1 **Time to bridge the gap between exploring and exploiting: prospects for utilizing**

2 **intraspecific genetic variation to optimise arthropods for augmentative pest control**

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20

21 **Short title**

22 Using genetic variation to improve biocontrol agents

23 **Abstract**

24 Intraspecific genetic variation in arthropods is often studied in the context of evolution and

25 ecology. Such knowledge, however, can also be very usefully applied for biological pest

26 control. Selection of genotypes with optimal trait values may be a powerful tool to develop

27 more effective biocontrol agents. Although it has repeatedly been proposed in the past, this

28 approach is currently still hardly applied in the commercial development of arthropod agents

29 for pest control. In this perspective paper, we call to take advantage of the increasing

30 knowledge on the genetics underlying intraspecific variation to improve biological control

31 agents. We first argue that it is timely now, because at present both the need and technical

32 possibilities for implementation exist, there is an: (1) increased economic importance of

33 biocontrol; (2) reduced availability of exotic biocontrol agents due to stricter legislation; and

34 (3) increased availability of genetic information on non-model species. We then present a

35 step-by-step approach towards the exploitation of intraspecific genetic variation for

36 biocontrol, outline that knowledge of the underlying genetic mechanisms is essential for

37 success, and indicate how new molecular techniques can facilitate this. Finally, we exemplify

38 this procedure by two case studies, one focussing on a target trait, offspring sex ratio, across

39 different species of hymenopteran parasitoids, and the other on a target species, the two-spot

40 ladybird beetle, where wing length and body colouration can be optimized for aphid control.

41 With this overview, we aim to inspire scientific researchers and biocontrol agent producers to

42 start collaborating on the use of genetic variation for the improvement of natural enemies.

43 **Keywords**

46

47 **Introduction**

48   In the development of new biological control agents, the *variation between species*, or

49   interspecific variation, has traditionally been used to select the most effective natural enemy.

50   In other words, different species are compared for their suitability as biological control

51   agents. Another source of variation is that *within species*, or intraspecific variation, but this is

52   hardly assessed in the current practice of augmentative biological pest control when selecting

53   for, or developing, arthropod natural enemies. There is ample evidence of such intraspecific

54   variation for traits important in biological control (Hopper et al., 1993; Lozier et al., 2008;

55   Wajnberg, 2010; Tabone et al., 2010; Nachappa et al., 2010; Wajnberg et al., 2012) which

56   may exist *between populations*, as well as *within populations*. In some species, this variation

57   is studied intensively to answer basic questions in ecology and evolution. Knowledge on

58   intraspecific variation could well be exploited to optimise the efficacy of existing natural

59   enemies, or to make new natural enemies more suitable for application in biological control.

60   This may be necessary when the characteristics desired for the application of a species in

61   biological control deviate from the average trait values in nature, for instance when the

62   climatic conditions of production or release of the biological control agent are different from

63   those that the organisms adapted to in their natural environment (e.g. White et al., 1970).

64   However, the presence of natural genetic variation in these traits provides the potential to

65   select for lower or higher trait values desired in biocontrol applications. The variation *between*

66   natural populations can be used to initiate the rearing with individuals from those populations

67   with properties closest to the desired ones ('strain selection or -choice'). In addition, or

68   alternatively, optimization of the performance can be reached by selecting those genotypes

69   across or *within* populations that are best suitable for biological control ('breeding selection').

70   Depending on the heritability of a trait (the proportion of the total variation between

71   individuals that is due to additive genetic variation, see Figure 1), prolonged selection over

3

72 generations can potentially shift the mean trait value in the cultured population to the value

73 desired for biological control (Figure 1). This response of trait value to selection is described

74 by the "breeder's equation" (Lush, 1943): $R=h^2S$, relating the change in mean trait value over

75 one generation of selection (R) to the selection differential (S) and the narrow sense

76 heritability (h^2).

77 This vintage idea of 'selective breeding' has been widely and successfully applied to breed

78 edible plants, animals, and ornamentals that are more productive, tasty, beautiful, or resistant.

79 The selection of strains or isolates is also a standard and crucial procedure in the development

80 of bacterial biopesticides (overviews in Kaushik, 2004; Chandler et al., 2010; recent examples

81 in Niassy et al., 2012). In contrast, this concept is hardly being used in the mass-production of

82 arthropod biological control agents (Hoy, 1990), despite the fact that is has been repeatedly

83 suggested to apply such 'genetic improvement' in the past decades (Hoy, 1986; Hopper et al.,

84 1993; Narang et al., 1993; Nunney, 2003). Several reasons might have hampered this

85 development, including financial, technical and legal limitations.

86 We state that it is currently time to reinvigorate the interest in this approach. We would like to

87 stimulate scientists working on fundamental questions regarding intraspecific natural

88 variation in arthropods to apply their knowledge for biocontrol and to inspire producers of

89 biological control agents to seek collaboration with such scientists to find solutions for the

90 current limits to biocontrol. Of course, selective breeding is only attractive and economically

91 feasible if no suitable natural enemies are available already. For example, in the 1970s a strain

92 of the parasitoid wasp *Aphitis lignanensis* tolerant to extreme temperatures was developed for

93 release in areas of California with such climate (White et al., 1970). The effectiveness of this

94 strain could never be properly tested because the species *Aphitis melinus*, which is naturally

95 adapted to such climatic conditions had already established in the area. White et al. (1970)

96 concluded that selective breeding should not be attempted when other adapted species or

97   strains are available. However, in cases where native natural enemies are suboptimal in

98   controlling a certain pest, selective breeding is can be economically feasible as long as the

99   benefits gained from the enhanced phenotype outweigh the costs of the selection- and

100   breeding programme.

101   We limit our perspective to the augmentative control, in which natural enemies are mass-

102   reared in biofactories for repeated releases in large numbers to obtain an immediate control of

103   pests (Van Lenteren, 2012). In contrast, classical biological control programs encompass the

104   long-term establishment of natural enemies in (agro)ecosystems. Although the methods

105   presented may be used to improve agents for classical biocontrol, the more complex dynamics

106   of natural ecosystems, and the evolutionary changes that may take place in the years after

107   release, make the targeted improvement of traits in these biological control agents more

108   challenging. Furthermore, we only consider the *exploitation of natural standing genetic*

109   *variation* (not epigenetic), and do not discuss the *generation of genetic variation*. The latter

110   may be induced by mutagenesis and transgenesis, whose application in biological control

111   recently has become technically more feasible with the development of CRISPR-Cas9

112   genome editing technologies (Sander & Joung, 2014). However, these approaches are subject

113   to stringent legislation and ecological risks, and are not expected to be applied widely in the

114   short term (Hoy, 2013; Webber et al., 2015).

115   We will first argue why it is currently necessary and feasible to implement this approach in

116   the development and production of mass-reared biological control agents. We then discuss

117   steps involved in the process from exploring to exploiting intraspecific genetic variation for

118   biological control, indicating how recent knowledge and techniques in genetics and genomics

119   can facilitate this. This approach is illustrated using two case studies of biological control

120   agents. As an example of an important biological control trait for which natural variation is

121   well studied, but only marginally applied, we then elaborate on offspring sex ratios in

122   hymenopteran parasitoids. We also use this topic to illustrate that advanced knowledge of the

123   underlying mechanisms regulating genetic variation is essential to successfully change trait

124   values for practical purposes. We finally present a case of an existing native biological control

125   agent, which has become more important since the ban of its exotic alternative, to illustrate

126   how selection on different traits can potentially improve this native species for its

127   performance in biocontrol. Hence, this paper will propose research avenues for collaborative

128   work on biocontrol agents, rather than providing tailor-made answers for every specific

129   problem.

130

Timeliness

131

A rising demand for biological control agents…

132

133   Augmentative biological control, and the integration of this method into traditional pest

134   control, has increased in popularity in the fight against arthropod pests in agriculture and has

135   professionalised in the last two decades (Van Lenteren, 2012). This is reflected by the

136   growing number of species of natural enemies available on the market, the development of

137   technologies to distribute natural enemies, and the refinement of biological control, for

138   example by combining different natural enemies (Van Lenteren, 2003, 2012). This trend is

139   likely to continue, because of (1) the growing awareness of undesirable effects on human- and

140   ecosystem health of pesticides (Enserink et al., 2013), and the associated more stringent

141   legislation on the use of these pesticides, (2) the evolution of pesticide-resistance in pest

142   species (Whalon et al., 2011), (3) the emergence of novel pests, by accidental or climate-

143   change associated introduction of exotic pest insects (Gornall et al., 2010) and (4) a positive

144   feedback loop of the use of biological control: when natural enemies are more commonly

145   released against one pest species, chemical control of another pest species may negatively

146 affect the performance of these biological control agents (Hussey & Bravenboer, 1971; Van

147 Lenteren, 2012).

148

149 *...but decreasing availability of species*

150 However, the number of species available for the development of new biological control

151 agents for augmentative release is becoming more and more restricted. Since many pests have

152 an exotic origin, and biocontrol agents are sourced from the native area of the pest, traditional

153 biocontrol agents are also often exotic. The recent Convention on Biological Diversity (see

154 www.cbd.int), which has resulted in the Nagoya protocol for Access and Benefit Sharing

155 (Secretariat of the Convention on Biological Diversity, 2011), limits the export of natural

156 enemies for biological control from many countries that have been a rich source of natural

157 enemies in the past (Cock et al., 2010; Van Lenteren et al., 2011). In addition, the United

158 Nations Food and Agriculture Organization guidelines for the export, shipping, import and

159 release of biological control agents demands a critical evaluation of imported species with

160 regard to the potential risks of releasing exotic natural enemies (IPPC, 2005). This legislation

161 results in increased costs of using exotic natural enemies. As a result, there is an on-going

162 trend towards utilizing more indigenous species for augmentative biological control: this

163 century, the number of indigenous natural enemies introduced to the market outnumbered the

164 exotic ones, reversing the trend of the past century (Van Lenteren, 2012).

165

166 *Improved knowledge and technology*

167 From a scientific perspective, the fields of genetics and genomics are developing rapidly, and

168 the costs of associated molecular methods are decreasing accordingly. This development is

169   speeding up the exploration of natural genetic variation of interest, and will also facilitate the

170   implementation of selection on this variation in the practice of biological control. From an

171   applied perspective, with an increased market, there is currently more money and knowledge

172   for the implementation of the required methods. This is reflected in the funding of initiatives

173   such as the Breeding Invertebrates for Next Generation BioControl Training Network

174   (BINGO-ITN, http://www.bingo-itn.eu/en/bingo.htm), in which academia, public and private

175   partners collaborate to improve the production and performance of natural enemies in

176   biological control by the use of genetic variation for rearing, monitoring and performance.

177   However, the current possibilities for industry to apply for intellectual property rights (IPR) to

178   protect insect strains improved by selective breeding are often limited to rearing and

179   application methods, which is an obstacle to industry investment in improving natural

180   enemies (Saenz-de-Cabezon et al., 2010). Similar difficulties regarding IPR on biological

181   material have been solved in the protection of new plant varieties using a system of breeders

182   rights (Plants, 1962). Developing an analogous insect breeders right system would help to

183   increase industry investment in improved strains and boost the application of genetic

184   techniques in biological control.

185

186   **How to exploit intraspecific variation**

187   *What source material?*

188   Utilizing natural variation to improve biological control is especially feasible for species

189   whose genetics and ecology have been extensively studied (Hoy, 1986), including many

190   predatory mites, parasitoids, and predatory ladybird beetles. Selecting genotypes best suited

191   for biological control requires a good characterization of standing intraspecific genetic

192   diversity for the traits of interest (Narang et al., 1993; Wajnberg, 2010) and the presence of

193 adequate genetic variation in the initial rearing culture is of key importance to the success of

194 selective breeding programmes (Johnson & Tabashnik, 1993). In that light, populations from

195 different geographical locations have sometimes been compared for their efficacy in

196 biological control, after which the most effective populations were selected for development

197 as biological control agents (Wajnberg, 2004).   While this approach is useful to select

198 biological control agents that match the climatic conditions where they will be deployed

199 (McDonald, 1976), it ignores the variation in standing genetic variation between populations,

200 limiting the potential for selective breeding. Instead, new cultures for selective breeding

201 should be founded by mixing large numbers of specimens from multiple geographical

202 locations, host species, host plants, or different habitats to maximise genetic variation

203 ((McDonald, 1976; Rhodes & Kawecki, 2009). Care should be taken to closely monitor the

204 fitness of newly established rearing cultures, to detect problems that could arise due to the

205 disruption of co-adapted gene complexes upon integrating individuals from diverse sources

206 (Mackauer, 1976; Nunney, 2003). Once a culture has established, additional measures are

207 likely needed to limit adaptation to the rearing environment (Sørensen et al., 2012). Several

208 authors have suggested methods to prevent this adaptation, such as the introduction of extra

209 biological stimuli (e.g. alternative hosts/prey) or the use of abiotic variation (e.g. temperature

210 fluctuations), all aiming to match the selection pressures in the culture to those experienced in

211 the field (Boller, 1972; Hopper et al., 1993; Nunney, 2003).

212

Which traits to target?

214 What trait to target for improvement in biocontrol has been one of the major questions in the

215 past (Hoy, 1986; Hopper et al., 1993; Whitten & Hoy, 1999) and may have hampered the

216 implementation of targeted selective breeding programs in biocontrol. To be successful for

217 augmentative biological control, biological control agents require efficient mass-rearing

218 before release, and should also be effective in controlling the pest species after release.

219 Optimisation will thus target traits related to their quality during production, to their pest-

220 control efficacy (resulting in a maximum reduction of pest population growth), or to both (but

221 sometimes there is a conflict of interest) (Bigler, 1989; Van Lenteren & Bigler, 2010). The

222 optimal set of trait values has often been debated in literature (e.g. Hoy 1986; Hopper et al.

223 1993; Whitten & Hoy 1999), and will vary according to 1) the biology of the natural enemy;

224 2) the biology of the pest; and 3) the agricultural system into which it is released (crop type,

225 pest species, target environment). To find target traits for selective breeding, the experience of

226 biocontrol producers could be complemented with sensitivity analyses of demographic

227 biocontrol agent-pest models (Godfray & Waage, 1991). Traits commonly featured for

228 optimisation are: climatic adaptation, habitat preference, synchrony with hosts, host-searching

229 capacity, specificity, dispersal ability, attack rate, longevity, non-diapause, female fecundity

230 and offspring sex ratio (Wajnberg, 2004, 2010). For many of these traits, genetic variation has

231 indeed been observed between and within populations for several biological control agents

232 (for reviews see Hopper et al. 1993; Wajnberg, 2004, 2010), providing scope for selective

233 breeding programs.

234

235 *How to analyse the genetic architecture of a target trait*

236 Once the target trait(s) for a species have been identified, knowledge of their genetic

237 architecture is essential to design the optimal selection programme that will yield the desired

238 trait values (Narang et al., 1993; Wajnberg, 2010). For example, when only a few loci affect

239 the trait, identification of these will help to select suitable individuals to start breeding from,

240 speeding up the selection process. Further information about interactions between alleles

241 (dominance, epistasis), will help to design efficient crossing schemes. In contrast, when

242 variation in the trait is controlled by multiple genetic loci and environmental conditions,

243 assessing the heritability will allow prediction of the response to selection in a breeding

244 program (i.e. the effective change in the phenotypic trait value in the next generation, see

245 Figure 1, for methods see: Falconer & Mackay, 1996; Wajnberg, 2004; Zwaan &

246 Beukeboom, 2005). For a full comprehension of the heritability of a trait, it could be

247 necessary to consider the effects of other heritable factors as well, such as epigenetic effects

248 and endosymbiotic organisms, which may interact with the gene to determine the phenotype

249 (e.g. Xie et al., 2008).

250 Knowledge on the genetic architecture is also needed to determine the scope for the selection

251 on a combination of target traits. The most efficient procedure (simultaneous selection,

252 sequential selection, or in parallel followed by crossing) depends on the nature of the

253 relationships between the traits, such as genetic linkage (genes are on the same chromosome),

254 pleiotropy (different traits are influenced by the same genes), and physical and energetic

255 trade-offs, which may hamper simultaneous selection on the combination (Davidowitz et al.,

256 2005).

257 Identification of the genetic architecture of traits is not a trivial task and involves several

258 different molecular and statistical tools, depending on the system that is being studied. A

259 prerequisite is the availability of genetic markers, such as the traditional but laborious

260 microsatellites or Amplified Fragment Length Polymorphisms (AFLPs) or the more modern

261 single nucleotide polymorphisms (SNPs) for the species under study. Current high-throughput

262 sequencing technologies now allow the fast and affordable generation of large amounts of

263 genomic information for any species (Ellegren, 2014), facilitating the discovery of such

264 markers. SNP discovery for non-model species can be even more effective when a pool of

265 individuals is sequenced at the same time (Pool-seq; Futschik & Schlötterer, 2010; Schlötterer

266   et al., 2014). A recent application of this technique to a laboratory population of the fly pupal

267   parasitoid *Nasonia vitripennis*, yielded more than 400,000 SNPs (van de Zande et al., 2014).

268   These markers are needed to link genomic regions to the phenotypes of interest, using either

269   classical quantitative trait loci mapping (QTL mapping, e.g. Lynch & Walsh, 1998), or more

270   advanced genetic mapping methods, such as Genome-Wide Association Studies (GWAS, e.g.

271   Gondro et al., 2013). While these linkage analyses involve complex statistic methodlogies,

272   they have sucessfully identified genomic regions associated with many traits (Mackay 2001;

273   for methods see Liu (1997), Lynch & Walsh, (1998), de Koning & Haley (2005). However,

274   care should be taken as QTL and GWAS studies can give an unrealistically simple view of the

275   genetic architecture (for critiques see Erickson et al. (2004) and Rockman (2012)), which can

276   complicate this step in selective breeding programs.

277

278   *How to select for the desired trait value?*

279   When the genetic architecture of the target trait is known, a suitable method can be chosen to

280   select and breed individuals with the desired trait values. Selection methods include the

281   selection of specific strains from a larger set of strains, artificial selection for a trait value,

282   hybridization of populations/strains, or introgression of a the desired trait or heritable element

283   (e.g. endosymbiont) in a different genetic background by targeted crossings and selection of

284   the offspring. Classical breeding techniques, based on the artificial selection of the most

285   optimal phenotypes, have the potential to greatly improve the performance of biological

286   control agents analogous to the results of animal and plant breeding in other agricultural

287   systems. However, this is a laborious procedure for complex life-history or behavioural traits,

288   which lack easily recordable morphological phenotypes (i.e. life-time fecundity, longevity,

289   egg maturation rates). In such cases, knowledge of the genomic regions underlying the traits

290   can facilitate the screening and selection process. Genetic markers linked to the trait of

291   interest can be used, both in an inventory of the natural variation for these traits among field

292   isolates, and in selecting the individuals used in breeding programs, i.e. marker-assisted

293   selection (MAS, Ribaut & Hoisington, 1998; Dekkers & Hospital, 2002) potentially saving a

294   lot of time. Genomic selection is an even more advanced way of using genomic data, in which

295   markers covering the whole genome (typically >50,000 markers) help to select the best

296   individuals to breed from (Meuwissen et al., 2001; Goddard & Hayes, 2009), thereby

297   increasing the accuracy of selection. Although this is a promising approach towards more

298   efficient breeding in future, the costs of large scale genome-wide genotyping are currently

299   still too high to be attractive for biological control producers.

300

301   *How to maintain genetic variation while selecting?*

302   Both in the process of the selection of individuals to start breeding from and in the

303   maintenance of the obtained selected culture, the loss of genetic variation is a risk. This is

304   inherent to all captive populations (Mackauer, 1976), but there are several ways to reduce loss

305   of genetic diversity, other than that of the target trait. These include starting with a large

306   population, keeping large numbers during breeding, outcrossing events, hybridization of

307   strains, and crossing inbred lines (Wajnberg, 1991; Bartlett, 1993; Hoekstra, 2003; Nunney,

308   2003). An example of a simple maintenance schedule that maximizes effective population

309   size in parasitoid cultures in the laboratory is given in Van de Zande et al. (2014) for the fly

310   pupal parasitoid *Nasonia vitripennis*. By keeping the population separated in multiple vials

311   that were mixed each generation (compartmentalization), the effective population size (N_e)

312   was kept at 236. This exceeds the recommendation to initiate and maintain natural enemy

313   cultures with an effective population size of $N_e>100$ (Roush, 1990; Bartlett, 1993; Nunney,

314   2003). This compartmentalization can readily be scaled to mass-breeding systems used by

315   biocontrol producers. When available, neutral genetic markers, such as microsatellites or

316   SNPs can be used to efficiently monitor genetic variation in natural enemy cultures. Current

317   trends in biological control regarding the quality of biological control agents can further

318   minimize the problem of genetic erosion. Advanced quality control procedures include

319   measuring multiple fitness components of the reared individuals, allowing the swift detection

320   of qualitative flaws (Leppla, 2003; Van Lenteren et al., 2003). When genetic erosion results in

321   lower fitness, this would soon be detected and interventions could be undertaken to restore the

322   genetic variation (e.g. by outcrossing).

323

324   *How to evaluate the success of selection?*

325   Several studies indeed report successful genetic improvement of desired traits in the

326   laboratory, indicating the feasibility of selective breeding (Whitten & Hoy, 1999). Examples

327   include the resistance to chemical pesticides in predatory mites and parasitoid wasps,

328   allowing their use in conjunction with insecticide treatments (Hoy, 1986; Rosenheim & Hoy,

329   1988; Johnson & Tabashnik, 1993), drought and temperature tolerance in predatory mites and

330   entomopathogenic nematodes (Hoy, 1985; Shapiro et al., 1997; Strauch et al., 2004; Salame et

331   al., 2010; Anbesse et al., 2012), and more female-biased sex ratios in parasitoids (Hoy &

332   Cave, 1986; Ode & Hardy, 2008). However, the efficacy of the selected strains in biological

333   control was then often not further tested in the field or greenhouse (Hoy, 1985). When a trait

334   of interest has successfully been improved in the laboratory, and a population can be

335   maintained in culture, the final step is to test under production- and field conditions whether

336   this is indeed translated into improved mass-rearing or biological control efficacy. Monitoring

337   the relative performance of improved strains after release has been done using traditional

338   neutral nuclear and mitochondrial markers (e.g. Kazmer & Luck, 1995; Hufbauer et al., 2004;

339   Coelho et al., 2016), but new population genomic methods allow for more detailed tracking of

340   the introgression of the genetic material into previously released populations (Stouthamer &

341   Nunney, 2014). Tracking the fate of improved strains and their associated alleles is important

342   to determine the success of selection programmes. Adaptation to laboratory conditions is

343   inherent to the captive breeding (Ackermann et al., 2001), and may alter the performance of

344   the natural enemies in biological control. Nevertheless, selective breeding of natural enemies

345   has produced strains that have proven to be successful in biological control after release by

346   allowing natural enemies to survive despite insecticide treatments (Hoy, 1986) or by

347   improving the responsiveness of entomopathogenic nematodes to their host insect (Hiltpold et

348   al., 2010), and a few examples of commercially available strains exist, including predatory

349   mites that have lost diapause through artificial selection on this trait stretching the season of

350   their application (Van Houten et al., 1995).

351

Example of a target trait: sex ratio in Hymenopteran parasitoids

353   In this section, we will illustrate the use of intraspecific variation in offspring sex ratios in

354   Hymenopteran parasitoids following the approach outlined above. Hymenopteran parasitoids

355   have a haplodiploid sex determination system (females are diploid and males are haploid)

356   which gives females full control over the sex of their offspring by fertilizing an egg or not

357   (Crozier, 1971; Cook & Crozier, 1995; Cook, 2002). This phenomenon is widely studied in an

358   evolutionary ecological context. In biological control programs, the sex of parasitoids is of

359   key importance, as only adult females will locate and parasitize the pest hosts. However,

360   optimizing the sex ratio of parasitoids will not only improve their efficiency when they are

361   released as biological control agents, it will also improve the mass-rearing process. The

15

362   production of large numbers of female parasitoids is particularly important for augmentative

363   biological control programs that release large numbers of mass-reared natural enemies to

364   control insect pest populations (Ode & Hardy, 2008). Managing and controlling the sex ratio

365   of parasitoids in augmentative biological control towards female-biased sex ratios can reduce

366   the costs of mass production in commercial insectaries. For example, in the egg parasitoid

367   *Gonatocerus ashmeadi* that attacks the glassy-winged sharpshooter, production costs could be

368   reduced by two-thirds when sex ratio was modified in favour of the number of females (Irvin

369   & Hoddle, 2006). For a plastic trait such as sex ratio, this modification can also be done by

370   altering the rearing conditions. However, in contrast to a genetically anchored modification,

371   such a condition-dependent modification will be lost upon release, reducing its effectiveness

372   in biocontrol practice. In principle, several genetic approaches are available to produce more

373   female-biased sex ratios when mass-rearing parasitoids for augmentative biological control,

374   which will be discussed below.

375

376   *Artificial selection*

377   Genetic variation in sex ratio adjustment of females has been found in several parasitoid

378   species (e.g. *N. vitripennis* Parker & Orzack, 1985; Orzack & Parker, 1986, 1990;

379   Pannebakker et al., 2008, 2011); *Muscidifurax raptor* (Antolin, 1992); *Heterospilus*

380   *prosopidis* (Kobayashi et al., 2003); *Uscana semifumipennis* (Henter, 2004); *Trichogramma*

381   spp. (Wajnberg, 1993; Guzmán-Larralde et al., 2014); *Asobara tabida* (Kraaijeveld & Alphen,

382   1995)). The presence of genetic variation for sex ratio makes this good source material, for

383   artificial selection on female-biased sex ratios. This has been done repeatedly, but such

384   selection has yielded mixed results. In one of the earliest reports, Wilkes (1947) managed to

385   reduce the number of females that exclusively produced male offspring from 36% to 2% after

16

386   8-10 generations of selective breeding in a culture of *Microplectron fuscipennis*, a pupal

387   parasitoid of sawflies. Simmonds (1947) reported a similar increase in the proportion of

388   females after only a few generations of selective breeding of the larval parasitoid *Aenoplex*

389   *carpocapsae*, and Parker & Orzack (1985) successfully altered the sex ratio of the fly pupal

390   parasitoid *N. vitripennis* in 13-15 generations. In contrast, Ram & Sharma (1977) failed to

391   alter the sex ratio of the egg parasitoid *Trichogramma fasciatum* in strains previously selected

392   for increased fecundity for 16 generations. This may well be explained by pleiotropic effects

393   of the genes coding for fecundity on genes involved in sex ratio, as was observed in *N.*

394   *vitripennis* when the genetic architecture was determined by QTL analysis (Pannebakker et

395   al., 2008, 2011). Prolonged selection for increased fecundity could have depleted the additive

396   genetic variation for sex ratio, preventing the intended simultaneous optimization of both

397   traits in a single strain. This illustrates the need to: (1) start selective breeding programs with

398   rearing cultures containing sufficient genetic variation for the trait of interest (Johnson &

399   Tabashnik 1993); (2) use a culturing scheme that maintains genetic variation (Nunney, 2003;

400   van de Zande et al., 2014), and (3) the importance of knowledge on interactions between the

401   genetic mechanisms involved.

402

403   *Using sex ratio distorters*

404   An alternative genetic approach to produce more female-biased sex ratios is the utilization of

405   natural sex ratio distorters that lead to a female-biased sex ratio (Stouthamer, 1993), i.e. a

406   form of strain choice/-selection. The endosymbiotic bacteria *Wolbachia* is the best studied sex

407   ratio distorter in parasitoid wasps and can manipulate the sex allocation pattern of the wasps

408   in several ways. The most drastic sex ratio alteration by *Wolbachia* is parthenogenesis

409   induction (PI), which results in all-female offspring (Stouthamer et al., 1990). PI-*Wolbachia*

410   are restricted to hosts with haplodiploid modes of reproduction (Stouthamer & Huigens,

411   2003), in which infected virgin females produce all-female offspring through gamete

412   duplication (Stouthamer & Kazmer, 1994; Gottlieb et al., 2002; Pannebakker et al., 2004),

413   resulting in the production of fully homozygous offspring (Suomalainen et al., 1987).

414   Biological control programs can obtain lines with sex ratio distorters either by selecting lines

415   from the field that carry sex ratio distorters or by artificially transferring sex ratio distorters

416   into preferred uninfected sexual parasitoid lines (Huigens et al., 2000; Tagami et al., 2001;

417   Huigens, de Almeida, et al., 2004). Both intraspecific and interspecific *Wolbachia*

418   transfection have already resulted in stable infections for multiple generations (Huigens, de

419   Almeida, et al., 2004; Zabalou et al., 2004).

420   Infection with PI-*Wolbachia* will increase the relative female production of infected lines,

421   providing a clear advantage to biological control programs. However, the potential fitness

422   effects of *Wolbachia* infections are not consistent across species and should be considered in

423   each case in practice (Russell & Stouthamer, 2010). Often, infection with PI-*Wolbachia*

424   results in a fitness costs to the infected female parasitoid (Stouthamer & Luck, 1993; Huigens,

425   Hohmann, et al., 2004). For example, females from infected *Trichogramma cordubensis* and

426   *T. deion* egg parasitoids have a lower fecundity and dispersal ability in the laboratory. In the

427   greenhouse, however, infected females parasitized more eggs than uninfected females, despite

428   the fitness cost of the infection (Silva et al., 2000). Interestingly, transfected lines of the egg

429   parasitoid *Trichogramma kaykai* varied significantly in fitness. While most lines showed a

430   decrease in fitness, several lines showed an increase in all fitness parameters (Russell &

431   Stouthamer, 2010), which would be exceptionally suitable for efficient mass-production.

432   In addition to an increased number of pest-controlling females in the population, infection

433   with PI-*Wolbachia* offers the possibility of advanced genotypic selection (Russell &

434   Stouthamer, 2010). Because PI-*Wolbachia* infected eggs will undergo gamete duplication,

435   fully homozygous females mated to males of a different genotype, will produce identical

436   heterozygous, *Wolbachia*-infected F1 daughters. If unmated, recombination in these daughters

437   will produce F2 daughters that are homozygous for an unlimited number of unique genotypes.

438   This allows selection of beneficial gene combinations in parasitoids for biological control

439   within two generations (Stouthamer, 2003; Russell & Stouthamer, 2010). This promising

440   technique is limited to those PI-*Wolbachia* infected wasps that still mate successfully, which

441   include a range of *Trichogramma* species.

442

443   *Maintaining female-biased laboratory populations*

444   The genetic mechanism of sex determination has a direct influence on the sex ratio produced

445   by a female parasitoid. In a number of parasitoids, sex is determined by the allelic

446   complementation at a single genetic locus (single locus Complementary Sex Determination or

447   sl-CSD). Unfertilized eggs always develop into males (hemizygous at the csd sex

448   determination locus), while fertilized eggs develop into females when the csd locus is

449   heterozygous, and into diploid males when homozygous (Cook, 1993b; Beukeboom & Perrin,

450   2014). The diploid males are often sterile or unviable, and constitute a considerable fitness

451   cost (Cook & Crozier, 1995; Zayed, 2004; Zayed & Packer, 2005). In biological control

452   programs, mass culturing of parasitoids with CSD can lead to the loss of genetic diversity at

453   this sex locus, which leads to an increase in the proportion of males produced in that culture

454   (Ode & Hardy, 2008; West, 2009). Several studies have indeed reported male biased

455   laboratory cultures (Platner & Oatman, 1972; Rappaport & Page, 1985; Smith et al., 1990;

456   Grinberg & Wallner, 1991; Johns & Whitehouse, 2004). This problem can be reduced by

457   maintaining parasitoid cultures at large population sizes to minimize the rate at which

458   diversity at the csd locus is lost (Stouthamer et al., 1992). Another approach is to maintain

459   parasitoid cultures as a large number of subpopulations. While diversity at the sex locus will

460   be reduced in each subpopulation, genetic diversity will be retained over the total parasitoid

461   culture (Stouthamer et al., 1992; Cook, 1993a; Nunney, 2003; van de Zande et al., 2014), thus

462   allowing the producer to maintain a viable proportion of females in the culture.

463

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

465   Predatory ladybirds are among the main natural enemies of aphids including many important

466   pest species of horticultural and ornamental crops. The use of ladybirds for augmentative

467   control is currently not very popular, due to the expensive mass-rearing and the variable

468   efficacy in biocontrol. However, attempts are ongoing to improve ladybirds for biological

469   control of aphids. Research in the past decade has provided scope for improved mass-rearing

470   by using cheaper artificial food (De Clercq et al., 2005; Jalali et al., 2009), and by altering the

471   rearing environment (Sørensen et al., 2013). Successful control, however, is thought to be

472   constrained by the tendency of the adult beetles to often fly away from the host plants without

473   returning (e.g. Gurney & Hussey, 1970; Hämäläinen, 1977; Lommen et al., 2008). Indeed, the

474   creation of flightless strains of the Asian *Harmonia axyridis* through selective breeding

475   (Ferran et al., 1998; Seko & Miura, 2013) has overcome this problem. However, the recent

476   ban on the use of the exotic *H. axyridis* in Europe, leaves Europe to use native species instead,

477   of which *Adalia bipunctata* is the most popular in biocontrol (Van Lenteren, 2012).

478   There are ample opportunities to improve this species as a biocontrol agent by our suggested

479   approach: there is excellent knowledge about its biology, covering its ecology, population

480   dynamics, behavioural and physiological traits (overviews in e.g. Hodek, 1973; Majerus,

481   1994; Dixon, 2000; Hodek et al., 2012), and the underlying genetics of several traits relevant

482   to biocontrol has been well studied. Below we will describe how selecting on genetic

483   variation in two traits of *A. bipunctata*, wing length and body colouration, could enhance the

484   performance of this native species in biological control.

485

Variation in wing length

487   There is a growing body of evidence that limiting the flight ability of ladybirds prolongs their

488   residence time on aphid-infested host plants and can thus enhance biological control efficacy

489   compared to conspecific winged controls (Ignoffo et al., 1977; Ferran et al., 1998; Tourniaire

490   et al., 1999; Weissenberger et al., 1999; Seko et al., 2008, 2014; Iguchi et al., 2012).

491   Therefore, the trait targeted for breeding selection was reduced flight ability. Interestingly,

492   some wild populations of *A. bipunctata* exhibit wing dimorphism, with "wingless" morphs

493   occurring rarely (Majerus & Kearns, 1989; Marples et al., 1993). In such individuals, both the

494   elytra and the flight wings are truncated, impairing the flight ability. Thanks to early classical

495   breeding experiments on this trait, it is known that this trait has a simple genetic architecture:

496   it is regulated by a recessive allele at a single locus (Marples et al., 1993; Ueno et al., 2004).

497   Wingless indiviuals possess two copies of this wingless allele (homozygote recessives). Using

498   this knowledge, winglessness can rapidly be fixed in laboratory populations. Individuals

499   possessing the recessive allele can be used as source material for a selective breeding program

500   focusing on this trait. Since the naturally occurring wingless morphs are rare, however, and

501   heterozygous individuals cannot visually be distinguished from wild types, field collected

502   wingless individuals were first crossed with a large number (hundreds) of wild collected

503   wildtype conspecifics to construct a breeding stock harbouring sufficient genetic variation to

504   prevent loss of fitness through inbreeding effects. Within three generations a pure-breeding

505   wingless population of individuals was indeed generated.

506   Evaluating the success of the selected stock, a greenhouse study proved an increased

507   residence time of wingless ladybirds on single pepper plants, compared to winged

508   conspecifics. Because the feeding behaviour was not altered by the wingless trait, this resulted

509   in better control of *Myzus persicae* aphids (Lommen et al., 2008). Releasing the wingless

510   stock on lime trees in an open, urban environment showed that this strain reduced the amount

511   of honeydew from lime aphids underneath the infested trees (Lommen et al., 2013). Together,

512   these preliminary experiments indicate that the selection of genetically wingless beetles

513   appears to be a promising direction to enhance the efficacy of biological control by *A.*

514   *bipunctata*.

515   Another requirement for the cost-effective use of wingless *A. bipunctata* is the feasibility of

516   economic mass-rearing. Although handling flightless ladybirds is much easier than those

517   capable of flight and saves costs of labour, producers of natural enemies have raised concerns

518   about the reduced fitness of wingless *A. bipunctata* (J. van Schelt, personal communication).

519   In contrast to the parasitoid sex ratio example described above, the enhanced biological

520   control efficacy achieved by selectively breeding for impaired flight, does not align with an

521   increased mass rearing efficiency. Instead, Ueno et al. (2004) indicated that wingless morphs

522   of *A. bipunctata* have a longer development time, a reduced life span, and a lower life-time

523   reproduction compared to their winged conspecifics. Lommen (2013) recently showed,

524   however, that artificial selection of more favourable genetic backgrounds from the standing

525   natural genetic variation in such wingless strains could improve mass-rearing. Laboratory

526   stocks of the wingless phenotype show large variation in the extent of wing reduction: though

527   all individuals are genetically 'wingless' and have the same genotype with two recessive

528   alleles for winglessness, there is a continuous range from individuals lacking all wing tissue

529   to those only missing the tip of the wings. Interestingly, this variation correlates with variation

530   in several fitness traits, with individuals missing less wing tissue performing better (Ueno et

531   al., 2004; Lommen, 2013). To investigate the potential to select such well-performing

532   wingless phenotypes with small reductions in wing length, the genetic architecture of the

533   variation was elucidated using classical quantitative genetics studies. It appears to be

534   regulated by at least two additional unknown genetic loci, but the phenotype is the result of

535   interactions between these genes and the environment (Lommen et al., 2005; Lommen, 2013).

536   This is reflected in the heritability (as determined by parent-offspring regression) of wing

537   length which is higher (h^2=0.64) at a rearing temperature of 19°C than at 29°C (h^2=0.29,

538   Lommen, 2013). Four generations of artificial selection within the wingless stock on only

539   slight wing reduction at 21°C yielded wingless stocks in which the majority of beetles had

540   only tiny reductions. Indeed, these showed a higher survival and reproduction than lines

541   oppositely selected for large reductions in wings. Moreover, wingless females mated more

542   successfully when they have less severe wing reductions (Lommen, 2013). Wingless lines

543   selected for slight reductions in their wings may not only improve the mass-rearing of

544   wingless *A. bipunctata*, but may additionally further improve aphid control, because of an

545   increased adult longevity.

546   In short, we see ample opportunity to exploit the intraspecific natural variation in wing length

547   of *A. bipunctata* to improve its performance as a biological control agent, both in its

548   suitability for mass-rearing and with respect to its control efficacy. The most promising option

549   for commercialization would be to develop a "wingless" strain consisting of beetles with only

550   slight wing truncations. This process would encompass the two levels of selection discussed

551   above: first, the qualitative wingless trait should be fixed in a laboratory culture of *A.*

552   *bipunctata*. This only requires a single copy of the wingless allele (which has, up to now,

553   been kept in culture), and three generations of rearing. Subsequently, this wingless laboratory

554   stock should be selected for quantitative expression of the trait to obtain the desired

555   phenotype with minimal wing reduction by selection over several generations. Since the trait

556   has an obvious and visible phenotype, no molecular marker is needed to keep track on the

557   presence of the trait. To prevent detrimental inbreeding effects during the selection process,

558   the numbers of individuals initially used to introgress the wingless locus into should be large.

559   The obtained laboratory cultures should then be kept large enough, or regularly outcrossed to

560   freshly sampled wild types, to maintain genetic variation in traits other than the wingless trait

561   (Wajnberg, 1991; Bartlett, 1993; Nunney, 2003).

562

563   *Variation in body colouration*

564   Variation in wing length of *A. bipunctata* is a potentially a rich source to improve biocontrol

565   by *A. bipunctata*. This is, however, a unique case of a rare mutation in some populations that

566   appears to be beneficial for biological control, but does not seem adaptive in natural

567   populations (Lommen, 2013). In contrast, there are many other traits in *A. bipunctata* that

568   exhibit large adaptive variation in natural populations in traits interesting for biological

569   control of which the genetic basis is well known. Colour polymorphism is such a trait that has

570   been studied extended, but has not been employed to optimise biocontrol. Within natural

571   populations, genetically distinct morphs have different amounts of melanisation of their dorsal

572   body parts, resulting in the coexistence of dark (melanised) and red (non-melanised) morphs

573   (Dobzhansky, 1924, 1933; Lusis, 1961; Majerus, 1994, 1998), which can serve as source

574   material for a selective breeding stock. The trait appears to be under natural selection by

575   climatic factors, with different colour forms having different relative fitness in different areas,

576   resulting in different frequencies of occurrence (Muggleton, 1978; Majerus, 1994; Brakefield

577   & de Jong, 2011). Because the darker coloured individuals (melanics) absorb solar radiation

578   more effectively than the lighter ones (non-melanics) (Lusis, 1961), the former reach higher

579   body temperatures and activities in colder climates (except in windy conditions where heat is

24

580　quickly lost) (de Jong et al., 1996), and, associated with this higher activity, have higher aphid

581　consumption rates, leading to better aphid control. Colour polymorphism is entirely under

582　genetic control, and the genetic architecture seems to involve a major locus with a series of

583　alleles, with those corresponding to melanic colourisation more dominant (Majerus &

584　Zakharov, 2000). Therefore, only a few generations of selection on colour are needed to

585　obtain separate pure-breeding melanic and non-melanic lines, and again the selection success

586　can directly be inferred from the visible phenotype, hence not requiring molecular markers.

587　Since climatic factors influence and limit the activity of natural enemies, they influence the

588　efficacy of pest control (Jalali et al., 2010). By releasing colour morphs of *A. bipunctata* that

589　maximise activity levels under the local climatic circumstances, biological control may be

590　optimized. In, for example, a greenhouse with an ambient temperature below the optimum

591　temperature for activity of *A. bipunctata*, but with abundant light, melanic ladybird beetles

592　may provide more efficient aphid control than non-melanics. On the other hand, in a windy

593　outdoor environment, the non-melanics may be more effective (de Jong et al., 1996).

594　Optimizing the activity levels of biocontrol agents through selective breeding of specific body

595　colours can be applied to a wider range of natural enemies. Variation in body melanisation is

596　common in insects and generally has a large genetic component (see e.g. True, 2003;

597　Wittkopp & Beldade, 2009; Van 't Hof & Saccheri, 2010; Ramniwas et al., 2013).

598　Interestingly, this has recently also been reported for parasitoids, where it indeed leads to

599　variation in levels of activity (Abe et al., 2013).

600

601　*Combining traits and environmental conditions*

602　We have described how selection on intraspecific genetic variation in two different traits

603　(wing length and body colouration) can produce lines with desired traits to improve biological

604   control by *A. bipunctata*. To optimise biological control, combinations of these traits could

605   easily be made according to the latest insights in the underlying genetics: winglessness and

606   melanism turn out to be only weakly genetically linked (Lommen et al., 2012), which allows

607   simultaneous selection on both traits. However, given the importance of gene-environment

608   interactions in this species, breeding conditions should be carefully chosen. In addition, a

609   proper cost-benefit analysis should be made early in the project to assess of the commercial

610   potential for wingless *A. bipunctata* in augmentative biological control. This involves a

611   comparison of selected and non-selected strains with the same origin and age under practical

612   rearing and application conditions.

613

614   **Conclusion**

615   In this paper, we have made a case for the exploitation of natural intraspecific genetic

616   variation to optimise and refine the use of natural enemies in augmentative biological control

617   of arthropod pests. We have argued that now is the right time to do so, because of: (1) an

618   increase in the use of augmentative biological pest control; (2) the reduced availability of

619   biological control agents for augmentation due to stricter legislation; and (3) the increased

620   availability of genetic information on non-model species (as illustrated in the sex-ratio case

621   study). Exploiting intraspecific natural variation for the optimization of natural enemies for

622   augmentative release is expected to meet with much fewer ethical and legislative issues than

623   the use of transgenics, imported exotic natural enemies or chemical insecticides. It also

624   complies with the current insights in sustainability of pest control. Therefore, we feel that this

625   approach deserves more attention than has been given to it so far. We have attempted to

626   sketch the implementation of selective breeding in a specific example of the ladybird to

627   illustrate the potential and limitations of this approach.

628 To develop a proof-of-concept showing that a genetic improvement strategy is widely

629 applicable in large-scale practice situations, a joint effort between scientists and practitioners

630 is urgently needed. In parallel, scientists should focus on (1) gaining in depth knowledge of

631 the genetic diversity within populations relevant to biological control (Wajnberg, 2004); (2)

632 the estimation of genetic parameters for haplodiploid species (Liu & Smith, 2000; Brascamp

633 & Bijma, 2014); and (3) identify traits that can be measured easily in the laboratory, which

634 can be predictive of field efficiency after release. Ultimately, using intraspecific natural

635 variation to optimise biological control agents will reduce the reliance of augmentative

636 biological control on the importation of non-native natural enemies. It will help to reduce the

637 environmental risks associated with this practice, and the dependency on other countries for

638 the acquisition of genetic resources.

639

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1091   **Figure legends**

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1093   **Figure 1.** Conceptual diagram of breeding selection illustrating the partitioning of phenotypic

1094   variance into genotypic variance and environmental variance. Top panel shows the frequency

1095   distribution of a hypothetical phenotypic trait in the parental generation (bold, large bell-

1096   shaped curve). The population as a whole consists of individual genotypes, represented by the

1097   small bell-shaped curves. Each of these genotypes has a different mean phenotypic value and

1098   variance. The difference between the means is influenced by genotypic variance, whereas the

1099   variance around the mean in each of the genotypes represents environmental variance. The

1100   former has a heritable component (additive genetic variance), whereas the latter does not. This

1101   is represented by the bottom panel, where the phenotypes with the lowest (in dark), and the

1102   highest (in light) phenotypic value in the parental generation have been selected, respectively.

1103   This leads to a shift to a lower, and a higher phenotypic mean value respectively in the

1104   downward- and upward selected offspring. This response is due to the selection on the

1105   genotypic component of the variance in the parental generation.

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Figure 1