SPECIAL ISSUE – IMPROVING PEST CONTROL: MASS REARING AND FIELD PERFORMANCE

It is time to bridge the gap between exploring and exploiting: prospects for utilizing intraspecific genetic variation to optimize arthropods for augmentative pest control – a review

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Abstract
Intraspecific genetic variation in arthropods is often studied in the context of evolution and ecology. Such knowledge, however, can also be very usefully applied to biological pest control. Selection of genotypes with optimal trait values may be a powerful tool to develop more effective biocontrol agents. Although it has repeatedly been proposed, this approach is still hardly applied in the current commercial development of arthropod agents for pest control. In this perspective study, we call to take advantage of the increasing knowledge on the genetics underlying intraspecific variation to improve biological control agents. We argue that it is timely now because at present both the need and the technical possibilities for implementation exist, as there is (1) increased economic importance of biocontrol, (2) reduced availability of exotic biocontrol agents due to stricter legislation, and (3) increased availability of genetic information on non-model species. We present a step-by-step approach towards the exploitation of intraspecific genetic variation for biocontrol, outline that knowledge of the underlying genetic mechanisms is essential for success, and indicate how new molecular techniques can facilitate this. Finally, we exemplify this procedure by two case studies, one focussing on a target trait – offspring sex ratio – across species of hymenopteran parasitoids, and the other on a target species – the two-spot ladybird beetle – where wing length and body colouration can be optimized for aphid control. With this overview, we aim to inspire scientific researchers and biocontrol agent producers to start collaborating on the use of genetic variation for the improvement of natural enemies.

Introduction

In the development of new biological control agents, interspecific variation has traditionally been used to select the most effective natural enemy. In other words, different species are compared for their suitability as biological control agents. Another source of variation is intraspecific, but this is hardly assessed in the current practice of augmentative biological pest control when selecting for, or developing, arthropod natural enemies. There is ample evidence of such intraspecific variation for traits important in biological control (Hopper et al., 1993; Lozier et al., 2008; Nachappa et al., 2010; Tabone et al., 2010; Wajnberg, 2010; Wajnberg et al., 2012), which may exist between as well as within populations. In some species, this variation is studied intensively to answer basic questions in ecology and evolution. Knowledge on intraspecific variation could be exploited to optimize the efficacy of existing natural

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enemies, or to make new natural enemies more suitable for application in pest control. Such enhanced suitability may be necessary if the characteristics desired for the application of a species in biological control deviate from the average trait values in nature, for instance, when the climatic conditions of production or release of the biological control agent are different from those organisms that are adapted to in their natural environment (e.g., White et al., 1970). The presence of natural genetic variation in these traits provides the potential to select for lower or higher trait values desired in biocontrol applications. Variation between natural populations may be used to initiate a rearing with individuals from populations with properties closest to the desired ones ('strain selection' or 'strain choice'). In addition, or alternatively, optimization of performance may be reached by selecting genotypes across or within populations that are best suited for biological control ('breeding selection'). Depending on the heritability of a trait (i.e., the proportion of the total variation between individuals that is due to additive genetic variation), prolonged selection over generations potentially shifts the

mean trait value in the cultured population to the value desired for biological control (Figure 1). This response of trait value to selection is described by the 'breeder's equation': $R = h^2S$, relating the change in mean trait value over one generation of selection (R) to the selection differential (S) and the narrow-sense heritability (h^2) (Lush, 1943).

This vintage idea of 'selective breeding' has been widely and successfully applied to breed more productive, tasty, beautiful, or resistant plants, animals, and ornamentals. The selection of strains or isolates is also standard procedure in the development of bacterial biopesticides (Kaushik, 2004; Chandler et al., 2010; Niassy et al., 2012). In contrast, this concept is hardly being used in the mass production of arthropod biological control agents, despite the fact that such 'genetic improvement' has been suggested repeatedly in the past decades (Hoy, 1986, 1990; Hopper et al., 1993; Narang et al., 1993; Nunney, 2003). Various reasons may have hampered this development, including financial, technical, and legal limitations. We state that it is time now to reinvigorate the interest in this approach. Our goal is to stimulate scientists working on
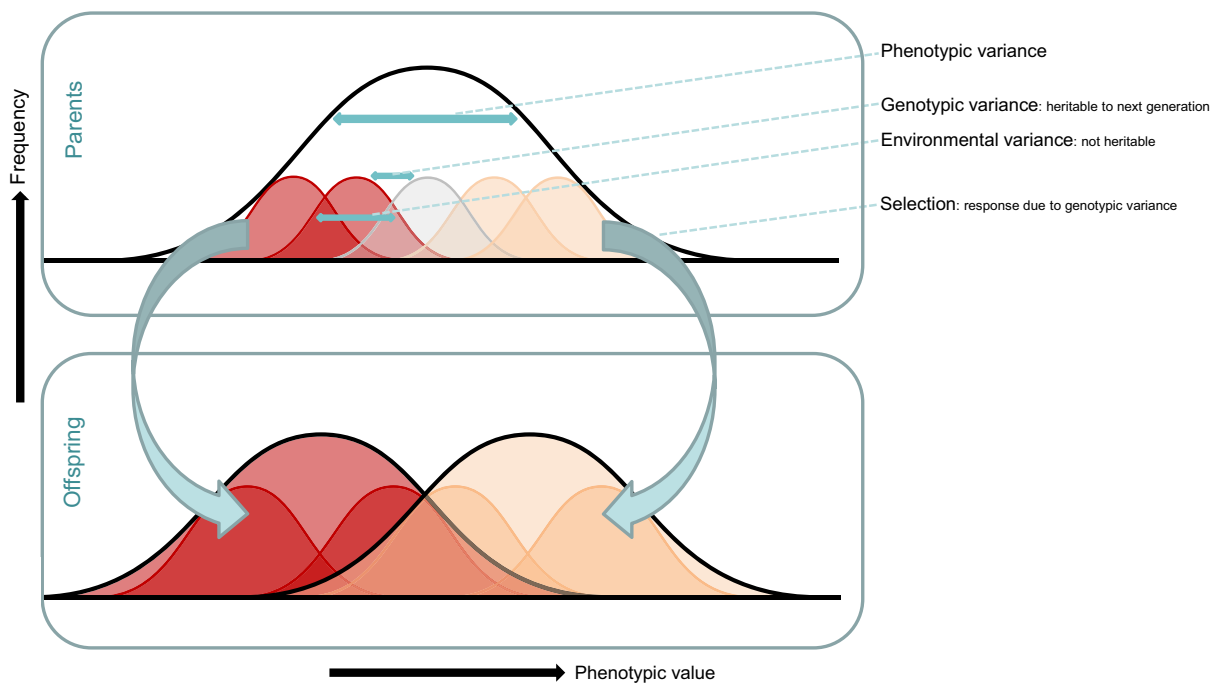

Figure 1 Conceptual diagram of breeding selection illustrating the partitioning of phenotypic variance into genotypic and environmental variance. The top panel indicates the frequency distribution of a hypothetical phenotypic trait in the parental generation (black bell-shaped curve). The population as a whole consists of individual genotypes, represented by the small bell-shaped curves. Each of these genotypes has a different mean phenotypic value and variance. The difference between the means is influenced by genotypic variance, whereas the variance around the means is environmental. The former has a heritable component (additive genetic variance), whereas the latter has not. This is represented by the bottom panel, where the phenotypes with the lowest (in dark) and the highest (in light) phenotypic values in the parental generation have been selected. This downward and upward selected offspring leads to a shift towards lower and higher phenotypic mean values, respectively. This response is due to the selection on the genotypic component of the variance in the parental generation.

fundamental questions regarding intraspecific natural variation in arthropods to apply their knowledge to biocontrol and to inspire producers of biological control agents to seek collaboration to find solutions for the current limits to biocontrol.

Of course, selective breeding is only attractive and economically feasible if no suitable natural enemies are available already. For example, in the 1970s a strain of the parasitoid wasp *Aphytis lignanensis* Compere tolerant to extreme temperatures was developed for release in areas of California (USA) (White et al., 1970). The effectiveness of this strain could never be tested properly because the species *Aphytis melinus* DeBach, which is naturally adapted to such climatic conditions, had already established in the area. White et al. (1970) concluded that selective breeding should not be attempted when other adapted species or strains are available. However, in cases where native natural enemies are suboptimal in controlling a certain pest, selective breeding can be economically feasible as long as the benefits gained from the enhanced phenotype outweigh the costs of the selection and breeding programme.

We limit our perspective to augmentative control, in which natural enemies are mass reared in biofactories for repeated releases in large numbers to obtain an immediate control of pests (van Lenteren, 2012). In contrast, classical biological control programmes encompass the long-term establishment of natural enemies in (agro)ecosystems. Although the methods presented may be used to improve agents for classical biocontrol, the more complex dynamics of natural ecosystems and the evolutionary changes that may take place in the years after release make the targeted improvement of traits in these control agents more challenging. Furthermore, we only consider the exploitation of natural standing genetic (not epigenetic) variation and do not discuss the generation of genetic variation. The latter may be induced by mutagenesis and transgenesis, whose application in biological control has become more feasible technically with the recent development of CRISPR-Cas9 genome editing technologies (Sander & Joung, 2014). However, these approaches are subject to stringent legislation and ecological risks, and are not expected to be applied widely in the short term (Hoy, 2013; Webber et al., 2015).

We first argue why it is currently necessary and feasible to implement this approach in the development and production of mass-reared biological control agents. We then discuss steps involved in the process from exploring to exploiting intraspecific genetic variation for biological control, indicating how recent knowledge and techniques in genetics and genomics can facilitate this. This approach is illustrated using two case studies of biological control agents. As an example of an important biological control

trait for which natural variation is well studied, but only marginally applied, we elaborate on offspring sex ratios in hymenopteran parasitoids. We also illustrate that advanced knowledge of the underlying mechanisms regulating genetic variation in offspring sex ratio is essential to change trait values for practical purposes. Finally, we present the case of the two-spot ladybird beetle, a native biological control agent that has become more important since the ban of its exotic alternative, to illustrate how selection on various traits potentially improve this native species for its performance in biocontrol. This study proposes research avenues for collaborative work on biocontrol agents, rather than providing tailor-made answers for every specific problem.

Timeliness

Rising demand for biological control agents...

Augmentative biological control, and its integration into traditional pest control, has increased in popularity in the fight against arthropod pests in agriculture and has professionalized in the last two decades (van Lenteren, 2012). This is reflected by the growing number of species of natural enemies available on the market, the development of technologies to distribute natural enemies, and the refinement of biological control, for example, by combining natural enemies (van Lenteren, 2003, 2012). This trend is likely to continue because of (1) the growing awareness of undesirable effects of pesticides on human and ecosystem health (Enserink et al., 2013), and the associated more stringent legislation on the use of pesticides; (2) the evolution of pesticide resistance in pest species (Whalon et al., 2011); (3) the emergence of novel pests, by accidental or climate change-associated introduction of exotic pest insects (Gornall et al., 2010); and (4) a positive feedback loop of the use of biological control: when natural enemies are more commonly released against one pest species, chemical control of another pest species may negatively affect the performance of these biological control agents (Hussey & Bravenboer, 1971; van Lenteren, 2012).

...but decreasing availability of species

However, the number of species available for the development of new biological control agents for augmentative release is becoming more and more restricted. As many pests have an exotic origin, and biocontrol agents are sourced from the native area of the pest, traditional biocontrol agents are often also exotic. The recent Convention on Biological Diversity (see www.cbd.int), which has resulted in the Nagoya protocol for Access and Benefit Sharing (Secretariat of the Convention on Biological Diversity, 2011), limits the export of natural enemies for

biological control from many countries that have been a rich source of natural enemies in the past (Cock et al., 2010; van Lenteren et al., 2011). In addition, the United Nations Food and Agriculture Organization guidelines for the export, shipping, import, and release of biological control agents demands a critical evaluation of imported species with regard to the potential risks of releasing exotic natural enemies (IPPC, 2005). This legislation results in increased costs of using exotic natural enemies. Thus, there is an on-going trend towards utilizing more indigenous species for augmentative biological control: since 2000, the indigenous natural enemies introduced to the market outnumbered the exotic ones, reversing the trend of the past century (van Lenteren, 2012).

Improved knowledge and technology

From a scientific perspective, the fields of genetics and genomics are developing rapidly and the costs of associated molecular methods are decreasing accordingly. This development is speeding up the exploration of natural genetic variation in interest, and will also facilitate the implementation of selection on variation in the practice of biological control. From an applied perspective, the increasing market implies the availability of more money and knowledge for the implementation of the required methods. This is reflected in the funding of initiatives such as the Breeding Invertebrates for Next Generation BioControl Training Network (BINGO-ITN, http://www.bingo-itn.eu/en/bingo.htm), in which academia, public partners, and private partners collaborate to improve the production and performance of natural enemies in biological control by the use of genetic variation. However, the current possibilities for industry to apply for intellectual property rights (IPR) to protect insect strains improved by selective breeding are often limited to rearing and application methods, which is an obstacle to industry investment in improving natural enemies (Saenz-de-Cabezon et al., 2010). Similar difficulties regarding IPR on biological material have been solved in the protection of new plant varieties using a system of breeders' rights (International Union for the Protection of New Varieties of Plants, 1962). Developing an analogous insect breeders right system would help to increase industry investment in improved strains and boost the application of genetic techniques in biological control.

How to exploit intraspecific variation

What source material?

Utilizing natural variation to improve biological control is especially feasible for species whose genetics and ecology have been studied extensively (Hoy, 1986), including many parasitoids, predatory mites, and predatory ladybird beetles. Selecting genotypes best suited for biological control requires good characterization of standing intraspecific genetic diversity for the traits of interest (Narang et al., 1993; Wajnberg, 2010) and the presence of adequate genetic variation in the initial rearing culture is of key importance to the success of selective breeding programmes (Johnson & Tabashnik, 1993). Populations from various geographical locations have sometimes been compared for their efficacy in biological control, after which the most effective populations were selected for development as biological control agents (Wajnberg, 2004). Although this approach is useful to select biological control agents that match the climatic conditions where they will be deployed (McDonald, 1976), it ignores the variation in standing genetic variation between populations, limiting the potential for selective breeding. Instead, new cultures for selective breeding should be founded by mixing large numbers of specimens from multiple geographical locations, host species, host plants, or different habitats to maximize genetic variation (McDonald, 1976; Rhodes & Kawecki, 2009). Care should be taken to closely monitor the fitness of newly established rearing cultures to detect problems that could arise due to the disruption of co-adapted gene complexes upon integrating individuals from diverse sources (Mackauer, 1976; Nunney, 2003). Once a culture has established, additional measures are likely needed to limit adaptation to the rearing environment (Sørensen et al., 2012), such as the introduction of extra biological stimuli (e.g., alternative hosts/prey) or the use of abiotic variation (e.g., temperature fluctuations), all aiming to match the selection pressures in the culture to those experienced in the field (Boller, 1972; Hopper et al., 1993; Nunney, 2003).

Which traits to target?

What trait to target for improvement in biocontrol has been one of the major questions in the past and may have hampered the implementation of targeted selective breeding programmes in biocontrol (Hoy, 1986; Hopper et al., 1993; Whitten & Hoy, 1999). For augmentative biological control to be successful, biocontrol agents require efficient mass rearing before release and they must be effective in controlling the pest after release. Optimization will thus target traits related to their quality during production, to their pest control efficacy (resulting in maximum reduction in pest population growth), or to both (Bigler, 1989; van Lenteren & Bigler, 2010). The optimal set of trait values has often been debated in literature (Hoy, 1986; Hopper et al., 1993; Whitten & Hoy, 1999), and will vary according to the biology of the natural enemy and the pest, as well as the agricultural system into which it is released

(crop type, pest species, target environment). To find target traits for selective breeding, the experience of biocontrol producers could be complemented with sensitivity analyses of demographic biocontrol agent–pest models (Godfray & Waage, 1991). Traits commonly featured for optimization are as follows: climatic adaptation, habitat preference, synchrony with hosts, host-searching capacity, specificity, dispersal ability, attack rate, longevity, non-diapause, female fecundity, and offspring sex ratio (Wajnberg, 2004, 2010). For many of these traits, genetic variation has indeed been observed between and within populations of a variety of biological control agents (for reviews, see Hopper et al., 1993; Wajnberg, 2004, 2010), providing scope for selective breeding programmes.

How to analyse the genetic architecture of a target trait

Once target traits for a species have been identified, knowledge of their genetic architecture is essential to design the optimal selection programme that will yield the desired trait values (Narang et al., 1993; Wajnberg, 2010). For example, if only a few loci affect the trait, identification of these will help to select suitable individuals to start breeding from, speeding up the selection process. Further information about interactions between alleles (dominance, epistasis) will help to design efficient crossing schemes. In contrast, if variation in the trait is controlled by multiple genetic loci and environmental conditions, assessing the heritability will allow prediction of the response to selection in a breeding programme (i.e., the effective change in the phenotypic trait value in the next generation, cf. Figure 1; for methods see Falconer & Mackay, 1996; Wajnberg, 2004; Zwaan & Beukeboom, 2005). For a full comprehension of the heritability of a trait, it may be necessary to consider the effects of other heritable factors as well, such as epigenetic effects and endosymbiotic organisms, which may interact with the gene to determine the phenotype (Xi et al., 2008).

Knowledge of the genetic architecture is also needed to determine the scope for selection on a combination of target traits. The most efficient procedure – simultaneous selection, sequential selection, or in parallel followed by crossing – depends on the nature of the relationships among the traits, such as genetic linkage (genes are on the same chromosome), pleiotropy (different traits are influenced by the same genes), and physical and energetic trade-offs, which may hamper simultaneous selection on the combination (Davidowitz et al., 2005).

Identification of the genetic architecture of traits is not a trivial task and involves several molecular and statistical tools, depending on the system that is being studied. A prerequisite is the availability of genetic markers, such as the traditional but laborious microsatellites or amplified

fragment length polymorphisms, or the more modern single nucleotide polymorphisms (SNPs) for the species under study. Current high-throughput sequencing technologies now allow the fast and affordable generation of large amounts of genomic information for any species, facilitating the discovery of such markers (Ellegren, 2014). SNP discovery for non-model species can be even more effective when a pool of individuals is sequenced at the same time (Pool-seq; Futschik & Schlötterer, 2010; Schlötterer et al., 2014). A recent application of this technique to a laboratory population of the fly pupal parasitoid *Nasonia vitripennis* (Walker) yielded more than 400 000 SNPs (van de Zande et al., 2014). These markers are needed to link genomic regions to the phenotypes of interest, using either classical quantitative trait loci mapping (QTL mapping; Lynch & Walsh, 1998), or more advanced genetic mapping methods, such as genome-wide association studies (GWAS; Gondro et al., 2013). Although these linkage analyses involve complex statistical methodologies, they have successfully identified genomic regions associated with many traits (Mackay, 2001; for methods see Liu, 1997; Lynch & Walsh, 1998; de Koning & Haley, 2005). However, care should be taken as QTL and GWAS studies can give an unrealistically simple view of the genetic architecture, which can complicate this step in selective breeding programmes (for critiques see Erickson et al., 2004; Rockman, 2012).

How to select for the desired trait value?

If the genetic architecture of the target trait is known, a suitable method can be chosen to select and breed individuals with the desired trait values. Selection methods include the selection of specific strains from a larger set of strains, artificial selection for a trait value, hybridization of populations/strains, or introgression of a desired trait or heritable element (e.g., endosymbiont) in a different genetic background by targeted crossings and selection of the offspring. Classical breeding techniques, based on the artificial selection of optimal phenotypes, have the potential to greatly improve the performance of biological control agents analogous to the results of animal and plant breeding in other agricultural systems. However, this is a laborious procedure for complex life-history or behavioural traits, which lack easily recordable morphological phenotypes (e.g., life-time fecundity, longevity, egg maturation rates). In such cases, knowledge of the genomic regions underlying the traits can facilitate the screening and selection process. Genetic markers linked to the trait of interest can be used, both in an inventory of the natural variation for these traits among field isolates, and in selecting the individuals used in breeding programmes, i.e., marker-assisted selection (MAS; Ribaut & Hoisington,

1998; Dekkers & Hospital, 2002), potentially saving a lot of time. Genomic selection is an even more advanced way of using genomic data, in which markers covering the whole genome (typically >50 000 markers) help to select the best individuals to breed from, thereby increasing the accuracy of selection (Meuwissen et al., 2001; Goddard & Hayes, 2009). Although this is a promising approach towards more efficient breeding in future, the costs of large-scale genome-wide genotyping are currently still too high to be attractive for biological control producers.

How to maintain genetic variation while selecting?

Both, in the process of selection of individuals to start breeding from and in the maintenance of the obtained selected culture, the loss of genetic variation is a risk. This is inherent to all captive populations (Mackauer, 1976), but there are several ways to reduce loss of genetic diversity, other than that of the target trait. These include starting with a large population, keeping large numbers during breeding, outcrossing events, hybridization of strains, and crossing inbred lines (Wajnberg, 1991; Bartlett, 1993; Hoekstra, 2003; Nunney, 2003). An example of a simple maintenance schedule that maximizes effective population size in parasitoid cultures in the laboratory is given in van de Zande et al. (2014) for the fly pupal parasitoid *N. vitripennis*. By keeping the population separated in multiple vials that were mixed each generation (compartmentalization), the effective population size (N_e) was kept at 236. This exceeds the recommendation to initiate and maintain natural enemy cultures with $N_e>100$ (Roush, 1990; Bartlett, 1993; Nunney, 2003). This compartmentalization can readily be scaled to mass-breeding systems used by biocontrol producers. If available, neutral genetic markers such as microsatellites or SNPs can be used to efficiently monitor genetic variation in natural enemy cultures. Current trends in biological control regarding the quality of biological control agents can further minimize the problem of genetic erosion. Advanced quality control procedures include measuring multiple fitness components of the reared individuals, allowing the swift detection of qualitative flaws (van Lenteren et al., 2003; Leppla, 2003). Genetic erosion resulting in lower fitness would soon be detected and interventions could be undertaken to restore the genetic variation (e.g., by outcrossing).

How to evaluate the success of selection?

Several studies report successful genetic improvement of desired traits in the laboratory, indicating the feasibility of selective breeding (Whitten & Hoy, 1999). Examples include the resistance to chemical pesticides in predatory mites and parasitoid wasps, allowing their use in conjunction with insecticide treatments (Hoy, 1986; Rosenheim &

Hoy, 1988; Johnson & Tabashnik, 1993), drought and temperature tolerance in predatory mites and entomopathogenic nematodes (Hoy, 1985; Shapiro et al., 1997; Strauch et al., 2004; Salame et al., 2010; Anbesse et al., 2012), and more female-biased sex ratios in parasitoids (Hoy & Cave, 1986; Ode & Hardy, 2008). However, the efficacy of the selected strains in biological control was often not further tested in the field or greenhouse (Hoy, 1985). If a trait of interest has successfully been improved in the laboratory, and a population can be maintained in culture, the final step is to test under production and field conditions whether this is indeed translated into improved mass rearing or biological control efficacy. Monitoring the relative performance of improved strains after release has been done using traditional neutral nuclear and mitochondrial markers (Kazmer & Luck, 1995; Hufbauer et al., 2004; Coelho et al., 2016), but new population genomic methods allow for more detailed tracking of the introgression of the genetic material into previously released populations (Stouthamer & Nunney, 2014). Tracking the fate of improved strains and their associated alleles is important to determine the success of selection programmes. Adaptation to laboratory conditions is inherent to captive breeding (Ackermann et al., 2001), and may alter the performance of natural enemies in biological control. Nevertheless, selective breeding of natural enemies has produced strains that have proven to be successful in biological control after release by allowing natural enemies to survive despite insecticide treatments (Hoy, 1986) or by improving the responsiveness of entomopathogenic nematodes to their host insect (Hiltpold et al., 2010), and a few examples of commercially available strains exist, including predatory mites that have lost diapause through artificial selection on this trait stretching the season of their application (van Houten et al., 1995).

Example of a target trait: sex ratio in hymenopteran parasitoids

In this section, we illustrate the use of intraspecific variation in offspring sex ratios in hymenopteran parasitoids following the approach outlined above. Hymenopteran parasitoids have a haplodiploid sex determination system (females are diploid and males are haploid) which gives females full control over the sex of their offspring by fertilizing an egg or not (Crozier, 1971; Cook & Crozier, 1995; Cook, 2002). This phenomenon is widely studied in an evolutionary ecology context. In biological control programmes, the sex of parasitoids is of key importance, as only adult females will locate and parasitize the pest hosts. However, optimizing the sex ratio of parasitoids will not only improve their effectiveness when they are released as

biological control agents, it will also improve the mass-rearing process. The production of large numbers of female parasitoids is particularly important for augmentative biological control programmes that release large numbers of mass-reared natural enemies to control insect pest populations (Ode & Hardy, 2008). Managing and controlling the sex ratio of parasitoids towards female-biased sex ratios can reduce the costs of mass production in commercial insectaries. For example, in the egg parasitoid *Gonatocerus ashmeadi* Girault that attacks the glassy-winged sharpshooter, production costs could be reduced by two-thirds when sex ratio was modified in favour of the number of females (Irvin & Hoddle, 2006). For a plastic trait such as sex ratio, this modification can also be done by altering the rearing conditions. However, in contrast to a genetically anchored modification, such a condition-dependent modification will be lost upon release, reducing its effectiveness in biocontrol practice. In principle, several genetic approaches are available to produce more female-biased sex ratios when mass-rearing parasitoids for augmentative biological control, which are discussed below.

Artificial selection

Genetic variation in sex ratio adjustment of females has been found in several parasitoid species – examples are *N. vitripennis* (Parker & Orzack, 1985; Orzack & Parker, 1986, 1990; Pannebakker et al., 2008, 2011), *Muscidifurax raptor* Girault & Sanders (Antolin, 1992), *Heterospilus prosopidis* Viereck (Kobayashi et al., 2003), *Uscana semifumipennis* Girault (Henter, 2004), *Trichogramma* spp. (Wajnberg, 1993; Guzmán-Larralde et al., 2014), and *Asobara tabida* (Nees) (Kraaijeveld & van Alphen, 1995). The presence of genetic variation for sex ratio makes this good source material for artificial selection on female-biased sex ratios. This has been done repeatedly, but such selection has yielded mixed results. In one of the earliest reports, Wilkes (1947) managed to reduce the number of females that exclusively produced male offspring from 36 to 2% after 8–10 generations of selective breeding in a culture of *Microplectron fuscipennis* Zetterstedt, a pupal parasitoid of sawflies. Simmonds (1947) reported a similar increase in the proportion of females after only a few generations of selective breeding of the larval parasitoid *Aenoplex carpocapsae* Cushman, and Parker & Orzack (1985) successfully altered the sex ratio of the fly pupal parasitoid *N. vitripennis* in 13–15 generations. In contrast, Ram & Sharma (1977) failed to alter the sex ratio of the egg parasitoid *Trichogramma fasciatum* (Perkins) in strains previously selected for increased fecundity for 16 generations. This may well be explained by pleiotropic effects of the genes coding for fecundity on genes involved in sex ratio, as was observed in *N. vitripennis* when the genetic

architecture was determined by QTL analysis (Pannebakker et al., 2008, 2011). Prolonged selection for increased fecundity could have depleted the additive genetic variation for sex ratio, preventing the intended simultaneous optimization of both traits in a single strain. This illustrates: (1) the need to start selective breeding programmes with rearing cultures containing sufficient genetic variation for the trait of interest (Johnson & Tabashnik, 1993); (2) the need to use a culturing scheme that maintains genetic variation (Nunney, 2003; van de Zande et al., 2014); and (3) the importance of knowledge on interactions between the genetic mechanisms involved.

Using sex ratio distorters

An alternative genetic approach to produce more female-biased sex ratios is the utilization of natural sex ratio distorters that lead to a female-biased sex ratio (Stouthamer, 1993), i.e., a form of strain choice/selection. The endosymbiotic bacterium *Wolbachia* spec. is the best studied sex ratio distorter in parasitoid wasps and can manipulate the sex allocation pattern of the wasps in several ways. The most drastic sex ratio alteration by *Wolbachia* is parthenogenesis induction (PI), which results in all-female offspring (Stouthamer et al., 1990). PI-*Wolbachia* are restricted to hosts with haplodiploid modes of reproduction (Stouthamer & Huigens, 2003), in which infected virgin females produce all-female offspring through gamete duplication (Stouthamer & Kazmer, 1994; Gottlieb et al., 2002; Pannebakker et al., 2004), resulting in the production of fully homozygous offspring (Suomalainen et al., 1987). Biological control programmes can obtain lines with sex ratio distorters either by selecting lines from the field that carry sex ratio distorters or by artificially transferring sex ratio distorters into preferred uninfected sexual parasitoid lines (Huigens et al., 2000, 2004a; Tagami et al., 2001). Both intraspecific and interspecific *Wolbachia* transfection resulted in stable infections for multiple generations (Huigens et al., 2004a; Zabalou et al., 2004).

Infection with PI-*Wolbachia* will increase the relative female production of infected lines, providing a clear advantage to biological control programmes. However, the potential fitness effects of *Wolbachia* infections are not consistent across species and should be considered in each case in practice (Russell & Stouthamer, 2010). Often, infection with PI-*Wolbachia* results in a fitness cost to the infected female parasitoid (Stouthamer & Luck, 1993; Huigens et al., 2004b). For example, females from infected *Trichogramma cordubensis* Vargas & Cabello and *Trichogramma deion* Pinto & Oatman egg parasitoids have a lower fecundity and dispersal ability in the laboratory. In the greenhouse, however, infected females parasitized more eggs than uninfected females, despite the fitness cost

of the infection (Silva et al., 2000). Interestingly, transfected lines of the egg parasitoid *Trichogramma kaykai* Pinto & Stouthamer varied significantly in fitness. Whereas most lines showed a decrease in fitness, several lines showed an increase in all fitness parameters (Russell & Stouthamer, 2010), which would make these lines exceptionally suitable for efficient mass production.

In addition to an increased number of pest controlling females in the population, infection with PI-*Wolbachia* offers the possibility of advanced genotypic selection (Russell & Stouthamer, 2010). Because PI-*Wolbachia*-infected eggs will undergo gamete duplication, fully homozygous females mated to males of a different genotype will produce identical heterozygous *Wolbachia*-infected F1 daughters. If unmated, recombination in these daughters will produce F2 daughters that are homozygous for an unlimited number of unique genotypes. This allows selection of beneficial gene combinations in parasitoids for biological control within two generations (Stouthamer, 2003; Russell & Stouthamer, 2010). This promising technique is limited to those PI-*Wolbachia*-infected wasps that still mate successfully, which include a range of *Trichogramma* species.

Maintaining female-biased laboratory populations

The genetic mechanism of sex determination has a direct influence on the sex ratio produced by a female parasitoid. In a number of parasitoids, sex is determined by the allelic complementation at a single genetic locus (single-locus complementary sex determination or sl-CSD). Unfertilized eggs always develop into males (hemizygous at the *csd* sex determination locus), whereas fertilized eggs develop into females when the *csd* locus is heterozygous, and into diploid males when homozygous (Cook, 1993b; Beukeboom & Perrin, 2014). The diploid males are often sterile or unviable, and constitute a considerable fitness cost (Cook & Crozier, 1995; Zayed, 2004; Zayed & Packer, 2005). In biological control programmes, mass culturing of parasitoids with CSD can lead to the loss of genetic diversity at this sex locus, which leads to an increase in the proportion of males produced in that culture (Ode & Hardy, 2008; West, 2009). Several studies have indeed reported male-biased laboratory cultures (Platner & Oatman, 1972; Rappaport & Page, 1985; Smith et al., 1990; Grinberg & Wallner, 1991; Johns & Whitehouse, 2004). This problem can be reduced by maintaining parasitoid cultures at large population sizes to minimize the rate at which diversity at the *csd* locus is lost (Stouthamer et al., 1992). Another approach is to maintain parasitoid cultures as a large number of subpopulations. While diversity at the sex locus will be reduced in each subpopulation, genetic diversity will be retained over the total parasitoid culture (Stouthamer et al., 1992; Cook, 1993a; Nunney, 2003; van de Zande et al., 2014), thus allowing the producer to maintain a viable proportion of females in the culture.

Example of a target species: the two-spot ladybird beetle

Predatory ladybirds are among the main natural enemies of aphids including many important pest species of horticultural and ornamental crops. The use of ladybirds for augmentative control is currently not very popular, due to the expensive mass rearing and the variable efficacy in biocontrol. However, attempts are ongoing to improve ladybirds for biological control of aphids. Research in the past decade has provided scope for improved mass rearing by using cheaper artificial food (De Clercq et al., 2005; Jalali et al., 2009), and by altering the rearing environment (Sørensen et al., 2013). Successful control, however, is thought to be constrained by the tendency of the adult beetles to often fly away from the host plants without returning (Gurney & Hussey, 1970; Hämäläinen, 1977; Lommen et al., 2008). Indeed, the creation of flightless strains of the Asian *Harmonia axyridis* (Pallas) through selective breeding (Ferran et al., 1998; Seko & Miura, 2013) has overcome this problem. However, the recent ban on the use of the exotic *H. axyridis* in Europe, leaves Europe to use native species instead, of which *Adalia bipunctata* (L.) is the most popular in biocontrol (van Lenteren, 2012).

There are ample opportunities to improve this species as a biocontrol agent by our suggested approach: much is known about its biology, covering its ecology, population dynamics, behavioural, and physiological traits (overviews in Hodek, 1973; Majerus, 1994; Dixon, 2000; Hodek et al., 2012), and the underlying genetics of several traits relevant to biocontrol has been well studied. Below we describe how selecting on genetic variation in two traits of *A. bipunctata* – wing length and body colouration – could enhance the performance of this native species in biological control.

Variation in wing length

There is a growing body of evidence that limiting the flight ability of ladybirds prolongs their residence time on aphid-infested host plants and can thus enhance biological control efficacy compared to conspecific winged ladybirds (Ignoffo et al., 1977; Ferran et al., 1998; Tourniaire et al., 1999; Weissenberger et al., 1999; Seko et al., 2008, 2014; Iguchi et al., 2012). Therefore, the trait targeted for breeding selection was reduced flight ability. Interestingly, some wild populations of *A. bipunctata* exhibit wing dimorphism, with 'wingless' morphs occurring rarely (Majerus

& Kearns, 1989; Marples et al., 1993). In such individuals, both the elytra and the flight wings are truncated, impairing the flight ability. Thanks to early classical breeding experiments on this trait, it is known that this trait has a simple genetic architecture: it is regulated by a recessive allele at a single locus (Marples et al., 1993; Ueno et al., 2004). Wingless individuals possess two copies of this wingless allele (homozygote recessives). Using this knowledge, winglessness can rapidly be fixed in laboratory populations. Individuals possessing the recessive allele can be used as source material for a selective breeding programme focusing on this trait. As the naturally occurring wingless morphs are rare, however, and heterozygous individuals cannot visually be distinguished from wild types, field-collected wingless individuals were first crossed with a large number (hundreds) of wild-collected wild-type conspecifics to construct a breeding stock harbouring sufficient genetic variation to prevent loss of fitness through inbreeding effects. Within three generations a pure-breeding wingless population of individuals was indeed generated (Lommen, 2013).

Evaluating the success of the selected stock, a greenhouse study proved an increased residence time of wingless ladybirds on single pepper plants, compared to winged conspecifics. Because the feeding behaviour was not altered by the wingless trait, this resulted in better control of *Myzus persicae* (Sulzer) aphids (Lommen et al., 2008). Releasing the wingless stock on lime trees in an open, urban environment indicated that this strain reduced the amount of honeydew from lime aphids, *Eucallipterus tiliae* (L.), underneath the infested trees (Lommen et al., 2013). Together, these preliminary experiments indicate that the selection of genetically wingless beetles is a promising direction to enhance the efficacy of biological control by *A. bipunctata*.

Another requirement for the cost-effective use of wingless *A. bipunctata* is the feasibility of economic mass rearing. Although handling flightless ladybirds is much easier than handling those capable of flight and saves costs of labour, producers of natural enemies have raised concerns about the reduced fitness of wingless *A. bipunctata* (J van Schelt, Koppert Biological Systems, pers. comm.). In contrast to the parasitoid sex ratio example, the enhanced biological control efficacy achieved by selectively breeding for impaired flight, does not align with increased mass-rearing efficiency. Instead, Ueno et al. (2004) reported that wingless morphs of *A. bipunctata* have a longer development time, a reduced life span, and a lower life-time reproduction compared to their winged conspecifics. Lommen (2013) recently showed, however, that artificial selection of more favourable genetic backgrounds from the standing natural genetic variation in such wingless strains could improve mass rearing. Laboratory stocks of the wingless phenotype display large variation in the extent of wing reduction: although all individuals are genetically 'wingless' and have the same genotype with two recessive alleles for winglessness, there is a continuous range from individuals lacking all wing tissue to those only missing the tip of the wings, while all phenotypes lack the ability to fly. Interestingly, this variation correlates with variation in several fitness traits, with individuals missing less wing tissue performing better (Ueno et al., 2004; Lommen, 2013). To investigate the potential to select such well-performing 'wingless' phenotypes with small reductions in wing length, the genetic architecture of the variation was elucidated using classical quantitative genetics studies. It appears to be regulated by at least two additional unknown genetic loci, but the phenotype is the result of interactions between these genes and the environment (Lommen et al., 2005; Lommen, 2013). This is reflected in the heritability of wing length (as determined by parent–offspring regression), which is higher ($h^2 = 0.64$) at a rearing temperature of 19 °C than at 29 °C ($h^2 = 0.29$; Lommen, 2013). Four generations of artificial selection within the 'wingless' stock on only slight wing reduction at 21 °C yielded 'wingless' stocks in which the majority of beetles had only tiny reductions. Indeed, these showed a higher survival and reproduction than lines selected for large reductions in wings. Moreover, 'wingless' females mated more successfully when they have less severe wing reductions (Lommen, 2013). 'Wingless' lines selected for slight reductions in their wings may not only improve the mass rearing of 'wingless' *A. bipunctata*, but also may additionally further improve aphid control because of an increased adult longevity.

In short, we see ample opportunity to exploit the intraspecific natural variation in wing length of *A. bipunctata* to improve its performance as a biological control agent, both in its suitability for mass rearing and with respect to its control efficacy. The most promising option for commercialization would be to develop a 'wingless' strain consisting of beetles with only slight wing truncations. This process would encompass the two levels of selection discussed above. First, the qualitative 'wingless' trait should be fixed in a laboratory culture of *A. bipunctata*. This only requires a single copy of the 'wingless' allele (which has, up to now, been kept in culture), and three generations of rearing. Subsequently, this 'wingless' laboratory stock should be selected for quantitative expression of the trait to obtain the desired phenotype with minimal wing reduction by selection over several generations. As the trait has an obvious and visible phenotype, no molecular marker is needed to keep track on the presence of the trait. To prevent detrimental inbreeding effects during the

selection process, the initial numbers of individuals used to introgress the 'wingless' locus into should be large. The obtained laboratory cultures should then be kept large enough, or regularly outcrossed to freshly sampled wild types, to maintain genetic variation in traits other than the 'wingless' trait (Wajnberg, 1991; Bartlett, 1993; Nunney, 2003).

Variation in body colouration

Variation in wing length of *A. bipunctata* is a potentially rich source to improve biocontrol by *A. bipunctata*. This is, however, a unique case of a rare mutation in some populations that appears to be beneficial for biological control, but does not seem adaptive in natural populations (Lommen, 2013). In contrast, there are many other traits interesting for biological control in *A. bipunctata* that exhibit large adaptive variation in natural populations and with a known genetic basis. Colour polymorphism is such a trait that has been studied extensively, but has not been employed to optimize biocontrol. Within natural populations, genetically distinct morphs have different amounts of melanization of their dorsal body parts, resulting in the co-existence of dark (melanized) and red (non-melanized) morphs (Dobzhansky, 1924, 1933; Lusis, 1961; Majerus, 1994, 1998), which can serve as source material for a selective breeding stock. The trait appears to be under natural selection by climatic factors, with different colour forms having different relative fitness in different areas, resulting in different frequencies of occurrence (Muggleton, 1978; Majerus, 1994; Brakefield & de Jong, 2011). Because the darker individuals (melanics) absorb solar radiation more effectively than the lighter ones (non-melanics) (Lusis, 1961), the former reach higher body temperatures and higher activity levels in colder climates (except in windy conditions where heat is quickly lost) (de Jong et al., 1996), leading to higher aphid consumption rates and better aphid control. Colour polymorphism is entirely under genetic control, and the genetic architecture seems to involve a major locus with a series of alleles, with those corresponding to melanic colourization more dominant (Majerus & Zakharov, 2000). Therefore, only a few generations of selection on colour are needed to obtain separate pure-breeding melanic and non-melanic lines, and again the selection success can directly be inferred from the visible phenotype, hence not requiring molecular markers.

As climatic factors influence and limit the activity of natural enemies, they influence the efficacy of pest control (Jalali et al., 2010). By releasing colour morphs of *A. bipunctata* that maximize activity levels under the local climatic circumstances, biological control may be optimized. In, for example, a greenhouse with ambient temperature below the optimum temperature for activity of *A. bipunctata*, but with abundant light, melanic ladybird beetles may provide more efficient aphid control than non-melanics. On the other hand, in a windy outdoor environment, the non-melanics may be more effective (de Jong et al., 1996).

Optimizing the activity levels of biocontrol agents through selective breeding of specific body colours can be applied to a wider range of natural enemies. Variation in body melanization is common in insects and generally has a large genetic component (True, 2003; Wittkopp & Beldade, 2009; van 't Hof & Saccheri, 2010; Ramniwas et al., 2013). Interestingly, genetically based variation in body colouration has recently been reported for parasitoids as well, where it also indeed leads to variation in activity levels (Abe et al., 2013).

Combining traits and environmental conditions

We have described how selection on intraspecific genetic variation in two traits (wing length and body colouration) can produce lines with desired traits to improve control by *A. bipunctata*. To optimize biological control, combinations of these traits could easily be made according to the latest insights in the underlying genetics: winglessness and melanism turn out to be only weakly linked genetically (Lommen et al., 2012), which allows simultaneous selection on both traits. However, given the importance of gene–environment interactions in this species, breeding conditions should be chosen carefully. In addition, a proper cost–benefit analysis should be made early in the project to assess the commercial potential for wingless *A. bipunctata* in augmentative biological control. This involves a comparison of selected and non-selected strains of the same origin and age under practical rearing and application conditions.

Conclusion

In this study, we have made a case for the exploitation of natural intraspecific genetic variation to optimize and refine the use of natural enemies in augmentative biological control of arthropod pests. We have argued that now is the right time to do so, because of: (1) an increase in the use of augmentative biological pest control; (2) the reduced availability of biological control agents for augmentation due to stricter legislation; and (3) the increased availability of genetic information on non-model species (as illustrated in the sex ratio case study). Exploiting intraspecific natural variation for the optimization of natural enemies for augmentative release is expected to meet with much fewer ethical and legislative issues than the use of transgenics, imported exotic natural enemies, or chemical insecticides. It also complies with the current insights

in sustainability of pest control. Therefore, we feel that this approach deserves more attention than has been given so far. We have attempted to sketch the implementation of selective breeding in a specific example of the ladybird to illustrate the potential and limitations of this approach.

To develop a proof-of-concept showing that a genetic improvement strategy is widely applicable in large-scale practice situations, a joint effort between scientists and practitioners is urgently needed. In parallel, scientists should focus on (1) gaining in-depth knowledge of the genetic diversity within populations relevant to biological control (Wajnberg, 2004); (2) the estimation of genetic parameters for haplodiploid species (Liu & Smith, 2000; Brascamp & Bijma, 2014); and (3) identify traits that can be measured easily in the laboratory, which can be predictive of field efficacy after release. Ultimately, using intraspecific natural variation to optimize biological control agents will reduce the reliance of augmentative biological control on the importation of non-native natural enemies. It will help to reduce the environmental risks associated with this practice, and the dependency on other countries for the acquisition of genetic resources.

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