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Are commercial stocks of biological control agents genetically depauperate? – A case study on the pirate bug *Orius majusculus* Reuter

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Abstract

Predatory arthropods are increasingly used in biological control of insect pests. For this purpose, control agents are produced commercially in large quantities for release in crops. The production stocks, however, may have undergone numerous population bottlenecks and may have been exposed to artificial selection pressures in the production facilities. Accordingly, commercial populations may be experiencing loss of genetic variation through inbreeding and genetic drift, which may reduce fitness and biocontrol efficiency. In the present study we investigated whether populations of the pirate bug *Orius majusculus* (Reuter) purchased from three European companies differed in a range of performance traits including predation rate, starvation tolerance, body size, locomotor activity, and heat tolerance. Furthermore, we crossed all populations pairwise and tested whether outcrossed F2 hybrid offspring had increased performance compared to the parental populations, as would be expected if they were genetically distinct and depauperate. Transcriptome sequencing (RNA-seq) revealed similar overall levels of genetic variation among commercial populations, but also evidence for genetic differentiation. Generally, females performed better across phenotypic traits than males. F2 hybrid offspring differed from parental populations in a highly trait- and sex specific manner. Although F2 hybrids performed better than parental populations in some traits, the results of the present study do not provide conclusive evidence that crossing of different commercial stock populations of *O. majusculus* improve the genetic quality and performance of this species as a biological control agent.

Keywords: *Orius majusculus*, cross-breeding, biological control agents, stress tolerance, predation rate, heterosis

1. Introduction

Biological control of insect pests is increasingly used in agriculture and horticulture to promote more environmentally sustainable, integrated pest management (Bale et al., 2008; Cock et al., 2010; Lamichhane et al., 2017). Accordingly, the worldwide application of biological control agents (BCAs) has increased from an area of ~17 million ha in 2003 to ~30 million ha in 2015 (van Lenteren et al., 2018; van Lenteren and Bueno, 2003). To improve the efficiency and impact of BCAs following release, different aspects concerning production avenues are currently being investigated (Morales-Ramos et al., 2013; Terblanche, 2014). For example, optimization of rearing diet (De Clercq et al., 2014) and rearing temperature (Cascone et al., 2015; Sørensen et al., 2013) in preparation for ambient conditions have been suggested to significantly increase the efficiency of BCAs upon release. However, thermal acclimation and priming with specific prey does not always improve prey consumption and other performance traits as desired (Helgadóttir et al., 2017; Jensen et al., 2017b).

Another approach that can potentially be exploited to increase the efficiency of arthropod predators in integrated pest management involves evaluating and improving the genetic quality of BCAs (Lommen et al., 2017; Szűcs et al., 2012). This includes 1) a focus on the origin of populations of BCAs ensuring high performance under the operating conditions, 2) artificial selection for traits of importance for their performance as BCAs, and 3) focus on maintaining high effective population sizes (Ne) to reduce inbreeding and random genetic drift, thus maintaining evolutionary potential. In the future, the biological control industry is likely to benefit strongly from experience and knowhow in animal breeding and quantitative genetics when it comes to employing effective selection procedures and control of inbreeding and genetic drift (Jensen et al., 2017a).

If a population has become inbred, this can be alleviated by introducing individuals from other populations of the same species or sub-species that are genetically distinct (Edmands, 2007;

Jensen et al., 2018; Whiteley et al., 2015). Several studies have successfully used this approach to aid in the rescue of threatened natural and domestic populations (Johnson et al., 2010; Westemeier, 1998). Often introducing new genetic material into a population leads to increased fitness in the offspring because alleles from one population can mask deleterious recessive alleles from the other population (Lohr & Haag 2015; Tallmon et al. 2004). Fitness often peaks in the F1 generation and decreases in subsequent generations (Edmands, 1999; Tallmon et al., 2004; Whiteley et al., 2015) due to recombination in meiosis (Tallmon et al., 2004). Therefore, the performance of the F2 hybrid or subsequent generations should be investigated to reveal if heterosis effects are persistent. However, introducing new genetic material into a population can also cause outbreeding depression if crossed populations have been separated for long and adapted to distinct environments (Edmands, 2007; Tallmon et al., 2004). Outbreeding depression can lead to reduced fitness due to breakdown of co-adapted gene complexes and local adaptation (Frankham et al., 2011; Weeks et al., 2011).

Several species of polyphagous predators, with large representation from the order Hemiptera, are mass-reared in commercial production facilities for use in biological control of arthropod pests (De Clercq et al., 2014; van Lenteren et al., 1997). Not least, pirate bugs from the genus *Orius* are important generalist predators in biological control (e.g. Bosco et al., 2008; Harwood et al., 2007; Xu et al., 2006). However, information regarding rearing conditions, origin of the population, and number of founder individuals are rarely readily available. Therefore, it is difficult to evaluate whether commercial stocks suffer from inbreeding or low genetic variation, and are maladapted to the environments under which they are to perform control of pest species (Sørensen et al., 2012). We propose that a focus on genetic aspects in rearing of BCAs will alleviate the performance of these agents.

We conducted common garden experiments on performance parameters in three different commercial populations of the pirate bug *Orius majusculus* (Reuter). This species is native to the

European continent and commercially produced as a natural enemy against agricultural pests, first of all aphids and thrips (Brødsgaard and Enkegaard, 1997; Malais and Ravensberg, 2003; van Lenteren et al., 1997). We compared the performance of F2 hybrid offspring obtained from pairwise crosses between parental, commercial populations. Predation rate, starvation tolerance, body size, locomotor activity, and heat tolerance were investigated. Further, we compared the levels of genetic differentiation and genetic variation (using RNA-seq) of the three commercial populations. Levels of genetic variation and differentiation were compared to results from a sample of wild *O. majusculus* collected in a maize field in Denmark.

We hypothesized that the commercial populations were phenotypically and genetically distinct because they have been held separately for many generations and probably have different geographical origin. For the same reasons, we expected commercial populations to harbor lower levels of genetic variation compared to the wild-caught individuals. Finally, we hypothesized that F2 hybrid individuals would outperform the parental populations due to heterosis.

2. Materials and Methods

2.1 Populations and maintenance

We obtained populations of *O. majusculus* from three European companies ($N \approx 500$ individuals per population): EWH BioProduction ApS (Tappernøje, Zealand, Denmark), Borregaard BioPlant ApS (Aarhus, Jutland, Denmark) and Bioplanet (Cesena, Emilia-Romagna, Italy), henceforth referred to as 'BioProduction', 'Borregaard' and 'Bioplanet', respectively. According to information provided by the companies, the three populations have been maintained separately at the distinct production facilities for at least 13 years. Upon receipt, each population was maintained in two plastic boxes (34 cm × 25 cm × 12 cm) with lids containing nylon-mesh covered aeration holes. The boxes

contained buckwheat (*Fagopyrum esculentum*, Moench) grain shells and paper napkin strips for structure. Water was provided in six 1.5 mL cotton plugged microtubes. The cultures were supplied with Mediterranean flour moth (*Ephestia kuehniella*, Zeller) eggs *ad libitum* as food, which were glued to a piece of paper using mayonnaise and replaced every second day. For oviposition, we provided fresh green bean (*Phaseolus vulgaris*, Linnaeus) pods, which were similarly replaced every second day, and moved to new boxes for the eggs to hatch. All populations and F2 hybrid offspring were bred and maintained at 23 °C and a photoperiod of 16:8 L:D in a climate cabinet (Binder model KBWF 720 E5.3, Tuttlingen, Germany).

2.2 F2 hybrid populations

To make pairwise hybrid offspring between the three commercial populations, we first collected 80 nymphs from each population. The nymphs were maintained individually in small plastic vials (93 mm × 23 mm diameter) with water-containing plaster of Paris bottoms, *E. kuehniella* eggs and a halved bean pod. As the nymphs reached the adult stage, individuals from each population were grouped by sex and maintained in larger vials (82 mm × 36 mm diameter) with up to 30 individuals in each. The vials were provided with *E. kuehniella* eggs *ad libitum*, water in cotton plugged 1.5 mL microtubes, and paper strips (1 x 5 cm) for structure. When all nymphs had reached the adult stage, six new populations were founded by mixing females from one population with males from another population in all possible population pair combinations (Table 1). When the resulting F1-hybrid offspring reached the adult stage, they founded an F2 generation. F2 individuals from reciprocal crosses between the same two stocks (i.e. males from stock 1 and females from stock 2 or males from stock 2 and females from stock 1) were mixed in equal numbers reducing the six populations to three experimental F2 populations. The three parental populations had similarly undergone two

generations in our laboratory in the same period, ensuring that common garden conditions were achieved. The distribution of age span was similar in all populations (Fig. S1).

2.3 Predation rate

We compared predation rate of *O. majusculus* of the six populations (N = 30 females and 30 males per population) by measuring consumption of *E. kuehniella* eggs over 48 h. Individuals were placed individually in petri dishes (55 mm diameter, 15 mm height) prepared with 1 mL of a 2% agar solution (20 g/L water) placed in the center as water supply. At least 40 *E. kuehniella* eggs were placed on the petri dish bottom around the agar. The experiment was performed in two blocks with 15 individuals per population and sex in each block. The individuals were starved for 24 h before the test. The number of empty eggshells was counted after 48 h as a measure of predation rate. Data from petri dishes where experimental individuals died during the experiment were excluded from the analysis.

2.4 Starvation tolerance

To measure starvation tolerance, individuals of *O. majusculus* from the six different populations (N = 30 females and 30 males per population) were placed individually in plastic vials (93 mm height × 23 mm diameter) with 4 mL of a 2% agar solution (20 g/L water) covering the bottom and providing water. The vials were plugged with foam stoppers and placed upside-down to reduce the risk of individuals getting stuck in the agar. Individuals were checked for mortality every 12 h and surviving individuals were transferred to new vials if the agar was shrinking due to evaporation or if any kind of microbial growth was observed on the agar. Dead individuals were collected and stored at -18 °C for later measurement of body size.

2.5 Body size

Pronotum size is a good proxy for body size because the pronotum does not change once adulthood is reached and is not affected by feeding state (Otti et al., 2009). All individuals (N = 30 females and 30 males per population) were placed on their back and photographed using a digital camera (Axiocam 105 color, Zeiss, Göttingen, Germany) connected to a stereomicroscope (Stemi 508, Zeiss, Göttingen, Germany). The width of the pronotum was measured to nearest µm using the software Zen 2.3 (Zeiss, Göttingen, Germany). Repeatability of measurements was verified by measuring the pronotum width a second time for 30 randomly selected individuals (linear regression: R^2 = 0.9953).

2.6 Activity

Locomotor activity of *O. majusculus* was investigated (N = 30 females and 30 males per population) using Drosophila Activity Monitors (DAM2, TriKinetics inc., Waltham, MA USA). Individuals were placed individually in glass tubes (0.5 cm diameter, 6.5 cm length) with a piece of dry pipe cleaner plugging one end and moist cotton wool plugging the other end providing moisture. The monitor recorded every passing of the tube center and data were logged every minute. The loaded monitors were placed in the climate cabinet with constant light and a temperature of 23 °C, and basal activity was measured over four hours. For analysis, data from the first hour was excluded to ensure that animals had settled down and that recordings represented basal voluntary activity.

2.7 Heat tolerance (CT_{max})

After recording basal activity at 23 °C for four hours (see section 2.6), the cabinet temperature gradually increased at a rate of 0.085 °C/min over the next four hours until reaching 44 °C. The temperature of maximal activity was determined for each individual as the temperature where the highest number of center crossings was recorded within a minute. The critical thermal maximum (CT_{max}) was determined for each individual as the temperature beyond which no further activity was registered.

2.8 RNA extraction and sequencing

To investigate whether the three commercial *O. majusculus* populations were genetically different, 100 live males, from each stock, as well as 100 males collected in a maize field in Northern Jutland, Denmark in August/September 2017 using a mouth aspirator, were frozen in liquid N_2 in pools of 20 individuals and stored at -80 °C until further processing.

RNA extraction was carried out using the TRIzol ™ Reagent according to the manufacturer's instructions with some modifications. One mL TRIzol reagent was added to a 2 mL prechilled (on ice) precellys tube containing 2 CK14 beads and 20 individuals of *O. majusculus*. The precellys tubes were placed in a Precellys mechanical homogenizer (Bertin Technologies, France) and subjected to 3 cycles of 10 sec (5-10 sec pause in between) homogenization at 6000 rpm. The samples were centrifuged for 5 min (12,000 g at 4 °C) and the supernatant was transferred to new tubes and 0.2 mL chloroform was added. The tubes were then vortexed and centrifuged for 15 min (12,000 g at 4 °C). The colorless aqueous upper phase (containing RNA) was transferred to a new tube containing 0.5 mL isopropanol, incubated on ice for 10 min and centrifuged for 10 min (12,000 g at 4 °C). The supernatant was discarded, and the pellet washed twice by adding 1 mL

75% ethanol, vortexing, and centrifuging for 5 min (12,000 g at 4 °C). The supernatant was discarded, and the tubes were left open for 5-10 min before the pellet was dissolved in 30 µL RNase-free water and incubated in a heat block at 60 °C for 15 min (vortexed 3 times). The RNA concentration of the samples was determined by NanoDrop ® Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE, USA) using absorption at 260 nm and the quality of the RNA was estimated by the A260/280 ratio as well as by analysis on a Tapestation 2200 (Agilent Technologies) using an RNA screen tape and evaluating the presence and integrity of 28S and 18S rRNA. Bearing in mind that insect RNA often contains a cryptic bond break in the 28S rRNA subunit, which we observed is also the case for *O. majusculus*, the standard RIN numbers cannot be used as quality measures (Winnebeck et al., 2010). The consistent presence of well defined, merged peaks co-migrating at the size of "normal" 18S rRNA, representing 28Sα and 28Sβ and 18S rRNAs, showing no "tail" of degradation following Tapestation analysis was taken as indication of high quality RNA extractions. The five samples representing each population was pooled into one sample and stored at -80 °C until preparation for sequencing.

RNA-seq sample preparation was carried out using the TruSeq® RNA Sample Prep v2 Low sample protocol (Illumina) strictly according to the manufacturer's instructions. Ten µg of total RNA (200 ng/µL) was used as input material to construct four libraries, each with a unique identification sequence, representing the four studied populations. Following sample preparation, the four libraries were evaluated on a Tapestation 2200 (Agilent Technologies) using a DNA ScreenTape to validate size distribution of the libraries. The concentration of DNA in the samples was measured using the Qubit ® 2.0 Fluorometer (Invitrogen, Life Technologies Corporation). Each library was diluted to a final DNA concentration of 10 nM using Illumina normalization buffer and pooled into a single sample. The pooled sample was subsequently diluted to 2 nM,

hybridized to the flow cell and sequenced on an Illumina HiSeq2000 (2009 series) system (Illumina, San Diego, USA) for 100 cycles.

2.9 Statistical analysis

2.9.1 Phenotypic data

The data obtained for the six populations in each of the investigated traits were generally not normally distributed (Shapiro-Wilk test, $P < 0.05$) and therefore proportional hazard and Wilcoxon tests were used. For each trait, the six populations were compared separately for females and males using a Wilcoxon test followed by a Wilcoxon each pair comparison. The effect of breeding (populations from companies constituting one group while the F2 hybrids constituting the other), population, sex and the population*sex interaction were analyzed using a proportional hazard test (effect likelihood ratio test). To account for any variability among the parental populations and the F2 hybrids the effect of population, sex and the interaction between them were nested within breeding before the proportional hazard tests were run. The level of heterosis in all six traits was assessed by calculating the phenotypic value of the F2 individuals relative to the mean of the parental populations. To test for the presence of heterosis the difference between the parental populations and the F2 hybrids for each trait and sex were compared using a Wilcoxon test (Table S1). Proportional hazard and Wilcoxon tests were performed in JMP 13.1.0 (SAS Institute Inc., Cary, North Carolina, USA). Two-tailed one-sample and unpaired t-tests were performed in Excel 2016 (Microsoft Corporation, Redmond, Washington, USA).

2.9.2 RNA-seq data

Following sequencing, 18,084,192 reads were imported in CLC Workbench v. 9.5.5, adaptor sequences removed, and the reads were trimmed using the following settings (low quality sequence

0.05; no ambiguous nt allowed; and resulting read length ≤ 50 nt). The resulting 17,840,270 reads were then *de novo* assembled into contigs in CLC workbench using the following settings (Mapping mode = Map reads back to contigs; Update contigs = Yes; Automatic bubble size = Yes; Minimum contig length = 150; Automatic word size = Yes; Perform scaffolding = Yes; Auto-detect paired distances = Yes; Mismatch cost = 2; Insertion cost = 3; Deletion cost = 3; Length fraction = 0.9; Similarity fraction = 0.95; Create list of un-mapped reads = Yes). 787 contigs that obtained a minimum of 10,000 reads of the combined samples were selected and used for mapping of the individual samples using Map Reads to Reference (CLC Workbench, settings: Masking mode = No masking; Match score = 1; Mismatch cost = 2; Cost of insertions and deletions = Linear gap cost; Insertion cost = 3; Deletion cost = 3; Length fraction = 0.9; Similarity fraction = 0.95; Global alignment = No; Non-specific match handling = Ignore; Output mode = Create stand-alone read mappings; Create report = Yes; Collect un-mapped reads = Yes). To eliminate potential bias in nucleotide diversity estimates stemming from differential sequencing depth, 5.8 $*10^6$ reads that mapped the selected contigs were subsamples from each sample and remapped to the 787 contigs using the same criteria as above. Variants within each sample was called using the Basic Variant Detection feature of CLC Workbench (Ploidy = 8; Ignore positions with coverage above = 100,000; Restrict calling to target regions = Not set; Ignore broken pairs = No; Ignore non-specific matches = Reads; Minimum coverage = 15; Minimum count = 1; Minimum frequency (%) = 2.0; Base quality filter = No; Read direction filter = No; Relative read direction filter = No; Read position filter = No; Remove pyro-error variants = No; Create track = Yes; Create annotated table = Yes). Note that ploidy setting has no influence on variant detection (see CLC workbench manual for further explanation). Only biallelic SNP variants with a MAF ≥ 2% were included in the further analysis. Nucleotide diversity (π) was calculated using the following formula (Nei and Li, 1979): $\sum_{ij}^{n} x_i x_j * \pi_{ij}$, where x_i and x_j are the respective frequencies of the i^{th} and j^{th} sequences and π_{if} is the number of

nucleotide differences per nucleotide site between the i^{th} and j^{th} sequences, and n is the number of sequences in the sample.

For biallelic SNPs, $x_i=1-x_j$ and $\pi_{if}=1$, and therefore the above equation can be simplified to: $\sum_{i=1}^{n} 2 * x_i * (1 - x_i)$, where x_i is the frequency of the observed SNP variant and n is the number of SNP sites. 95% confidence intervals were calculated by assuming that overall π can be considered as n times the mean value of π at a single site. Hence, the 99% confidence intervals can be calculated as: $X \pm (\frac{12,58 * \sigma}{\sqrt{n}})$. To assess the samples relatedness, 5029 SNP sites which was heterozygous in all samples were selected and principal component analysis of the samples were conducted using ClustVis (Metsalu and Vilo, 2015).

3. Results

3.1 Predation rate

Offspring from crosses between populations had higher predation rate than parental populations ($\chi^2_{1,360}$ = 5.91, $P < 0.05$). This result was driven by a significant difference among males ($\chi^2_{1,159}$ = 5.92, $P < 0.05$) where the crosses generally had a higher predation rate than the parental populations (Fig. 1A). Females from the parental populations and the F2 hybrids showed no difference in predation rate ($\chi^2_{1,176}$ = 0.08, $P = 0.78$; Fig. 1A). Females generally had higher predation rate than males across populations ($\chi^2_{2,360}$ = 71.42, $P < 0.001$; Fig. 1A). There was no significant interaction between population and sex on predation rate ($\chi^2_{4,360}$ = 2.79, $P = 0.59$; Fig. 1A).

Different levels of heterosis for predation rate were seen in females and males from crosses between the parental populations (Table S1). The significant effect of crossing observed in males were driven by the crosses BioProduction x Borregaard and Borregaard x Bioplanet where the offspring had significantly higher predation rate than the average of males from the parental

populations (BioProduction x Borregaard: $t_{27} = 3.24$, $P < 0.01$; Borregaard x Bioplanet: $t_{27} = 2.58$, $P < 0.05$; Table S1), but no significant difference between offspring from crossing individuals from BioProduction and Bioplanet and the parental populations (BioProduction x Bioplanet: $t_{28} = 0.61$, $P = 0.55$; Fig. 1A and Table S1). In females no significant heterosis was observed (Table S1).

3.2 Starvation tolerance

Overall, starvation tolerance of offspring from crosses was significantly higher than the average of the parental populations ($\chi^2_{1,359} = 15.95$, $P < 0.001$; Fig. 1B). There was a general tendency for the Bioplanet population to have a lower starvation tolerance than populations from the other companies (Fig. 1B). Females generally had a higher starvation tolerance than males ($\chi^2_{2,359} = 187.44$, $P < 0.001$; Fig. 1B). There was also a significant interaction between population and sex on starvation tolerance ($\chi^2_{4,359} = 12.11$, $P < 0.05$), indicating that the difference in starvation tolerance between the sexes depended on population.

Overall the females showed no difference in starvation tolerance between the parental populations and the F2 hybrid populations ($\chi^2_{1,179} = 0.274$, $P = 0.10$, Fig. 1B). The males, however showed a significant difference in starvation tolerance ($\chi^2_{1,180} = 11.42$, $P < 0.001$) with the crossbred populations being able to tolerate starvation for a longer period (Fig. 1B). Even though females generally did not show heterosis, the females from the cross Borregaard x Bioplanet had a significantly higher starvation tolerance ($t_{29} = 2.59$, $P < 0.05$) than the mean of the parental populations (Table S1). In the other two crosses, no significant difference in female starvation tolerance was found when compared to the mean of the parental populations (BioProduction x Borregaard: $t_{29} = 0.99$, $P = 0.33$; BioProduction x Bioplanet: $t_{29} = 1.77$, $P = 0.09$; Table S1). The difference in starvation tolerance between males from a cross and the mean of males from the parental populations was significant in the crosses BioProduction x Bioplanet ($t_{29} = 9.09$, $P < 0.001$)

and Borregaard x Bioplanet (t_{29} = 3.21, P < 0.01; Table S1). Males from the cross BioProduction x Borregaard showed no indication of heterosis (t_{29} = 0.74, P = 0.47; Table S1).

3.3 Body size

Offspring from crosses overall had smaller pronotum width than individuals from the parental populations in both sexes ($\chi^2_{1,359}$ = 3.90, P < 0.05; Fig. 1C). Females generally had wider pronotums than males in all populations ($\chi^2_{2,359}$ = 287.24, P < 0.001; Fig. 1C). We observed no significant interaction between population and sex on pronotum width ($\chi^2_{4,359}$ = 8.77, P = 0.07). There was no overall difference between the parental populations and offspring from crosses for neither females ($\chi^2_{1,179}$ = 1.62, P = 0.20) nor males ($\chi^2_{1,180}$ = 1.74, P = 0.19, Fig. 1C). However, when investigating individual contrasts, we found that females from the cross Borregaard x Bioplanet were significantly larger than the mean of females from the parental populations (t_{29} = 4.75, P < 0.001; Table S1). Similarly, males from the cross Borregaard x Bioplanet were significantly larger (t_{29} = 2.96, P < 0.01) than the mean size of males from the parental populations (Table S1). In contrast, both sexes from the crosses BioProduction x Borregaard and BioProduction x Bioplanet were significantly smaller than the mean of the parental populations (Females: t_{29} = -4.49, P < 0.001 and $t_{29,30}$ = -6.26, P < 0.001, respectively; Males: t_{29} = -3.91, P < 0.001 and t_{29} = -3.85, P < 0.001, respectively; Table S1).

3.4 Basal activity

Individuals from the parental populations generally had a higher level of basal activity than the offspring from the crossed populations ($\chi^2_{1,358}$ = 14.41, P = 0.001; Fig. 1D). There was no significant difference in the activity level between females and males ($\chi^2_{2,358}$ = 3.88, P = 0.14; Fig.

1D). However, there was a significant interaction between population and sex on the basal level of activity ($\chi^2_{4,358} = 11.69$, $P < 0.05$). For both sexes the activity level was significantly lower in offspring from the crosses compared to the parental populations (Females: $\chi^2_{1,180} = 11.84$, $P < 0.001$; Males: $\chi^2_{1,178} = 7.93$, $P < 0.01$, respectively; Fig. 1D).

The females from all three crosses had a significantly lower basal level of activity compared to the parental populations (BioProduction x Borregaard: $t_{29} = -8.19$, $P < 0.001$; BioProduction x Bioplanet: $t_{29} = -2.05$, $P < 0.05$; Borregaard x Bioplanet: $t_{29} = -2.88$, $P < 0.01$; Table S1). The same tendency was observed for males, although only significant for the cross BioProduction x Bioplanet as compared to the mean of the parental populations ($t_{29} = -2.28$, $P < 0.05$; Table S1). In the other two crosses, no significant difference in male basal activity level was found when compared to the mean of the parental populations (BioProduction x Borregaard: $t_{28} = -1.20$, $P = 0.24$; Borregaard x Bioplanet: $t_{29} = -1.62$, $P = 0.12$; Table S1).

3.5 Temperature of maximal activity

The temperature of maximal activity did not differ between parental and offspring from crossed populations ($\chi^2_{2,356} = 0.01$, $P = 0.93$; Fig. 1E). Females generally had maximal activity at a higher temperature than males across populations ($\chi^2_{2,356} = 34.76$, $P < 0.001$; Fig. 1E, Fig. S2). There was no significant interaction between population and sex on the temperature where activity peaked ($\chi^2_{4,356} = 0.31$, $P = 0.99$).

The lack of difference between the parental and F2 hybrid populations were confirmed within sexes (Females: $\chi^2_{1,177} = 0.08$, $P = 0.78$; Males: $\chi^2_{1,179} = 0.747$, $P = 0.39$; Fig. 1E). However, females from the cross BioProduction x Borregaard had a significantly higher peak activity temperature ($t_{29} = 6.54$, $P < 0.001$) than females from the parental populations indicating heterosis

in this hybrid population (Table S1). In the other two hybrid populations, no significant difference in peak activity temperature was found when compared to the mean of the parental populations (BioProduction x Bioplanet: $t_{28} = 1.14$, $P = 0.26$; Borregaard x Bioplanet: $t_{27} = -1.27$, $P = 0.22$; Table S1). None of the male F2 offspring from crosses had a significantly different peak activity temperature when compared to the mean of the parental populations (BioProduction x Borregaard: $t_{28} = 1.13$, $P = 0.90$; BioProduction x Bioplanet: $t_{29} = -1.92$, $P = 0.07$; Borregaard x Bioplanet: $t_{29} = -0.17$, $P = 0.87$; Table S1).

3.6 Critical thermal maximum

The critical thermal maximum (CT_{max}) did not differ between parental and crossbred populations ($\chi^2_{1,356} = 0.69$, $P = 0.41$; Fig. 1F). Females generally had a higher CT_{max} than males ($\chi^2_{2,356} = 63.07$, $P < 0.001$; Fig. 1F, Fig. S2). There was a significant interaction between population and sex on CT_{max} ($\chi^2_{4,356} = 22.52$, $P = 0.001$; Fig. 1F). Overall the females showed no difference in CT_{max} between the parental and F2 offspring populations ($\chi^2_{1,177} = 0.67$, $P = 0.42$; Fig. 1F). The males, however, showed a significant difference in CT_{max} ($\chi^2_{1,179} = 9.208$, $P < 0.01$) with the F2 hybrid populations having a lower CT_{max} than parental populations (Fig. 1F). Even though females generally did not show heterosis, the females from the cross BioProduction x Borregaard had a significantly higher CT_{max} ($t_{29} = 3.66$, $P < 0.01$) than the mean of the parental populations (Table S1). The other two crosses showed no difference in CT_{max} when compared to the mean of the parental populations (BioProduction x Bioplanet: $t_{28} = -0.83$, $P = 0.41$; Borregaard x Bioplanet: $t_{27} = -1.11$, $P = 0.28$; Table S1). In males none of the crosses had a significantly different CT_{max} compared to the mean of the parental populations (BioProduction x Borregaard: $t_{28} = 1.06$, $P = 0.30$; BioProduction x Bioplanet: $t_{29} = -0.36$, $P = 0.72$; Borregaard x Bioplanet: $t_{29} = 0.93$, $P = 0.36$; Table S1).

3.7 Genetic differences of parental populations

Overall nucleotide diversity (π) in the three parental commercial populations was on average 14% lower than in the wild caught sample with only minor differences among commercial populations (Fig. 2). Pearson correlation analysis of the nuleotide diversity showed that the wild caught sample was most differentiated from the commercial populations, and that the Bioplanet and Borregaard populations were very similar (Table 2). The BioProduction population did show an intermediate similarity to the two other commercial populations (Table 2). PCA analysis on SNP frequencies on sites polymorphic across all three commercial populations and the wild caught sample confirmed these conclusions (Fig. S3).

4. Discussion

There are numerous challenges associated with production of arthropods for biological control, including genetic aspects related to inbreeding and genetic drift, and selection of appropriate genotypes for continuation of the population (Mackauer, 1976). In this study, we tested the hypothesis that the F2 hybrid offspring from commercial populations of *O. majusculus* had increased performance compared to their parental populations, caused by possible inbreeding depression in the parental populations. Secondly, we investigated whether the three commercial populations were genetically distinct and differed in levels of genetic variation. Finally, we quantified levels of genetic variation in a wild-caught sample and compared it to the commercial populations.

We found that the three commercial populations were phenotypically distinct in some traits, whereas in other traits we found no difference between populations. The F2 hybrid populations outperformed the parental populations in some traits, e.g. starvation tolerance, and often the population effects were sex-specific. Across the six traits assessed in this study, significant heterosis was observed in five cases in males (three different traits) and four cases in females (four different traits) (Table S1). Results from the RNA-seq analysis showed genetic differentiation between all three commercial populations, most pronounced between the BioProduction and the Borregaard and Bioplanet populations (Table 2, Fig. S3). The genetic analysis also revealed similar levels of genetic variation within the three commercial populations, but as expected lower variation compared to the wild population (Fig. 2).

Heterosis has previously been observed in the ragwort flea beetle, *Longitarsus jacobaeae* (Waterhouse), which is used as a BCA to control tansy ragwort, *Jacobaea vulgaris* (Gaertner), in North America (Szűcs et al., 2012). In their study, the F2 hybrids between two genetically and phenotypically divergent populations of *L. jacobaeae* from Switzerland and Italy had a higher fecundity than individuals from the parental populations (Szűcs et al., 2012). Although a significant increase in performance (potentially due to heterosis) was observed for some traits in our study, the results revealed moderate and highly trait- and sex-specific effects of crossing the commercial populations. The lack of strong heterosis could be explained by large population sizes in the rearing facilities of the commercial populations such that they are not genetically depauperate. Further, the molecular genetic data suggests that the Borregaard and Bioplanet populations are not strongly genetically differentiated and consequently we would expect least heterosis from a cross between these two populations (Table 2, Fig. S3) (but see discussion below).

We observed heterosis for starvation tolerance in three of six cases (Table S1). Starvation tolerance is likely an important trait for BCAs in environments with variable and unpredictable prey

availabilities, e.g. in agroecosystems early in the season. For this trait, F2 hybrid females from the cross Borregaard x Bioplanet and males from the cross BioProduction x Bioplanet, and Borregaard x Bioplanet, showed heterosis. F2 hybrid females from the cross BioProduction x Bioplanet also showed heterosis (although not statistically significant). This tendency, combined with the results showing that individuals from Bioplanet have a low starvation tolerance (Fig. 1B), indicate that it is advantageous for this population to be crossed with another population to increase starvation tolerance.

Some heterosis is also observed for predation rate (Fig. 1A, Table S1). This trait is proposedly important for the efficiency of BCAs and therefore the results suggest that crossing populations may be a valuable tool in biological control of pest species. However, using a mobile prey like aphids instead of sessile moth eggs would have required the BCAs to locate and attack the prey, which might have led to a different conclusion. This is clearly speculative but studies suggest that the expression of heterosis, as well as inbreeding depression, is typically more severe under more challenging environmental conditions, i.e. such as when animals need to locate, capture and kill the prey (Parsons, 1971; Reed et al., 2012). Thus, we advocate that future studies investigating benefits associated with heterosis in relation to biological control include studying the impact of environmental stress and more complex (but ecologically relevant) biotic scenarios.

We show that effects of crossing are highly trait specific. For example, the F2 hybrid offspring from crosses between BioProduction x Bioplanet and Borregaard x Bioplanet had high starvation tolerance but lower basal activity levels compared to the parental populations (Fig. 1B and D). This exemplifies a positive tradeoff but also the importance of assessing multiple traits when evaluating impacts of heterosis on BCA efficiency.

Even though crossbreeding of BCA populations can have a positive impact on the efficiency of BCAs in some traits, it is important to assess what negative consequences it might have on other

traits e.g. due to outbreeding depression. This is illustrated from a case in New Zealand, where a Moroccan strain of the parasitoid wasp, *Microctonus aethiopoides* (Loan) is used to control a weevil, *Sitona discoideus* (Gyllenhal), in alfalfa, *Medicago sativa* (Linnaeus) (Goldson et al., 1990). Their results showed that crossing two strains of *M. aethiopoides* resulted in lower parasitism on *S. discoideus* (Goldson et al., 2003). Thus, outbreeding may have negative effects on the performance of BCAs, which is also observed in several traits in our study (Table S1).

Heterosis was observed in nine cases across both sexes (Table S1), five of which were in the F2 hybrids from the cross between the Borregaard and Bioplanet populations. Interestingly, these two were the most similar populations according to the nucleotide diversity measures (Table 2), and therefore heterosis would be expected to be least pronounced in F2 offspring from this cross. However, the diversity analysis was made using the common SNP sites as estimators for relatedness. These sites will not impact heterosis directly, because all populations are already heterozygous in these sites. Indeed, heterosis is more likely to be caused by the complementation of homozygous deleterious sites in one of the populations. Therefore, the observed heterosis could stem from genetic variation that is fixed differently among the Borregaard and Bioplanet populations. However, given the similarity in SNP frequencies in common polymorphic sites (Fig. 2 and S3), and the fact that the Borregaard and Bioplanet populations share more observed polymorphic sites (77%) than they do with the wild (58% for both) and BioProduction (68% and 64%), this seems unlikely to have happened in more than a few SNP sites. Thus, the background for the observed heterosis is presently unidentified.

5. Conclusions

Out of the six investigated traits, predation rate, starvation tolerance and body size (large individuals are expected to consume more food) are considered particularly important for effective

biological control. Although the population differences in this study are not always strong, most of the cases where heterosis was observed (seven out of nine) were found in these three traits suggesting a genetic potential to improve performance of the BCA. Effects of crossing parental lines were generally weak and highly trait- and sex-specific (Table S1). We propose that further research investigating heterosis by environmental interactions are needed in order to optimize the quality of BCAs. Unfortunately, we do not have phenotypic data from a wild population or from hybrids between wild and commercial populations. Due to different levels of genetic variation and selective history, hybrids between wild and commercial populations might show stronger responses than those observed in this study. Whether such crosses might express stronger heterosis or improved biological control efficiency compared to the commercial populations requires further investigations.

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Figure captions

Fig. 1. Trait values for populations of *Orius majusculus* originating from three different companies and the crosses between them. (A) Predation rate (number of eaten *E. kuehniella* eggs per hour), (B) Starvation tolerance (days), (C) Pronotum width (mm), (D) Basal activity (center crossings per minute), (E) Temperature of maximal activity (°C) and (F) Critical Thermal maximum (°C). The boxes show median and 10^{th}, 25^{th}, 75^{th} and 90^{th} percentiles. Within each panel, results for both females and males are shown. The P-values presented in the plots are from a Wilcoxon test testing for difference between the performance of the populations within each sex. Different capital letters indicate significant differences in performance between the populations based on a Wilcoxon each pair test ($\alpha = 0.05$) within each sex. The dotted lines indicate the median across the three parental populations and across the three crosses. An asterisk indicates a significant difference between the median of the parental populations and the cross.

Fig. 2. Nucleotide diversity (π) $\pm$ 99 % confidence interval for each of the three commercial populations and the wild sample.

Tables

Table 1. Overview of all possible crosses between the populations from the three companies including the number of males and females used from each company.

Cross between companies **Females / males**	**Number of individuals used** **Females /males**
BioProduction / Borregaard	20 / 20
Borregaard / BioProduction	26 / 26
BioProduction / Bioplanet	20 / 6
Bioplanet / BioProduction	17 / 17
Borregaard / Bioplanet	25 / 6
Bioplanet / Borregaard	16 / 16

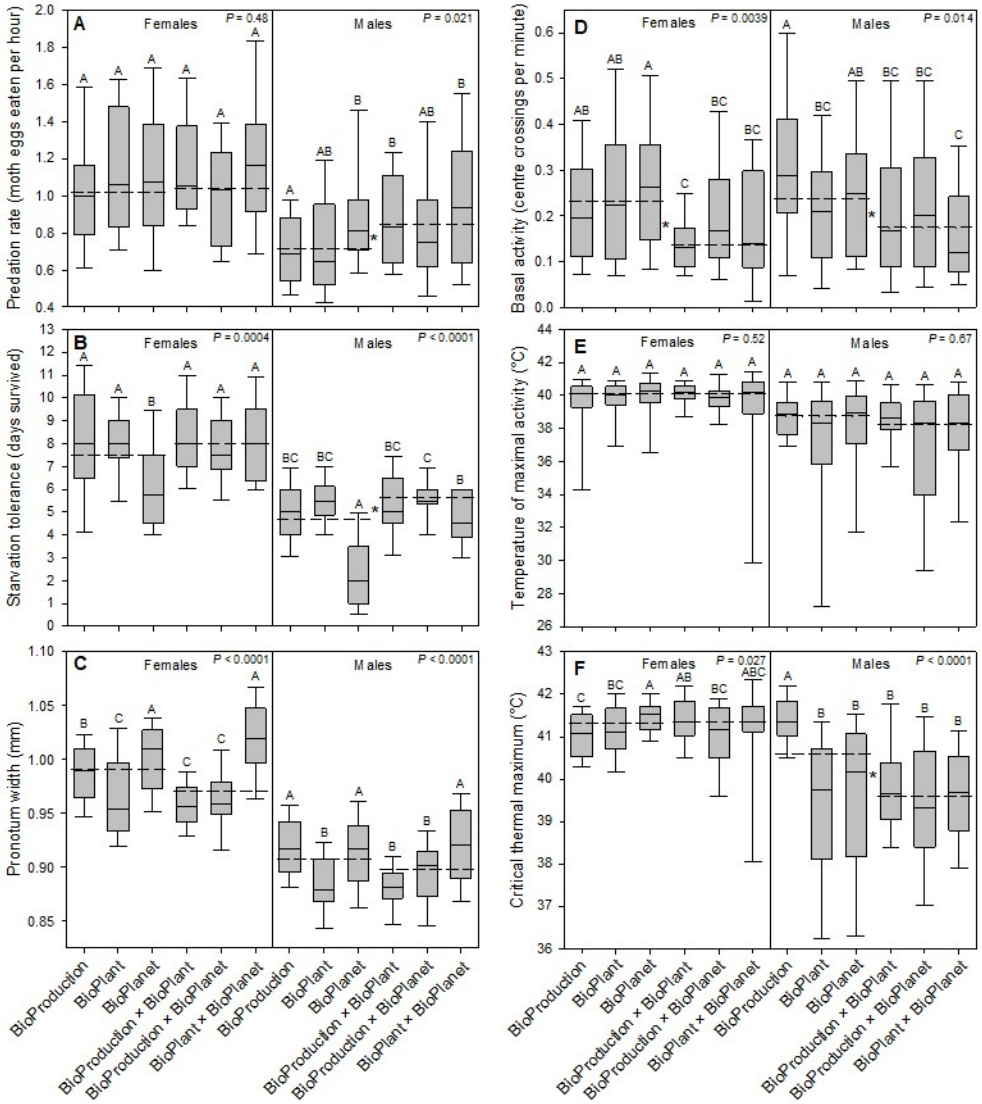
Table 2. Pearson correlation coefficients (Pearson's r) of the nucleotide diversity between the three commercial populations.

	Borregaard	Bioplanet	Wild
BioProduction	0.46	0.47	0.32
Borregaard		0.90	0.27
Bioplanet			0.27

T.N.K., L.B.R., K.J., J.G.S., M.H., and J.O. conceived and designed the experiments, L.B.R., K.J., K.L.N., E.S., and T.N.K. collected and analyzed the data, and all authors contributed to writing the manuscript.

Highlights

- Commercial stocks of biological control agents may be genetically depauperate
- Cross-breeding of pirate bugs might improve the performance of this species
- Highly trait and sex specific effects of crossing were observed
- Hybrid vigor was often observed for consumption, size and starvation resistance
- Commercial stocks had low genetic variation compared to a wild population


A
Females
P = 0.48
Males
P = 0.021
Predation rate (moth eggs eaten per hour)
B
Females
P = 0.0004
Males
P < 0.0001
Starvation tolerance (days survived)
C
Females
P < 0.0001
Males
P < 0.0001
Pronotum width (mm)
D
Females
P = 0.0039
Males
P = 0.014
Basal activity (centre crossings per minute)
E
Females
P = 0.52
Males
P = 0.67
Temperature of maximal activity (°C)
F
Females
P = 0.027
Males
P < 0.0001
Critical thermal maximum (°C)
BioProduction
BioPlant
BioPlanet
BioProduction × BioPlant
BioProduction × BioPlanet
BioPlant × BioPlanet