rate from the expected according to a commonly used growth curves (Benet, personal communication).

Not a single male prawn was found in any of the ponds, The lowest survival in the all-female WW population was 67.9%, and the highest, 98.1%. The survival rates for the all-female WZ population also fell within this range (Table 2). For the prawns, there were no significant differences between the two types of polyculture in terms of average survival ($P = 0.17$), total yield ($P = 0.67$) and growth rate ($P = 0.67$).

At harvest, the vast majority of female prawns of both genotypes were found to be virgin (81% for WZ, 90% for WW), with the remainder being classified as virgin undergoing ovarian development (9% for WZ, 4% for WW), spent (8% for WZ, 4% for WW) or spent undergoing ovarian development (2% for both) (Fig. 2A and B). Only a single egg-carrying female was found in all the all-WW female ponds at harvest, suggesting that the spent females had probably dropped their unfertilized eggs prior to harvest The chances to observe such a female are low, probably due to the fact that unfertilized females drop the eggs within 24–48 h from laying (Sagi et al., 1986).

As may be expected for all-female prawn populations, size variation was limited, with large animals (weighing ≥ 27 g) comprising ~6% of the harvest for both genotypes and medium-sized animals (weighing 15–27 g) comprising 77% and ~70% of the harvest for the WW and WZ populations, respectively (Fig. 2C, D). The mean weights of the prawns in the two types of culture did not differ significantly ($P = 0.58$), being

Table 2
Post-harvest data for WZ and WW female prawn populations under polyculture conditions.

Prawn parameter	WZ prawns (N = 3 ponds)	WW prawns (N = 3 ponds)
Survival (%)	83.4 ± 2.8	80 ± 9.2
Yield (kg/ha)	599.6 ± 93.8	552.2 ± 41
Final body weight (g)	19.2 ± 0.22	17.6 ± 0.18
SGR (%/days)	4.1 ± 0.1	4.0 ± 0.04

Values in the Table are means ± SE.

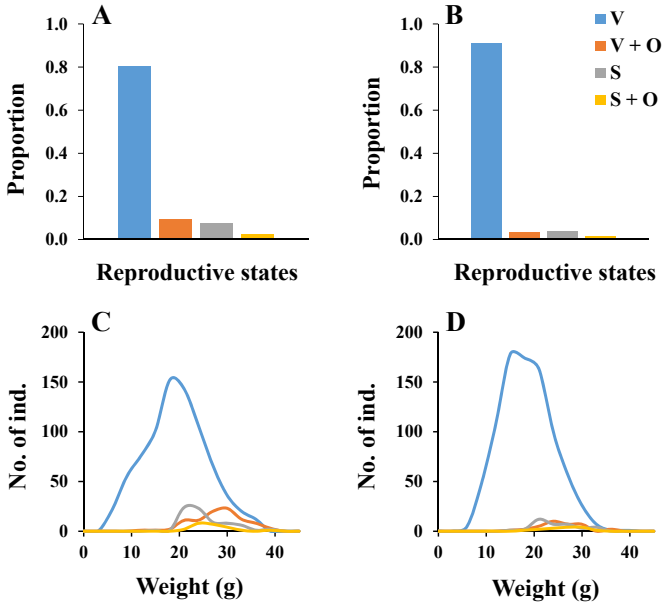

Fig. 2. Proportion (top) and weight distribution (bottom) of representative samples from three ponds of all female WZ (A and C, n = 979) and WW (B and D, n = 953) prawn populations grown under polyculture conditions according to reproductive states. The samples were combined from three replicates (ponds) for each population. S - spent; S + O - spent with developed ovaries; V - virgin; V + O - virgin with developed ovaries.

Table 1
Postharvest data for fish grown in polyculture ponds with all-female prawns.

Fish parameter	In polyculture with WZ prawns (N = 3 ponds)	In polyculture with WW prawns (N = 3 ponds)	Pond with no prawns[a]
Survival (%)	70.9 ± 13.2	76.2 ± 9.9	87.9 ± 2.6
Yield (kg/ha)	9047 ± 1472.6	9630 ± 1096.7	8966
Average weight (g)	620.3 ± 0.95	609.3 ± 0.4	600 ± 2.7
SGR (%/days)	1.4 ± 0.04	1.35 ± 0.01	1.48

Values in the Table are means ± SE. The experiment was performed in triplicate. There were no significant differences in any of the parameters between the fish grown in polyculture with WW and WZ prawns.

[a] Data from a former experiment in the same facility with the same tilapia line (Benet, personal communication; SE were not given for yield and SGR).

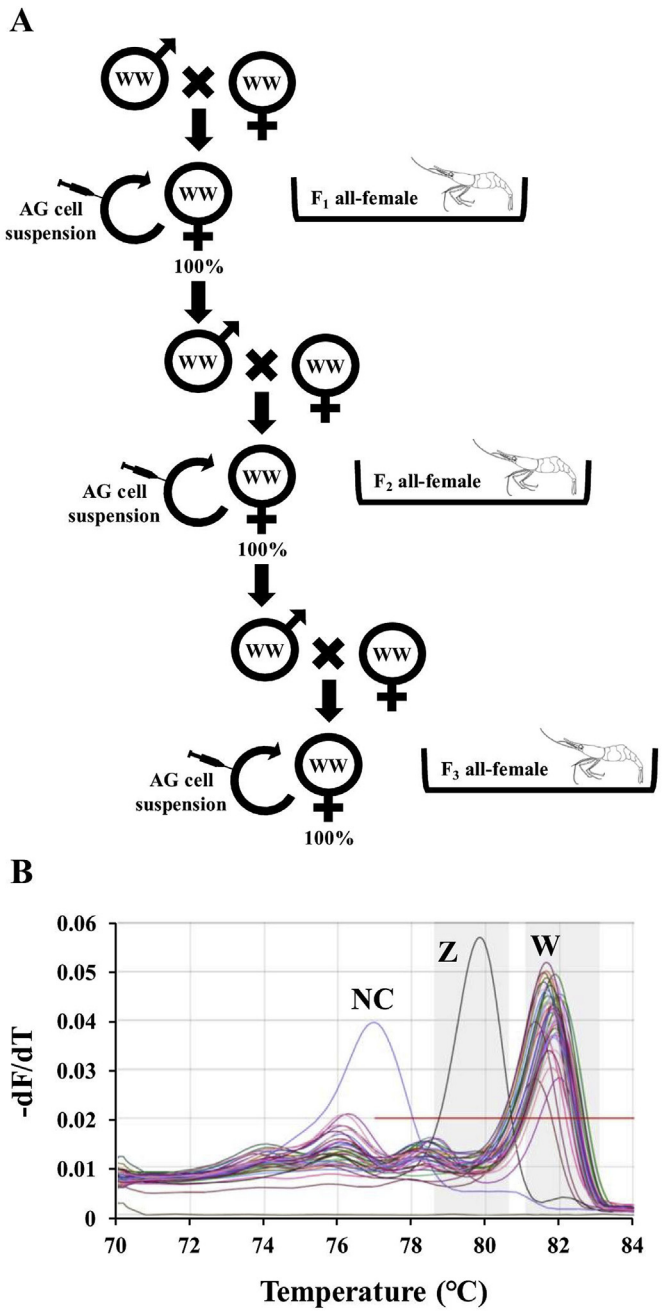

Fig. 3. Three generations of WW all-female populations without the Z chromosome were achieved (A). Genotyping of sex chromosomes is presented through melt curve analysis of real-time quantitative PCR of the second generation. Representative animals (n = 70) from all the ponds containing the WW females were randomly sampled twice: the first sampling point was at 5 days post hatching (n = 24) and the second, at PL_1 (after metamorphosis, n = 46). The 'NC' peak represents a negative control (primer dimer), the 'Z' peak represents a Z chromosome marker as a control, and the 'W' peak represents a W chromosome marker.

19.4 ± 3.2 g and 17.6 ± 1.1 g for WZ and WW, respectively (Table 2). Moreover, the same pattern of size variation was observed for each of the triplicates, with no significant differences ($P > 0.05$) (Fig. 2). The weights of the virgin prawns in the WZ and WW populations (ranging between 5 g and 40 g in both WZ and WW populations, Fig. 2C, D) were not normally distributed ($P < 0.001$), but both were positively skewed. For the other reproductive states, with prawns ranging in weight from 17 g to 40 g in the WZ population and 15 g to 36 g in the WW population, the weight distribution was positively skewed for the spent WZ females, but that for the spent WW females ($P = 0.21$) followed a normal distribution, as was also the case for the weights of all the spent females with developed ovaries ($P = 0.9$ for WZ, $P = 0.47$ for WW) and the virgins with developed ovaries ($P = 0.09$ for WZ, $P = 0.97$ for WW).

Finally, the genomic validation confirmed that WW prawn reproduction (without the Z chromosome) had been achieved for all three generations (Fig. 3A). The existence of W chromosomes alone in the population was validated for WW animals through a melt curve analysis of qPCR. In all samples, representative results (from the second generation) showed a peak at ~81.5 °C ('W' in Fig. 3. B), representing the W chromosome marker. Only a single peak for the Z chromosome marker at 79.5 °C ('Z' in Fig. 3B) was found in a normal female, which served as a positive control. Similar results were also obtained for the other generations (data not shown).

4. Discussion

In aquaculture, the advantages of polyculture have been extensively documented, including improved water conditions and waste recycling through proper use of the different ecological niches in the pond, enhanced growth, and higher product yield, particularly when the addition of the second crop requires only minimal amounts of feed and a minimal effort on part of the grower (Cruz et al., 2008; Martínez-Porchas et al., 2010; Uddin et al., 2007; Wang and Lu, 2016). In particular, it has previously been shown that for a prawn-tilapia polyculture system the periphyton substrate contributes to both the survival and the growth of both species (Uddin et al., 2007); this finding can certainly be utilized for further improvement of such cultures in the future.

With more farmers integrating prawns into tilapia ponds, we may expect profitable new markets to emerge, as was recently indicated by Rodrigues and coauthors (Rodrigues et al., 2019). Under the polyculture conditions of the present study, the fish showed no significant differences between the ponds in terms of SGR, yield and weight at harvest (Table 1) and also exhibited similar aquaculture performance to that reported previously for cultures grown without prawns (Benet, personal communication). These findings suggesting that the introduction of the prawns to the tilapia ponds did not cause any disturbance of growth or survival of the fish are in keeping with previous studies reporting similar positive results for prawn–tilapia polyculture (Cruz et al., 2008; Wang and Lu, 2016). With respect to the prawns, significant growth was achieved in the present study with no additional prawn feeding: All the feed was supplied as floating tilapia feed, according to fish biomass, and thus the direct availability of the feed to the prawns was minimal, if any. The largest prawns reached ~40 g, and the prawn size distribution was positively skewed (Fig. 2. C and D), which further emphasizes the advantages of such polyculture keeping with other studies investigating prawn–tilapia polyculture (Cruz et al., 2008; Uddin et al., 2007). In global terms, since tilapia is one of the most widely cultivated fish crops, the addition of prawns with no additional feed or labor requirements can increase food production, reduce waste, and improve the quality of this important traditional fish species vs monoculture tilapia ponds.

However, prawn culture under the polyculture conditions of the present study did not follow the same pattern of growth as that in a previous large-scale monoculture experiment in the same earthen-ponds (Levy et al., 2017). In comparison with that previous study, the survival rates and SGR were reduced in the present study from 89.1 to 83.4% and 4.4 to 4, respectively; the average weight of the prawns was reduced from 39.1 to 19.1 kg; and the yield dropped correspondingly from 1405 to 599 kg. The all-female prawns in the present study reached smaller body size at a similar density of 4 prawns per m^2, even though there was only a 4-day difference in the grow out period between the two studies. The differences in performance could thus be attributed to the fact that the monoculture prawns in the previous study

were fed with sinking pellets according to prawn biomass (whereas no prawn feed was supplied in the current study) and/or to the high feeding efficiency of the Chitrelada line of tilapia used in the present study. In addition, the cases of wild spawning that did occur in the present study, despite the mostly all-male tilapia culture in all the ponds, might have further increased competition with the prawns (Milstein et al., 2000). There is therefore a need for future experiments to test the hypothesis that a prawn–tilapia polyculture could be optimized by supplying an additional designated feed for the prawns and preventing wild spawning of the fish.

A particularly important aspect of the current study is that it constituted the first field study of WW (lacking the Z chromosome) all-female prawn populations produced by a recently developed novel biotechnology (Levy et al., 2016). As mentioned above, the mating of WW females and WW neo-males produces only WW progeny in each consecutive generation, in contrast to WZ female and neo-male crosses, which produce mixed populations (25% males and 75% WZ and WW females), as shown in Fig. 1 (Levy et al., 2016; Levy et al., 2019). Three generations without the Z chromosome were validated by qPCR, with the results indicating the lack of Z chromosomes in all females tested. In aquaculture, such prawns could be used to produce a continuous all-female line with no need to genomically test the progeny of each generation. The all-female WW populations were found to be viable and almost identical to WZ all-female populations in most major aquaculture criteria, including survival rate, size uniformity, SGR, and yield per hectare. The high similarity between *M. rosenbergii* WW and normal female (WZ) populations gives legitimacy for the use of WW females in aquaculture without compromising the environment, particularly in areas where the species is non-native.

The ecological risks of farming non-native species have been revealed in many studies on invasive species, including decapods, and on the extensive damage they can cause to the local environment. The lack of the Z chromosome can offer an ecological advantage in that escapees of WW monosex populations of *M. rosenbergii* farmed in many places throughout the world (FAO, 2018; New, 1990) would not be able to reproduce and become invasive, as was recently found in Brazil and East Africa for mixed prawn populations (Kuguru et al., 2019; Loebmann et al., 2010). It would be virtually impossible for WW female prawns to multiply in the wild, and it is not likely that *M. rosenbergii* WW female escapees would crossbreed with any other *Macrobrachium* species (Savaya-Alkalay et al., 2018b). Thus, damage to native species and long-term ecological damage to biodiversity and the environment could be prevented. On the contrary, WW *Macrobrachium* prawns could actually be exploited for environmental tasks as efficient biocontrol predators against disease-causing snails (Savaya-Alkalay et al., 2018a).

From a basic biological perspective, the consecutive generations of homogametic all-male prawns (ZZ populations without the W chromosome, previously produced in our laboratory) (Shpak et al., 2017) and all-female WW populations (without the Z chromosome, described in the present study) call for further study of sexual determination and differentiation in decapods and their controlling mechanisms. The homogametic monosex populations completely lacking one of the sex chromosomes call into question the content of the sex chromosomes in such systems. Perhaps despite the presence of sex chromosomes, the entire tool kit responsible for sexual development in the prawn is located on the autosomal set of chromosomes. The resent sequencing of an M. rosenbergii phased genome (Levy et al., 2019) will enable needed future research to unveil the content of the sex chromosomes in search for sex determining genes.

Declaration of competing interest

None of the authors have a conflict of interest in respects to this research paper.

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