

Research Article

The *IAG* gene in the invasive crayfish *Procambarus clarkii* – towards sex manipulations for biocontrol and aquaculture

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Abstract

The red swamp crayfish *Procambarus clarkii* (Girard, 1852) is considered a voracious invasive species that gained this reputation through the displacement of native crustaceans and is threatening the biodiversity of many freshwater ecosystems through Europe, Africa and Asia. At the same time, this crayfish is an important aquaculture species since it is easy to grow and has high production rates. It is crucial to develop tools to control and prevent invasive populations to reconcile conservation objectives with aquaculture goals. Thanks to recent biotechnological progress in crustacean monosex aquaculture, we present here novel insights in crayfish reproductive biology that might pave the way for the production of monosex populations through “IAG-switch”-manipulation based on the identification of the *P. clarkii* insulin-like androgenic gland hormone (IAG) encoding gene. Our data indicate that this manipulation can be used to create “neo-females”, which produce all-male progeny. This approach is a promising avenue for increasing food production in regions where crayfish is cultured, while greatly reducing risk of invasion in case of accidental release in the wild. We also explored potential use of stocking neofemale crayfish to control invasive populations by generating a strong bias in male-to-female sex-ratio. Specifically, we developed a simple demographic model and used it to investigate whether and under what assumptions stocking neofemale *P. clarkii* can cause the eradication of an invasive population. Our work suggests that all-male crayfish production could be further developed towards an effective control of wild invasive populations as part of an integrated pest management approach.

Key words: all-male population, insulin-like androgenic gland hormone (IAG), invasive species, integrated pest management

Introduction

The red swamp crayfish *Procambarus clarkii* (Girard, 1852) is known to be an invasive species in more than twenty countries, spread over five continents, with a major environmental footprint caused by multiple traits (Loureiro et al. 2015). For example, this crayfish is capable of changing the

bio-physical conditions of its habitat (Gherardi 2006) causing considerable environmental and economic damage (Guan and Wiles 1997) and potentially shifting species biodiversity irreparably (Hobbs et al. 1989). The reasons for *P. clarkii*'s great success as an invasive species, are threefold. First, this species has the typical biological traits of highly reproductive “*r*-selected” species, i.e., small body size and early sexual maturity, high fecundity and fast somatic growth, a generalist and opportunistic feeding behaviour with a broad dietary spectrum, all leading to a high population growth rate (Lindqvist and Huner 1999; Paglianti and Gherardi 2004). Second, *P. clarkii* has a very high tolerance for extreme environmental conditions such as those found in temporary streams and polluted habitats (Gherardi et al. 2000). Third, it is a carrier of the devastating pathogenic oomycete *Aphanomyces astaci* that does not harm *P. clarkii* but triggers devastating epidemic outbreaks in native crayfish in the invasion regions (Dieguez-Uribeondo and Söderhäll 1993).

The rapid expansion of invasive crayfish populations into new territories over the last few decades has caused significant ecological impacts via loss of native crustaceans and aquatic biodiversity (Kerby et al. 2005; Lodge et al. 2005; Gherardi et al. 2011). Understanding how to reconcile crayfish aquaculture production with the conservation of native aquatic biodiversity is thus of primary importance. In fact, once escaped, the control of *P. clarkii* invasive populations in Europe has proven to be a daunting task: numerous management efforts have been attempted including chemical, physical, and biological measures as well as regulations against importation and transportation. Although various biocides were tested against the invasive crayfish, none were successful as an effective control tool because they needed to be used repeatedly and in large quantities across the extensive crayfish habitat, producing two major drawbacks: namely, the prohibitive intervention cost of expensive chemicals and, because of lack of specificity, potentially devastating effects on the native fauna and flora (Anastácio 2000; Quaglio et al. 2002; Cecchinelli et al. 2012; Loureiro et al. 2015). Another control strategy that has been widely implemented is the harvest of crayfish using traps, nets, electro-fishing and the draining of ponds. When applied extensively and continuously for a long time, these strategies were able to control crayfish populations but rarely able to extirpate them. Consequently, as soon as the control effort decreased, invasive populations quickly rebounded due to the high fecundity of the species (Westman et al. 1978; Skurdal and Qvenild 1986; Holdich et al. 1999; Kerby et al. 2005; Gherardi et al. 2011). Toxin-producing microbes and disease-causing microorganisms have also been considered as agents of biological control, but the most successful approach so far has been the use of predatory fish such as eels, burbot, perch, bass and pike. However, their introductions in water bodies where they are not native may lead in turn to undesired ecological effects caused by predation on

non-target species (Westman 1991; Holdich et al. 1999; Frutiger and Müller 2002; Aquiloni et al. 2010; Freeman et al. 2010). Without a widely applicable single strategy to extirpate crayfish populations, the most promising approach to controlling crayfish invasion is currently integrated pest management (IPM). IPM was successfully implemented in the early 2000s, for example, to control an invasive population of the rusty crayfish *Faxonius rusticus* (Girard, 1852) in Sparkling Lake, Wisconsin, USA. The strategy decreased recruitment by combining an intensive trapping effort targeting large, highly fecund crayfish with fishing restrictions on the native fish species smallmouth bass and rock bass, allowing them to prey on smaller crayfish. Another successful IPM experiment recently carried out in Italy achieved 85% reduction of invasive *P. clarkii* in two years by integrating intensive trapping with SMRT (sterilized males release technique). SMRT which is performed by X-ray radiation, although seems like a promising avenue for *P. clarkii* control, is only partially effective, reporting ~ 40% reduction in fertility of treated males (Aquiloni et al. 2009; Cecchinelli et al. 2012; Piazza et al. 2015).

Following aquaculture expansion in many countries, *P. clarkii* is currently the most cosmopolitan crayfish, found in every continent except Australia and Antarctica (Huner 2002). It is thus crucial to create new technologies that allow cost-effective and environmentally safe crayfish aquaculture that maximizes profit and food production while simultaneously minimizing the risk of unwanted invasions. Monosex production of *P. clarkii* has shown potential as a means to leverage the industrial aspects of this crayfish; in particular, all-male crayfish aquaculture is expected to increase incomes with respect to mixed-sex populations because energy intake is devoted mostly to somatic growth instead of reproduction. Moreover, monosex production is by its nature safer than mixed sex aquaculture in regions where *P. clarkii* is non-native; even if crayfish accidentally escape from aquaculture facilities into natural habitat, they are unable to establish a growing, permanent population due to the lack of sexual reproduction. Additionally, because monosex crayfish minimize risk of invasion, they can be marketed alive at a higher price, thus further increasing farmers' income. As observed already for other crayfish species and for the freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879), monosex production seems more profitable than mixed-sex (Ventura and Sagi 2012).

Monosex production in the latter species was achieved through the use of advanced molecular tools such as RNA interference (RNAi) to manipulate the androgenic gland (AG), a masculine specific gland (Ventura et al. 2012). These tools do not include genetically modified organisms, chemicals or hormones (Fire et al. 1998). Instead, RNAi manipulation uses a natural cellular mechanism of interference at the RNA level. To manipulate gene expression, double-strand RNA (dsRNA) molecules that are complementary to the natural mRNA produced in the crustacean are inserted and evoke

targeted enzymatic degradation of the specific mRNA thus preventing further translation of the protein (in our case insulin-like androgenic gland hormone [IAG]). This mechanism is termed temporal gene silencing (Fire et al. 1998) and has been successfully used in various crustaceans (Sagi et al. 2013). The AG secretes IAG which is responsible for masculine trait differentiation and maintenance. Manipulations of the AG and IAG in decapods cause a variety of sexual shifts, from a temporary shift in secondary sex characteristics (Rosen et al. 2010) up to a full and functional sex reverse from a normal male to a so called neo-female [genetic males that morphologically resemble females and produce eggs (Ventura et al. 2012)]. In this respect the IAG functions as a switch: a lack of IAG in males produces neo-females. Since male prawns and crayfish carry homogametic sex chromosomes (ZZ) and females are heterogametic (WZ), a cross between a normal male (ZZ) and a neo-female (ZZ) produces 100% male progeny (Ventura and Sagi 2012). AG manipulations were also reported in *P. clarkii* via injections of AG cells in premature males and females, but did not result in a full and functional sex reversal (Taketomi and Nishikawa 1996); These studies were conducted more than two decades ago without the use of current molecular tools. Given the recent biotechnological advances which allow the production of freshwater prawn monosex populations via IAG-switch manipulations (Ventura and Sagi 2012; Levy et al. 2016), it is our hypothesis that similar biotechnology can be applied for crayfish species such as *P. clarkii* with technical modifications of the delivery method.

This study has two major objectives, first, to lay the molecular foundations for monosex crayfish production; second, to develop a simple demographic model of crayfish and use it to explore whether stocking neo-females could be effective for controlling invasive populations in the wild.

To lay the foundation for monosex production our specific objectives were to identify the location of the AG, sequence the IAG, and examine the expression patterns of the IAG in *P. clarkii* (Pc-IAG). In addition, we conducted an experimental short-term silencing experiment to demonstrate that molecular manipulation of the IAG could pave the way to produce neo-females.

A novel environmental application for monosex crayfish is suggested: control of invasive populations in the wild by skewing the sex ratio of the invasive population. A demographic model is presented in order to determine whether and under what alternative assumptions on population growth rate and crayfish life expectancy neo-female stocking can be an effective agent of biological control. Knowledge gaps are discussed where more research is needed in the future.

Materials and methods

General approach

In order to lay the foundation towards a technology for all-male populations based on the IAG-switch manipulation in *P. clarkii*, a multistep process

was required. Since this was the first study carried out in our laboratory with *P. clarkii*, a species validation was the first step, in order to assure that the specimens we acquired were indeed *P. clarkii* and not any other *Procambarus* species. A brief physiological study using histology was essential to correctly identify the AG. Next, we extracted RNA from the AG tissue, in order to sequence the *P. clarkii* insulin-like androgenic gland factor (*Pc-IAG*). Obtaining this sequence was the primary step towards molecular manipulation. In order to understand the timing and location of the future manipulation, once the sequence was available, we studied its expression patterns, both spatial (in which tissues it is expressed) and temporal (when does the expression begins among juvenile crayfish). Timing of initial expression is crucial for a successful long-term manipulation. Finally, based on the above information a POC was achieved through an experimental short-term silencing, that was comprised of three steps: preparation of the silencing agent, dsRNA, *in-vivo* injection to animals and RNA extraction for real-time PCR to assess silencing efficiency, followed by a statistical analysis. All above steps were considered as a POC for the IAG-switch manipulation that permitted the exploration of its application in the demographic model.

Animals

Procambarus clarkii were maintained at Ben-Gurion University of the Negev under the following conditions: food comprising shrimp pellets (Rangen Inc., Buhl, ID, USA, 30% protein) was supplied *ad libitum* three times a week. Six hundred (600) liter tanks were biomechanically filtered using 110 liter filters. Water quality was assured by circulating the entire volume through a biofilter maintaining all the required water physicochemical parameters, as described before (Khalaila et al. 2001).

Histology

AGs were dissected from mature males, together with the attached terminal ampulla. Five μm -thick sections were prepared and stained by hematoxylin and eosin as previously described (Manor et al. 2007).

Molecular study

All crayfish dissected in the study were anesthetized in ice cold water for 5 min prior to dissection. Species determination was based on a molecular analysis using PCR for the amplification of *P. clarkii* 16S ribosomal RNA gene sequence (accession no. DQ666844.1). Total RNA was extracted and cDNA was prepared with the EZ-RNA Total RNA Isolation Kit, used according to the manufacturer's instructions (Biological Industries). First-strand cDNA was synthesized by reverse transcription using the VersoTM cDNA Kit (Thermo Fisher Scientific Inc.) with 1 μg of total RNA. REDTaq

Table 1. Primers used in the present study.

Primer use	Primer name	Forward 5' to 3'	Reverse 5' to 3'
Species determination and positive control for RT-PCR	<i>P. clarkii</i> 16S	CATTGGGAACTAAAGGCCGC	TCAACATCGAGGTCGCAAAC
RT-PCR	Pc-IAG_F1	CCAAACATTACTGAAACTGAT	
RT-PCR	Pc-IAG_F2	AACCTTCTGGTGGACTTCGACTG	
Temporal and spatial expressions	Pc-IAG	TAGATAACCTTCTGGTGGACTTCG	TGTCTGTGTTGGCTTGACG
Real-time target gene	<i>QPc-IAG</i>	TCAGGACGAGTGTTCGAATTA	GCTCCCTCACCTCACCAG
Real-time housekeeping gene	<i>QP.clarkii COI</i>	GAGGCTTTTGGGACATTAGGT	TCTACATCCATCCCTACCGTAAA
RACE	Pc_RACE	ACTGATGAGGATCGGAAGATGGCGT	CTCTGGCCGCATAGTGCAGATGAGT

ReadyMix PCR Reaction Mix (Sigma) was used for PCR amplification, according to the manufacturer's instructions. All primers for this study are listed in Table 1. PCR products were separated on agarose gels, bands were excised, purified and cloned as previously described (Manor et al. 2007). The obtained sequences were compared to known sequences using the BLAST algorithm.

P. clarkii insulin-like androgenic gland factor (*Pc-IAG*)

To ease the identification of the AG in *P. clarkii*, an endocrine manipulation comprising the removal of the X-organ Sinus Gland complex, causing hypertrophy of the AG (hAG) was performed on six mature males, as previously described (Khalaila et al. 2001). RNA from the hAG was extracted as described above and cDNA was prepared and amplified by PCR, as previously described using the SMARTer RACE kit (Clontech). PCR was conducted using specific forward primers (see Table 1) based on the IAG sequences of other crayfish (*Cherax quadricarinatus* [Von Martens, 1868] and *Cherax destructor* [Clark, 1936] see Supplementary material Table S1) and the Universal Primers Mix (UPM) provided with the kit as a reverse primer. The sequence of the 5' end of *Pc-IAG* was obtained by 5' RACE using Pc_RACE reverse (see Table 1) and UPM. The PCR products were cloned and sequenced as described above. A Phylogenetic analysis was conducted using MEGA, version 6.0. This analysis considered 15 available IAGs of crustacean species (listed in Table S1) and an insulin precursor from *Aplysia californica* (J.G. Cooper, 1863) as an out-group. Evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 5,000 replicates was taken as representing the evolutionary history of the selected mature IAGs among the taxa analysed.

Tissue specificity

The spatial expression of *Pc-IAG* was examined by PCR using cDNA prepared from several mature male tissues (Hepatopancreas, Muscle, Testis,

AG, Green gland and Eye) and female tissues (Hepatopancreas, Muscle, Ovary, Green gland and Eye) as previously described (Manor et al. 2007). The cDNA was then amplified by PCR using specific *Pc-IAG* forward (nt 165–189) and reverse (nt 575–594) primers. *P. clarkii* 16s rRNA served as a positive control (see Table 1).

Temporal expression of *Pc-IAG*

In order to study the timing of initial expression of the target gene, specimens were collected from a berried female on a weekly basis. Ten specimens were collected and maintained in an RNA SAVE solution (Biological Industries), starting from the hatching day, until eight weeks post hatching. Total RNA from 80 specimens was extracted and reverse transcribed to cDNA as described above. The cDNA was then amplified by PCR using the above mentioned primers (see Table 1).

Short-term silencing experiments

Double-Stranded RNA Preparation

dsRNA based on the *Pc-IAG* open reading frame was *in-vitro* synthesized using a TranscriptAid T7 high yield transcription kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. Quality of dsRNA was assessed on an agarose gel and quantified with a NanoDrop spectrometer (Thermo Fisher Scientific Inc.). The dsRNA was maintained at -80°C until used, as described previously (Ventura et al. 2009).

In-vivo injection of dsRNA

Mature male crayfish (10.8 ± 0.81 g) were divided into three groups, as follows: treatment – *Pc-IAG* dsRNA-injected ($n = 8$), 1st control – exogenous Remebee (RB) (Beeologics) (Sharabi et al. 2015) dsRNA-injected ($n = 8$) and 2nd control (intact) ($n = 4$). The 1st control is essential in order to assure that the dsRNA does not cause any effect when it is not specific to the target gene. The males were injected once with 5 μg of dsRNA/g body weight into the sinus adjacent to the fifth walking leg (Shechter et al. 2008). Based on previous studies (Rosen et al. 2010), two days after the injection, the males were dissected and the bases of the fifth walking legs including the AGs were isolated.

RNA extraction and real-time RT-PCR

RNA was extracted from the AGs of males used in the *in-vivo* experiment. Total RNA was isolated and First-strand cDNA was synthesized as described above. Relative Quantification (RQ) of *Pc-IAG* transcript levels were obtained using the *QPc-IAG* primers (Table 1) with the FastStart Universal Probe Master (Rox) (Roche Diagnostics GmbH) and Universal Probe Library Probe #25 (Roche). *P. clarkii* cytochrome C oxidase subunit I

(*Pc*-COI, accession no. AY151521.1), which was used as a normalizing gene, was also quantified using the forward and reverse primers Q *P. clarkii* COI as presented in Table 1 with the above-mentioned mix and Universal Probe Library Probe #136 (Roche). Reactions were performed with the ABI Prism 7300 sequence detection system (Applied Biosystems).

Statistical analysis

Data are expressed as median, minimum and maximum (Box Plot). Due to the nature of the experiment, the analyzed groups were relatively small and not normally distributed. Thus, non-parametric tests had to be used. The effect of dsIAG injection was analyzed with a Kruskal-Wallis test in STATISTICA 12.0.

*The biological control of invasive *P. clarkii* populations: a demographic model*

We developed a family of simple demographic models of increasing complexity to investigate whether *P. clarkii* neo-females can be used to control an invasive crayfish population by altering sex ratio and driving *P. clarkii* females to local extinction. These models track the dynamics of crayfish in a closed, homogenous population (i.e., with no immigration or emigration) in a small lake where the spatial dynamics can be ignored as first approximation. The first building block is a logistic model of an invasive crayfish population with density-dependent recruitment, namely (Model #1):

$$dP/dt = \varphi \cdot (1 - \gamma P) \cdot F - \mu P = \varphi \cdot (1 - \gamma P) \cdot \frac{1}{2} P - \mu P \quad (1)$$

where $P=M+F$ is total population abundance (number of males + females in the waterbody), φ the per-capita recruitment rate, μ the per capita mortality rate, γ a parameter accounting for density-dependent reduction in recruitment exerted through competition for space, resources and/or cannibalism, the coefficient $\frac{1}{2}$ accounts for 1:1 sex ratio in the absence of neo-females ($F=M= \frac{1}{2}P$). Model (1) does not account for age/size structure in the population and assumes that births of this highly fecund species are not synchronized in a narrow reproductive season, which can be representative of invasive crayfish population at low latitudes (Gherardi 2006).

According to model (1), the crayfish population eventually reaches a long-term non-trivial (i.e., positive) equilibrium P_{eq} , hereafter referred as the population carrying capacity K , that can be derived by setting $dP/dt \equiv 0$ and solving for P , namely:

$$P_{eq} = K = (\varphi - 2\mu)/\varphi\gamma$$

As stocking neo-female alters population sex ratio, we extended model (1) so as to track the dynamics of males, females and neo-females, respectively,

and to account for the fact that neo-females generate an only-male progeny, namely (Model #2):

$$dF/dt = \varphi \cdot (1 - \gamma P) \cdot \frac{1}{2}F - \mu F \quad (2a)$$

$$dM/dt = \varphi \cdot (1 - \gamma P) \cdot (\frac{1}{2}F + N) - \mu M \quad (2b)$$

$$dN/dt = W - \mu N \quad (2c)$$

where W is the number of neo-females stocked per unit time, N is the density of neo-females, $P = M + F + N$ is population size. Note that in the case $W = N = 0$, this model (here after referred to as model #2) collapses to model (1). Model #2 can be solved analytically to derive (i) long-term equilibria as a function of stocking rate W and the other model parameters, and (ii) the minimum value of the stocking rate W_{th} leading to extirpation of the females' population.

In addition to analytical solutions, we also simulated population dynamics by integrating numerically eq.s (2) with the package *lsoda* in R Cran (Soetaert et al. 2010) and investigated transient dynamics for the following control scenarios: (a) stocking neo-female only, (b) intensive harvesting of crayfish only; and (c) an integrated strategy based on both crayfish fishing and neo-female stocking.

For numerical simulations, the model was parameterized as follows: *P. clarkii* mean life expectancy at birth (LE) was set to 1.5 years according to Huner (Huner 2002), and mortality rate μ was computed as $1/LE$, i.e., the inverse of life expectancy at birth (Cohen 2010). Initial population size $P = F + M$ was set to 2 with sex ratio 1:1, namely one male and one female, and $N = 0$. Recruitment rate φ in the logistic models (1) and (2) is an aggregate parameter accounting for per capita fecundity (number of eggs per reproductive female per reproductive event, ranging between 300–500 (Gherardi 2006) and probability for eggs to hatch, for larvae to survive to the post-larval (PL) stage and for PLs to emerge as reproductive individuals. These parameters are affected by environmental conditions (such as temperature, salinity, water quality), resources/productivity of the water body, habitat type, ecological interactions (predators, competitors, pathogens and parasites) that maybe be highly variable from site to site and are difficult to estimate in the wild. Anyway, their aggregated value, summarized in the reproductive rate φ , determines the rate of grow of the population at very low densities in the initial phases of invasion, i.e., when the effect of density-dependent regulation of population size, due to intraspecific competition for space or resources, is still marginal. *P. clarkii* has proved to be a very successful invasive species with high population growth rates thanks to its peculiar life history traits, including early maturity at small body size, high per-capita fecundity at any body size, high tolerance to a wide range of environmental conditions and generalist and opportunistic feeding habits (Gherardi 2006). Field observations (Harper et al. 2002) and

Table 2. Model parameters used for simulations.

Parameter	Symbol-formula	Value [unit]	unit
Life expectancy	LE	1.5	years [y]
Natural Mortality rate	$\mu = LE^{-1}$	0.66	y^{-1}
Carrying capacity at equilibrium	K	1,000	Individuals
Pop. turnover	$\mu \cdot K$	55	Ind. per month
Minimum stocking density	$W_{th} = \mu \cdot K / 3$	18	Ind. per month
Stocking density for simulations	$W = 2W_{th}$	36	Ind. per month
Fishing mortality	$\eta = 2 \cdot \mu$	1.33	y^{-1}
Initial conditions	F, M, N	1, 1, 0	individuals
Finite growth rate over one year			
2x → Fecundity rate	φ_1	2.76	y^{-1} Ind. ⁻¹
→ Density dependent parameter	$\gamma_1 = (1 - 2 \cdot \mu / \varphi_1) / K$	$5.10 \cdot 10^{-4}$	
4x → Fecundity rate	φ_2	4.17	y^{-1} Ind. ⁻¹
→ Density dependent parameter	γ_2	$6.76 \cdot 10^{-4}$	
8x → Fecundity rate	φ_3	5.59	y^{-1} Ind. ⁻¹
→ Density dependent parameter	γ_3	$7.58 \cdot 10^{-4}$	

modelling studies (Martelloni et al. 2012) show that invasive crayfish can exhibit a 4-fold increase in population size in one year at low population densities. Here, in order to account for site-specific variations in population growth rate and for uncertainty in the estimation of this parameter, we analyzed three cases ($i = 1, 2$ and 3) corresponding to a population of invasive crayfish capable of a 2, 4 or 8 fold increase in population size in one year at low densities, and we set the per-capita recruitment rates φ_i accordingly. For each of the three cases, the density-dependency parameter γ was set so that the long-term carrying capacity K of the undisturbed population is equal to 1,000 individuals, namely:

$$\gamma_i = (\varphi_i - 2\mu) / (1000 \varphi_i) \quad i = 1, 2, 3$$

At the lower bound of population growth rate analyzed in this paper (i.e., 2x increase in population size at low density in a year starting from one male and one female crayfish), the unfished crayfish population takes ca. 15 years to approach carrying capacity. At the upper bound of population growth (i.e., 8x increase in population size at low density in a year), the crayfish population takes ca. five years to approach carrying capacity. A summary of model parameters is reported in Table 2.

Control and extirpation policies were simulated for 50 years (unless stated otherwise) as follows: in all the scenarios we started with 2 individuals, one male and one female, and simulated population growth for 15 years, the minimum time needed for the slow growing population to approach carrying capacity. Then, in the first control scenario (a), neo-female crayfish were stocked for 15 years (i.e., from year 15 to 30) with stocking rate W equal to twice the minimum density W_{th} to achieve female elimination. We assumed that the population of females could be considered extirpated if female abundance dropped below 0.1% females abundance at carrying capacity. We then simulated the population for additional 20 years (i.e., from year 30 to year 50) to assess the rebounding potential, i.e., whether

the population was able to recover and bounce back to its natural carrying capacity once neo-females stocking ended in year 30.

In the second control scenario (b), we simulated initial population growth for the first fifteen years as above, then we assumed to harvest crayfish for 15 additional years (with no neo-female stocking) and, finally, we simulated population dynamics for additional twenty years after ending crayfish harvesting. To account for the additional fishing mortality η , we extended model #1 as follow (Model #3):

$$dF/dt = \varphi \cdot (1 - \gamma P) \cdot \frac{1}{2}F - (\mu + \eta) F \quad (3a)$$

$$dM/dt = \varphi \cdot (1 - \gamma P) \cdot \frac{1}{2}F - (\mu + \eta) M \quad (3b)$$

and we set fishing mortality $\eta = 2 \cdot \mu$ for the four population growth cases, representing a case of high fishing intensity equivalent to a reduction of two third of mean life expectancy at birth.

Finally, we simulated integrated control, i.e., a scenario in which neo-female stocking and crayfish harvesting occurred at the same time, namely (Model #4):

$$dF/dt = \varphi \cdot (1 - \gamma P) \cdot \frac{1}{2}F - (\mu + \eta) F \quad (4a)$$

$$dM/dt = \varphi \cdot (1 - \gamma P) \cdot (\frac{1}{2}F + N) - (\mu + \eta) M \quad (4b)$$

$$dN/dt = W - (\mu + \eta) N \quad (4c)$$

Models 2–4 were deliberately kept as simple as possible, so that to be analytically tractable and to identify general properties over a wide range of feasible model parameters. Nonetheless, we also relaxed some of the underlying hypotheses on model 2–4 so as to account also for (i) strong seasonality in the reproductive rate (i.e., recruitment occurring only in a restricted reproductive season – model #5), (ii) a small, constant immigration rate (e.g., one female per year), to represent contribution of nearby crayfish populations that are not subject to control and are weakly connect to the target one through a complex river network (model #6) and (iii) simple population structure, i.e., two stage classes, unreproductive juvenile and reproductive adults (model #7). These additional simulations showed that the general conclusions derived by analytically solving model 2–4 are valid under a wide set of assumptions. The R script for the simulations is available as online supplementary information.

Results

Identification of the AG

Histological sections reveal that the AG in *P. clarkii* is located next to the sperm duct (Figure 1A) adjacent to the base of the fifth walking legs. The sperm duct wall is rich in muscle fibres and the nuclei of the AG cells are clearly visible (Figure 1B).

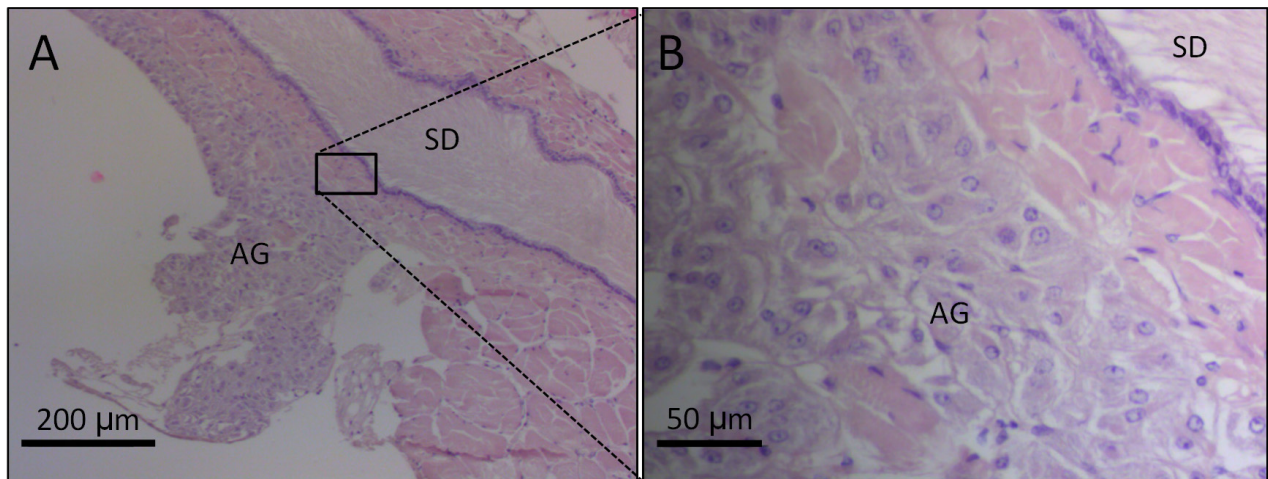


Figure 1. Histological sections of the sperm duct and AG of a mature *Procambarus clarkii* male stained with Hematoxylin and Eosin. The left picture (A) shows the sperm duct (SD) and the androgenic gland (AG). Picture B zooms in into the AG where the nuclei of the AG cells are seen. Photo by EDA.

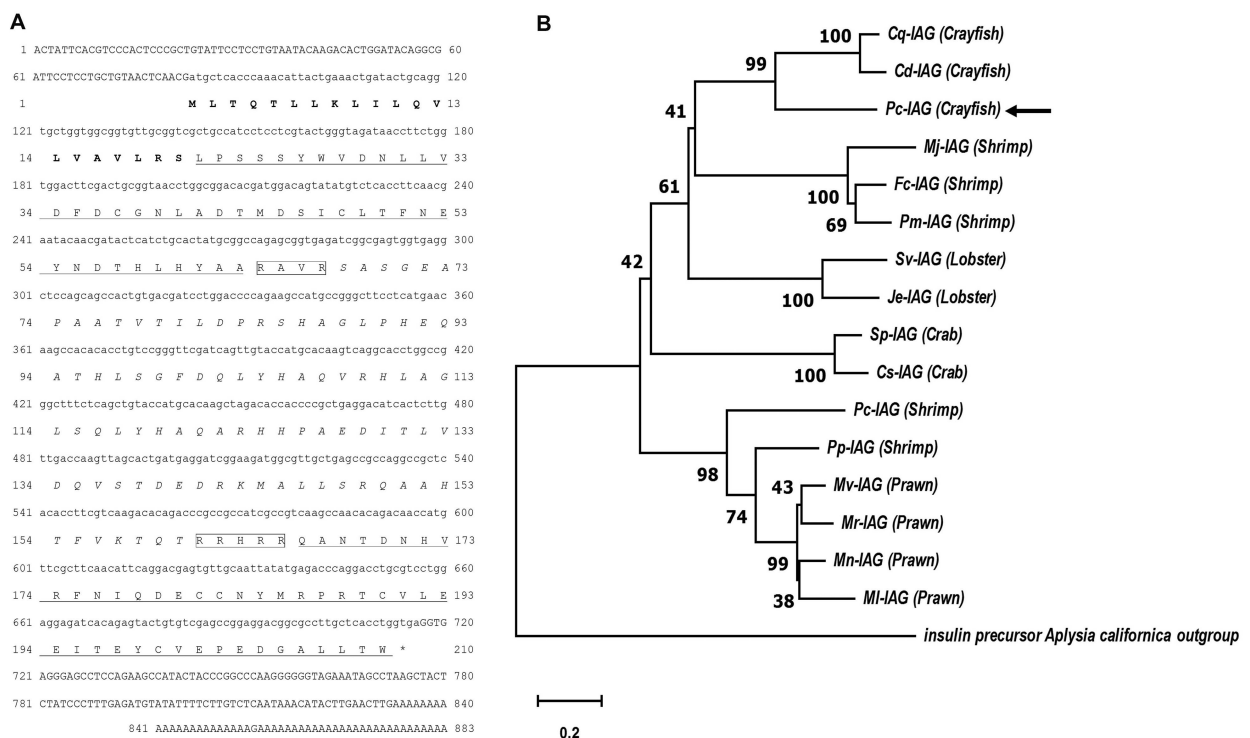


Figure 2. *Pc-IAG* sequence and bioinformatics analyses. (A) The *Procambarus clarkii* IAG gene and its deduced amino acid sequence. *Pc-IAG* cDNA sequence and deduced *Pc-IAG* protein. The amino acids of the signal peptide (encoded by nucleotides 85 to 145) are shown in bold. The putative B and A chains are underlined and the putative C peptide is *italicized*. The predicted arginine C-proteinase cleavage sites are boxed. The stop codon is marked with an asterisk. (B) Phylogenetic tree of the IAGs. The tree is based on the CLUSTAL W algorithm of 15 IAGs from decapod crustacean species, calculated and presented by MEGA 6. An insulin precursor from *Aplysia californica* serves as an out-group. *Pc-IAG* is marked with an arrow. The numbers on the junctions represent the percentage of attempts, reflecting the specific divergence within 5,000 replicates, while the bar represents the number of amino acid substitutions per site.

The full-length cDNA of Pc-IAG: Encoding sequence and phylogenetic analysis of the deduced peptide

Full-length *Pc-IAG* cDNA was found to be 833 bp-long (Figure 2A, Accession number KT343750). The sequence was isolated from a hAG by means of RT-PCR using degenerate primers (Table 1), followed by 5' and 3'

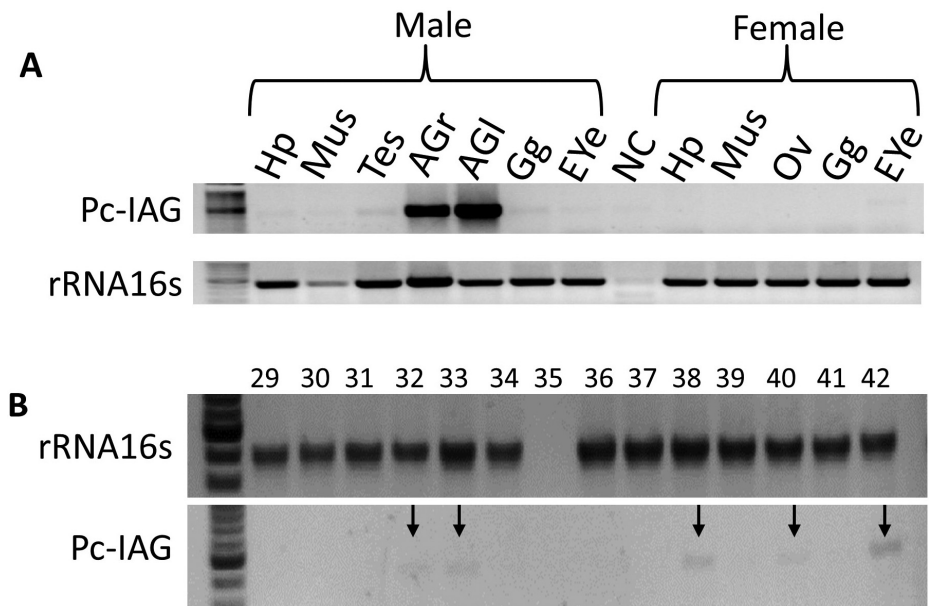


Figure 3. Spatial and temporal expression of *Pc-IAG*. (A) Presents the expression of *Pc-IAG* in several tissues of a mature male and female. HP (Hepatopancreas), Mus (muscle), Tes (Testis), AGr (Androgenic gland right side), AGI (Left side), Eye and the Negative control (NC) had no cDNA template. The Bottom gel presents the positive control of the housekeeping gene 16s rRNA. (B) *Pc-IAG* expression in 14 animals from the temporal expression experiment. The numbers represent animal ID, every week 10 animals were sampled, therefore animals 29 and 30 are from the third week after hatching, animals 31–40 are from the fourth week.

RACE. *Pc-IAG* consists of an open reading frame (ORF) of 633 bp flanked by a 5' UTR (103 bp) and a 3' UTR (167 bp) containing the putative polyadenylation site AATAAA. The *Pc-IAG* ORF was also predicted by ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). A 25 amino acid-long signal peptide (Figure 1A, bold letters) was predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP>). The predicted *Pc-IAG* ORF encodes a preprohormone in the following linear order: a signal peptide, B chain, C peptide, and A chain. The B and A chains of *Pc-IAG* are thought to be connected by two putative inter-chain disulfide bridges which are suggested to be formed between Cys12 and Cys23 residues of the B chain and Cys17 and Cys34 of the A chain. Two other cysteine residues located in the A chain, Cys16 and Cys25, are suggested to form an intra-chain disulfide bridge. Two putative cleavage sites of RAVR and RRHRR at amino acids 67 and 164 are suggested to excise the C peptide.

A phylogram generated using neighbour-joining methods segregated the different decapod IAGs in accordance to their genus (Figure 2B). The different clades in the phylogram, reflecting the similarities of the proteins in the different species, were found to correlate with the different groups of decapods (Shrimp, Prawn, Crayfish, Crab and Lobster).

Spatial and temporal expression of Pc-IAG

The expression of *Pc-IAG* was found solely in the AG tissue (Figure 3A), at the base of the 5th walking legs of males as described above. The temporal expression of *Pc-IAG* commences at the fourth week after the hatching date.

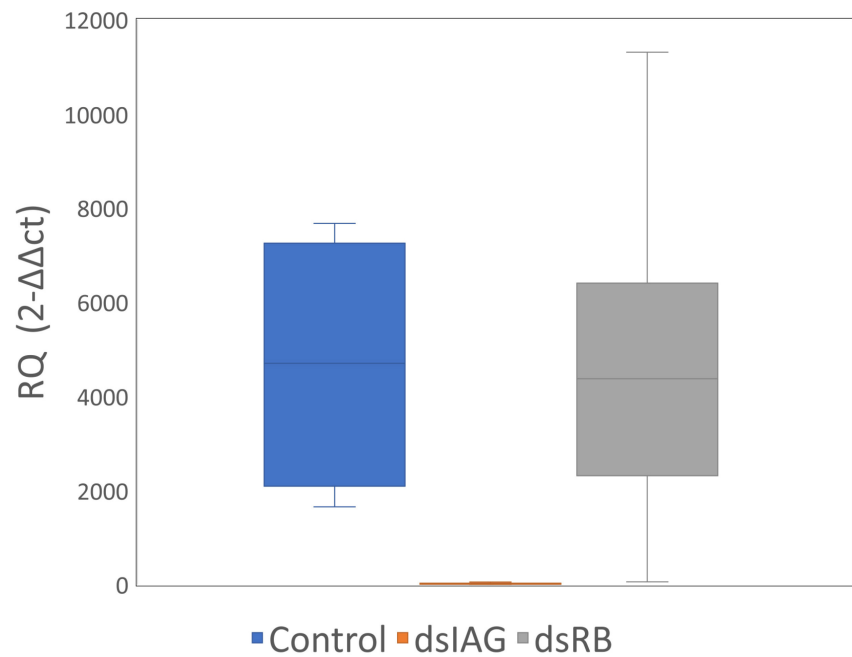


Figure 4. Levels of *Pc-IAG* transcripts following in vivo dsRNA injections in male crayfish. Relative *Pc-IAG* transcript levels were quantified in crayfish males by real-time RT-PCR following short-term silencing. Three different groups were tested, two injected with either ds*Pc-IAG* (n = 8) or ds*RB* (n = 8) and one intact group that was not injected at all (n = 4). The groups were found to be statistically different [Kruskal-Wallis statistic: H (df = 2, N = 20) = 13.725, p = 0.001]. Error bars represent SEM

The first animals to show expression were animal numbers 32, 33, 38 and 40 (Figure 3B, black arrows). The average weight of these animals at week #4 was 75 ± 5 mg and their average length was 16.7 ± 0.2 mm. All animals that were taken for sampling earlier did not show any expression as shown in Figure 3B, therefore data were not shown.

RNAi experiment

Short-term silencing experiment quantified by Real-Time RT-PCR was performed using RNA extracted from the AG's of treated males. Significant reduction (~ 98%) in *Pc-IAG* transcript levels, as compared with control groups (Kruskal-Wallis test: H (2, N = 20) = 13.72 p = .001; Figure 4) was recorded. Specifically, the relative quantification (RQ) values noted in the experimental group injected with ds*Pc-IAG* (22.6 ± 5.6 ($2^{-\Delta\Delta ct}$)) were at least 200-fold lower than the intact group (4684.7 ± 1335.7 ($2^{-\Delta\Delta ct}$)) and exogenous injected group (4754.5 ± 1254.6 ($2^{-\Delta\Delta ct}$)). Raw data is presented in supplemental file Table S2-Short-term silencing results.

A preliminary experiment is presented in Figure S4, showing similar results.

Population modelling - Use of neo-female as biological control agent for invasive P. clarkii population

Analytical solutions.

The long-term equilibrium of the demographic model with neo-female stocking was derived by setting the right-hand side of model #3 to zero and

solving for N and $M > 0$ and $F \geq 0$. As long as neo-females are continuously stocked (i.e., $W > 0$ and constant in time), the long-term equilibria— N_{eq} , F_{eq} , M_{eq} —can be derived as follows:

$$N_{eq} = W/\mu; F_{eq} = \frac{1}{2} (K-3W/\mu); M_{eq} = \frac{1}{2} (K+W/\mu) \text{ if } W < W_{th} = \mu \cdot K/3 \quad (6a)$$

$$N_{eq} = W/\mu; F_{eq} = 0; M_{eq} = \varphi \cdot N_{eq} \cdot (1-\gamma \cdot N_{eq}) / (\mu + \gamma \cdot \varphi \cdot N_{eq}) \text{ if } W \geq W_{th} = \mu \cdot K/3 \quad (6b)$$

Therefore, the long term equilibrium F_{eq} is a linear and decreasing function of W that equals zero when $W \geq W_{th} = \mu \cdot K/3$, i.e. when the stocking rate W equals or exceeds one third of the population turnover rate at carrying capacity, with $\mu \cdot K$ being approximately the number of prawns dying per unit time. The overall population size in the long-term is:

$$P_{eq} = F_{eq} + M_{eq} + N_{eq} = K \quad \text{if } W \leq \mu \cdot K/3 \quad (7a)$$

$$P_{eq} = M_{eq} + N_{eq} > K \quad \text{if } W > \mu \cdot K/3 \quad (7b)$$

Stoking neo-females reduces the number of genetic females (eq.s 6) and increases that of males, as expected, but long-term population size equals the carrying capacity K as long as $W \leq \mu \cdot K/3$, or exceed it when $W > \mu \cdot K/3$. Consequently, stocking neo-females long enough at densities equal or above W_{th} can eliminate females locally; once there are no females left in the population, it is possible to stop stocking neo-females, and, in the long run, the remnant male population decreases to extinction.

Numerical simulations

Numerical simulations showed that, for the values of model parameters reported in Table 2, it was not possible to drive the female population to extinction by stocking neo-females for 15 years only, regardless the population growth rate (Figure 5, first row, panels a, d, g). Female extirpation could be achieved only by stocking neo-females for longer periods (e.g., 25 years, Figure S1 in online supplementary information). In agreement with the analytical solution, population size during the stocking phase significantly exceeded the natural carrying capacity K of the waterbody, the slower the rate of growth of the population, the larger the population surplus with respect to the natural carrying capacity. In the case of the slowest growing population ($i = 1$), total population size during the stocking phase exceeded the carrying capacity by more than 40% (Figure 5a). According to eqs (6b), the population surplus with respect to the natural carrying was even larger for higher turnover rates, i.e., for higher mortality rates μ .

Numerical simulations of the alternative strategy, i.e. intensive harvesting only with fishing mortality $\eta = 2\mu$, showed that harvesting was effective in reducing population size (Figure 5, second panel row), but it led to crayfish elimination only in the case of a slow growing population (Figure 5b). For all the other cases, at the end of the fishing period the population bounced

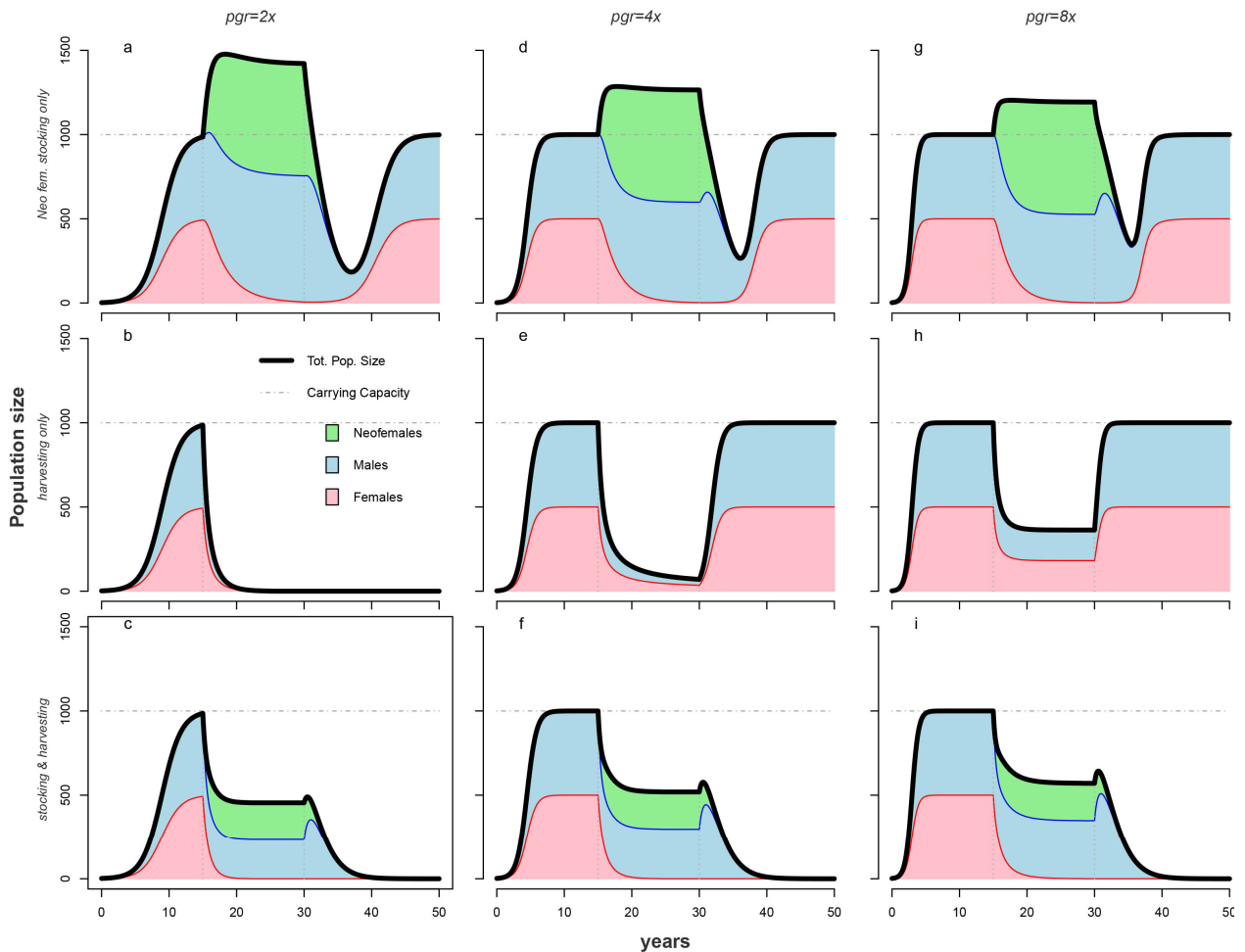


Figure 5. Invasive crayfish population dynamics: results of model simulations for three control strategies, namely neo-female stocking only (first row), intensive harvesting only (second row) and integrated control with neo-female stocking and intensive harvesting (third row), and for an annual population grow rate (pgr) at low densities equal to 2, 4 and 8 for panels in first, second and third column respectively. All the other model parameters are set as described in Methods.

back to its natural carrying capacity (Figure 5b, e, h). Note that fishing mortality η was held constant regardless the value of the intrinsic growth rate ϕ_i of the population. Therefore, fishing mortality was proportionally higher and more impacting for slow growing populations (Figure 5b) than for fast growing populations (Figure 5h).

Simulations showed that the most effective strategy to speed up and possibly achieve female extirpation and, at the same time, to avoid overshooting the carrying capacity for fast growing populations was to integrate neo-female stocking ($W > 0$) with intensive harvesting of the population (Figures 5f and 5i).

Additional analyses showed that accounting for strong seasonality in reproduction (model #5, Figure S2) or for population age/size structure did not change the qualitative results of models #2–4, whereas the presence of immigration (model #6, Figure S3) obviously prevented extirpation of the invasive crayfish population.

Discussion

Production of monosex all-male populations of *P. clarkii* has a dual purpose: first, to control *P. clarkii* invasive populations and second, for the aquaculture industry as a responsible non-invasive product that is expected to increase yield (Conde and Domínguez 2015). This work lays the foundations for the production of *P. clarkii* monosex populations by describing the AG, sequencing *Pc-IAG* and performing *in vivo* loss-of-function experiments through RNAi. We showed that the “IAG-switch” could be manipulated by temporarily blocking the expression of *Pc-IAG* as an a priori step towards a full and functional sex-reverse manipulation as done in another crustacean species (Ventura et al. 2012). There are still major biotechnological challenges remaining before the successful commercial production of all-male *P. clarkii* populations, as neo-females must be generated through long-term silencing experiments. In *M. rosenbergii* for example, few RNAi microinjections at an early post-larval stage, cause such a complete and irreversible such sex-shift from males to neo-females (Ventura et al. 2011, 2012). Determining the precise timetable for such an intervention is mandatory in order to complete the development of this biotechnology. The short-term silencing experiments conducted in this study are the first step and a partial POC that the avenue of RNAi is a promising research direction. Once the POC will be completed via long-term RNAi manipulations with the availability of neo-females brood-stock – mass production of *P. clarkii* all-male will be in reach. In order to meet the enormous needs of *P. clarkii* aquaculture industry (More than 1 million Tons in China alone (FAO 2017)), more efficient delivery methods such as oral delivery, are desired (Coy et al. 2012; Yu et al. 2013). Since a *P. clarkii* female produces only dozens to hundreds of eggs at each reproduction cycle (in contrast to thousands that are produced in *M. rosenbergii*) more neo-females are needed to produce large numbers of monosex offspring. Thus, the oral delivery technique is of major importance in this species as it would allow to perform the manipulation on multiple specimens in parallel, instead of individual microinjections (as being done successfully in the all-male *M. rosenbergii* industry) (Savaya-Alkalay and Sagi 2016).

The temporal expression pattern we revealed in juvenile specimens (Figure 3B) suggests that *Pc-IAG* expression occurs as early as four weeks after hatching; therefore, the “IAG-switch” manipulation must be performed earlier. Since microinjection into such small animals (< 75 mg) is a tedious and challenging procedure, new methods for the delivery of the silencing agent are desired. Development of an oral delivery method could allow intervention at an earlier stage than the zygote formation, such as the oocyte formation stage in the ovary. Each decapod crustacean group (crabs, prawns, shrimp, crayfish and lobsters) has a typical life-cycle and sexual differentiation pattern, therefore, the window of opportunity for

sexual differentiation manipulation must be identified separately for each target species. Consequently, tailor-made biotechnology is necessary for every species (or genus) (Ventura and Sagi 2012).

This study explored whether neo-female crayfish can be used to control invasive *P. clarkii* in the wild by skewing the sex ratio of an isolated population toward the progressive elimination of females. The modelling analysis showed that stocking neo-females at one third or more of the turnover rate of the population for a long enough period could theoretically lead to the elimination of genetic females and, consequently, to the long-term extirpation of the invasive crayfish population. Anyway, the main drawback of this control strategy is that neo-female stocking requires, to be effective, to overshoot crayfish's natural carrying capacity for years. This is clearly a paradoxical, although transient, side effect of the control strategy: as the goal of eradication is to minimize the ecological damage caused by invasive populations of *P. clarkii*, artificially augmenting the crayfish population above its natural carrying capacity for such a long time could lead to potentially devastating impacts on the conservation of native biodiversity. Therefore, neo-females stocking alone cannot be a viable solution for the control of invasive crayfish populations.

However, neo-female stocking can be part of an integrated control strategy when harvesting alone is unable to control a fast growing, invasive, isolated crayfish population. This result is consistent with the general consensus that integrated pest management, i.e., the combined use of different control strategies, represents the best option for controlling invasive populations (Dumbauld et al. 2006; Loureiro et al. 2018). In addition, we expect that the cost-effective generation of neo-females will boost commercial production of monosex *P. clarkii* while minimizing the risk of additional crayfish invasions.

Although we tried to capture the fundamental processes driving crayfish dynamics, our modelling analysis is not free of caveats and limitations. For instance, crayfish's fecundity and survival are likely modulated by age and/or size, so a finer description of population structure with multiple age/size classes would introduce more realism in the model (Martelloni et al. 2012). reproduction and recruitment of newly born crayfish might be also regulated by more complex density-dependent functions than the one assumed with logistic growth in our models. While more detailed models are necessary to capture specific aspects of crayfish population dynamics and to fine tune control strategies and determine optimal timing and abundance of stocking as well as integrated fishing effort, the results of our simulations accounting for seasonality in reproductive rate and a simplified population structure show that the general conclusions of our analyses are quite robust to alternative hypotheses about model structure.

The most important limitation in our modelling exercise was the lack of population spatial structure: refuges and semi-isolated, weakly connected

populations are generally the norm, rather than the exception, in a complex landscape or in a network of streams and ponds, especially when crayfish invade wide geographical regions. Therefore, it might be difficult in practice, if not impossible, to extirpate the whole population down to the last female, whether by stocking females or fishing out crayfish, as the survival and immigration of just few females from nearby habitats would allow the population to rebound when the restocking and/or harvesting programs end (Figure S3) (Loureiro et al. 2015). Therefore, the challenges of stocking neo-females (or harvesting crayfish) in all suitable patches on a mosaic of weakly connected local populations would make these control strategies less effective than projected by theoretical simulations, making full extirpation difficult, if not unfeasible. However, if a species-specific silencing agent could be directly dispersed in the invaded environment, existing juvenile wild males could be potentially sex-reversed into neo-females in their natural environment, maximizing the production of all-male progeny. Such intervention would skew the populations sex ratio without boosting the invasive population above its natural carrying capacity – a possibility worth future analysis via field and laboratory experiments.

In conclusion, *P. clarkii* stands out as one of the most damaging and difficult to control invasive species, therefore novel approaches for its control and managements are required, following also the recent genetic pest management suggested for invasive species. In addition, since it has a prominent role in aquaculture, monosex production may be of major interest for that rapidly growing industry. Our results in this study provide a foundation for the future production of *P. clarkii* monosex populations, both for environmental applications and aquaculture industry. We call for further research on the manipulation of sexual differentiation in *P. clarkii* in order to achieve the desired full and functional sex-reversal of males into neo-females that produce all-male progeny.

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Conflict of interest

The authors declare no conflicts of interest

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Supplementary material

The following supplementary material is available for this article:

Figure S1. Eradication achieved with a longer stocking period.

Figure S2. Simulation outcome with seasonal fluctuations in crayfish reproduction.

Figure S3. Simulation outcome with constant crayfish immigration.

Figure S4. Levels of *Pc-IAG* transcripts following in vivo dsRNA injections in male crayfish.

Table S1. Gene bank accession numbers.

Table S2. Short term silencing results.

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