

# Triploid bumblebees indicate a direct cost of inbreeding in fragmented populations

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## Abstract

Hymenopteran species with single-locus complementary sex-determination (sl-CSD) face an additional cost of inbreeding because of a loss of diversity at the sex-determining locus. Laboratory studies of a range of Hymenoptera have found that a small percentage of diploid males produce viable diploid sperm, and that if these males mate, then the resultant females produce triploid offspring that are sterile. Here, we use microsatellite markers to determine the frequency of triploid individuals of *Bombus muscorum* and *B. jonellus* in a model island system. Triploids were found in populations of both species. Observed triploid frequencies of up to 8% were detected, and estimated total frequencies peaked at 20% with respect to normal diploid workers. For both species, triploid frequency was negatively correlated with surrogates of population size, providing direct evidence for inbreeding in small populations. Populations limited to <~15 km<sup>2</sup> of suitable habitat were particularly likely to harbour triploids. Estimated total triploid frequencies were higher in *B. muscorum* than in *B. jonellus*, perhaps due to the greater dispersal range of the latter species. Implications for the conservation of rare social hymenopterans are discussed.

**Keywords:** conservation biology, conservation genetics, ecosystem services, habitat degradation, inbreeding, insects

Received 6 May 2011; revision received 4 May 2012; accepted 15 May 2012

## Introduction

Many hymenopterans use a reproductive system called arrhenotokous parthenogenesis in which females develop from fertilized diploid eggs, whereas unfertilized eggs produce males (but see Beukeboom *et al.* 2007). The sex of an individual depends on one of several sex-determination mechanisms (Cook 1993; van Wilgenburg *et al.* 2006), best understood of which is the single-locus complementary sex-determination system (sl-CSD) thought to be ancestral to the Hymenoptera (Crozier 1977; Periquet *et al.* 1993; Crozier & Pamilo 1996). Under this system, individuals that are heterozygous at the poly-allelic sex locus develop into females, whilst hemizygous (or homozygous) individuals develop into males (Cook & Crozier 1995). These males generally have low viability, are unable to mate or are

sterile (reviewed in Cowan & Stahlhut 2004). There are additional knock-on costs for females that choose to mate with diploid males, because few fertile offspring result. Both inbreeding and genetic drift in small populations increase the frequency of 'matched-matings' (Zayed & Packer 2001). The resultant diploid male production (DMP) is thought to substantially increase the extinction proneness of inbred populations (Zayed & Packer 2005).

In species with sl-CSD, the high fitness costs of DMP should favour mechanisms which minimize inbreeding, particularly in social species where diploid males are produced at the expense of female workers (Cowan & Stahlhut 2004). This is perhaps the reason why, in the Hymenoptera, both sexes commonly disperse from the natal area prior to mating (Michener 1974; Antolin & Strand 1992), and why males and reproductive females are frequently produced asynchronously or leave the nest at different times (Holldobler & Bartz 1985; Holldobler & Wilson 1990). In bumblebees, young males are forced

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from the colony by the workers after 4 or 5 days, presumably to avoid sib-mating (Plowright & Pallett 1979). Additionally, in wasps, bumblebees and other eusocial bees (*Lasioglossum* sp.) kin-recognition systems result in preferential mating between unrelated individuals (Smith 1983; Smith & Ayasse 1987; Foster 1992; Ode *et al.* 1995).

Despite these mechanisms, diploid males have been detected in more than 40 species of social hymenopterans (van Wilgenburg *et al.* 2006). Two species of bumblebees have been studied in the laboratory to date; *B. terrestris* (Duchateau *et al.* 1994; Duchateau & Marien 1995; Ayabe *et al.* 2004) and *B. atratus* (Garófalo & Kerr 1975; Plowright & Pallett 1979), and in accordance with sI-CSD expectations, 50% of brother-sister matings led to colonies which produced diploid males at the expense of half of the workers. However, recent studies in small populations of rare bumblebees found very few diploid males despite low inferred population size, so the extent of DMP and the consequences for wild populations remain uncertain (Darvill *et al.* 2006; Ellis *et al.* 2006). Many rare bumblebee species now exist only in small isolated populations, so elucidating the importance of genetic factors in their continuing declines is a conservation priority.

Some diploid males of a range of hymenopteran species succeed in mating and produce triploid offspring. Triploids are sterile and therefore the resulting colonies are unable to reproduce, so although the cost of the initial matched mating is delayed, it remains a reproductive dead-end. Triploids have now been found in several Hymenoptera species including sawflies, wasps, ants and bees (Table 1). The presence of triploids indirectly indicates that diploid males were produced in the previous generation (Crozier & Pamilo 1996), which is significant because in some social species, diploid males are not detectable at all stages of the colony cycle (Pamilo *et al.* 1994; Henshaw *et al.* 2002).

The low frequencies of bumblebee diploid males found to date may be the result of high diversity at the sex-determining locus, or inbreeding-avoidance mechanisms such as sex-allele signalling (Paxton *et al.* 2000). Alternatively, by screening males caught incidentally during worker sampling, previous population genetic studies may have underestimated true diploid male frequencies, perhaps because DMP colonies are short-lived (Whitehorn *et al.* 2009). Here, we determine the frequency of triploid individuals within previously studied populations of *B. muscorum* and *B. jonellus* in a model island system. Additionally, we establish whether triploid frequencies correlate with population size, as predicted by mutation-drift equilibrium expectations (Yokoyama & Nei 1979).

**Table 1** Hymenopteran species in which triploids have been detected

Taxon	Origin	References
Sawflies		
<i>Athalia</i>	Lab	Naito & Suzuki 1991
<i>rosae ruficornis</i>		
<i>Neodiprion nigroscutum</i>	Lab	Smith & Wallace 1971 cited in Ayabe <i>et al.</i> 2004
Wasps		
<i>Bracon hebetor</i>	Lab	Bostian 1934; Torvik 1931; Whiting 1961
<i>Habrobracon 'pectinophorae'</i>	Lab	Inaba 1939, cited in Whiting 1961
<i>Cotesia vestalis</i>	Lab	de Boer <i>et al.</i> 2007
<i>Ropalidia revolutionalis</i>	Wild	M. Henshaw, unpublished in Liebert <i>et al.</i> (2004)
<i>Polistes fuscatus</i>	Wild	Liebert <i>et al.</i> 2004
<i>Polistes dominulus</i>	Wild	Liebert <i>et al.</i> 2004, 2005
<i>Polistes aurifer</i>	Wild	Liebert <i>et al.</i> 2004
Ants		
<i>Solenopsis invicta</i>	Wild	Krieger <i>et al.</i> 1999
<i>Tapinoma erraticum</i>	Wild	Cournault & Aron 2009
<i>Camponotus</i> sp. 5 (tetraploid)	Wild	Imai <i>et al.</i> 1977
<i>Crematogaster</i> sp. 2	Wild	Imai <i>et al.</i> 1977
Bees		
<i>Apis mellifera</i>	Lab	Chaud-Netto 1975
<i>Bombus atratus</i>	Lab	Garófalo & Kerr 1975 cited in Ayabe <i>et al.</i> 2004
<i>Bombus terrestris</i>	Lab	Duchateau & Marien 1995; Ayabe <i>et al.</i> 2004
	Wild	Nagamitsu & Yamagishi 2009
<i>Bombus florilegus</i>	Wild	Takahashi <i>et al.</i> 2008

Adapted and expanded from Liebert *et al.* (2004).

## Materials and methods

### Sample collection and DNA treatment

During June to September in 2003–2005, a total of 1074 *Bombus muscorum* (976 ♀ and 98 ♂) and 768 *B. jonellus* (764 ♀ and 4 ♂) were collected from 17 islands in the Inner and Outer Hebrides (Scotland, UK) (Table 2, Fig. 1). The islands visited spanned a range of sizes and degrees of isolation. Further sampling was conducted at Staffa on 8th September of 2007 with the twin aims of obtaining larger samples of males and a sample from relatively late in the season. An additional 108 *B. muscorum* (63 ♀ and 47 ♂) and 119 *B. jonellus* (58 ♀ and 61 ♂) were collected. Samples were preserved in pure ethanol and stored at ambient temperature.

Total DNA was extracted from macerated tarsi using the HotShot protocol (Truett *et al.* 2000). Samples were

**Table 2** The number of triploids detected in each of the populations. Also shown are the total sample sizes, average heterozygosity ( $H_E$ ), allelic richness, population size (category) and habitat area. For *B. muscorum*, allelic richness and  $H_E$  were calculated using all loci except B131. Allelic richness values are standardized to the smallest sample size (which for both species is 25)

Population	<i>Bombus muscorum</i>					<i>Bombus jonellus</i>				
	Sample size	Triploids detected	Allelic richness	$H_E$	Habitat area (km <sup>2</sup> )	Sample size	Triploids detected	Allelic richness	$H_E$	Habitat area (km <sup>2</sup> )
Barra	50 ♀ 3 ♂	–	3.10	0.39	67	82 ♀ 0 ♂	–	8.63	0.77	65
Mingulay	49 ♀ 1 ♂	–	2.99	0.37	5	34 ♀ 1 ♂	2 ♀	7.50	0.70	5
Muldoanich	25 ♀ 6 ♂	–	3.63	0.42	1	–	–	–	–	–
Pabbay	38 ♀ 16 ♂	1 ♀	3.33	0.40	3	36 ♀ 0 ♂	–	7.55	0.73	3
Sandray	59 ♀ 1 ♂	1 ♀	3.05	0.37	4	25 ♀ 0 ♂	–	9.00	0.76	4
Monachs	50 ♀ 49 ♂	4 ♀	2.22	0.31	2	–	–	–	–	–
Uists	87 ♀ 3 ♂	–	3.32	0.40	400	92 ♀ 0 ♂	–	9.30	0.75	221
Colonsay	67 ♀ 0 ♂	–	3.21	0.42	32	28 ♀ 0 ♂	–	10.24	0.76	30
Lunga	36 ♀ 6 ♂	–	3.43	0.51	1	38 ♀ 0 ♂	–	9.90	0.74	1
Staffa 2004	56 ♀ 0 ♂	4 ♀	3.33	0.48	0.5	48 ♀ 0 ♂	2 ♀	8.19	0.70	0.5
Iona	50 ♀ 5 ♂	–	4.22	0.55	6.5	–	–	–	–	–
Canna	62 ♀ 3 ♂	–	3.11	0.43	5.75	45 ♀ 0 ♂	1 ♀	9.58	0.76	5.75
Coll	70 ♀ 0 ♂	–	3.46	0.50	73	45 ♀ 1 ♂	1 ♀	9.05	0.74	51
Eigg	64 ♀ 2 ♂	–	3.30	0.53	10.75	36 ♀ 1 ♂	–	9.87	0.76	10.8
Muck	53 ♀ 0 ♂	1 ♀	2.91	0.43	4.25	47 ♀ 0 ♂	–	8.81	0.75	4.25
Rum	43 ♀ 1 ♂	1 ♀	2.91	0.45	13	32 ♀ 0 ♂	–	10.00	0.75	27
Tiree	119 ♀ 2 ♂	–	3.27	0.50	75.25	54 ♀ 1 ♂	–	8.18	0.72	24
Skye	–	–	–	–	–	43 ♀ 0 ♂	–	10.03	0.75	200
Mull	–	–	–	–	–	37 ♀ 0 ♂	–	10.68	0.77	200
Mainland <sup>†</sup>	–	–	–	–	–	42 ♀ 0 ♂	–	10.02	0.76	>200
Staffa 2007	63 ♀ 47 ♂	–	2.99	0.42	0.5	58 ♀ 61 ♂	–	7.75	0.75	0.5

<sup>†</sup>A mainland sample of *B. jonellus* was collected from the west coast Scotland. It was not possible to estimate a habitat area for this population as there was no distinct boundary to the available habitat.

genotyped at up to eight microsatellite loci (from B132, B131, B118, B100, B96, B10, B11, B124, B126; Estoup *et al.* 1995, 1996). Loci were amplified and visualized following Darvill *et al.* (2006). A detailed analysis of genetic diversity and structuring in this same data set is presented in Darvill *et al.* (2010). In brief, for *B. jonellus*, sister pairs were identified and all but one sister removed from the data set using KINSHIP v1.3.1, but this was not possible for *B. muscorum* as low genetic diversity prevented reliable identification of sisters. Genetic diversity was assessed by calculating Nei's unbiased heterozygosity (Nei 1987) and allelic richness using FSTAT.

#### Detection of triploids

Microsatellite loci were visualized on a slab-gel sequencer (ABI PRISM<sup>TM</sup> 377), and fragment sizes were manually scored using Genotyper (Applied Biosystems) or were screened on an ABI 3730 capillary DNA sequencer (The Sequencing Service, University of Dundee) and visualized using STRAND (Veterinary Genetics Laboratory, University of California at Davis, <http://www.vgl.ucdavis.edu/informatics/strand.php>). If scoring was uncertain, individuals were reamplified and re-run. If this appeared to

confirm the first run, the score was recorded. If runs were inconsistent, then the sample was rerun up to five times until two consecutive runs gave the same genotype.

Triploids were identified by the presence of three distinct alleles at one or more loci. The presence of abnormally asymmetric peak intensities in heterozygote genotypes was also deemed indicative (although not conclusive), following Liebert *et al.* (2004, 2005). Where three peaks consistently appeared at one or more loci and abnormally asymmetric heterozygote genotypes were present at others, the individual was recorded as a putative triploid.

Repeat DNA extractions were then carried out to control for the possibility of contamination, and the process of PCR amplification and genotyping was repeated at all loci. All putative triploids were subject to a minimum of three extractions and four runs, and only if these produced consistent results were they deemed to be triploid, following Liebert *et al.* (2004).

#### Estimating total triploid frequencies

The probability of a triploid having three different alleles at one or more loci, and therefore of it being

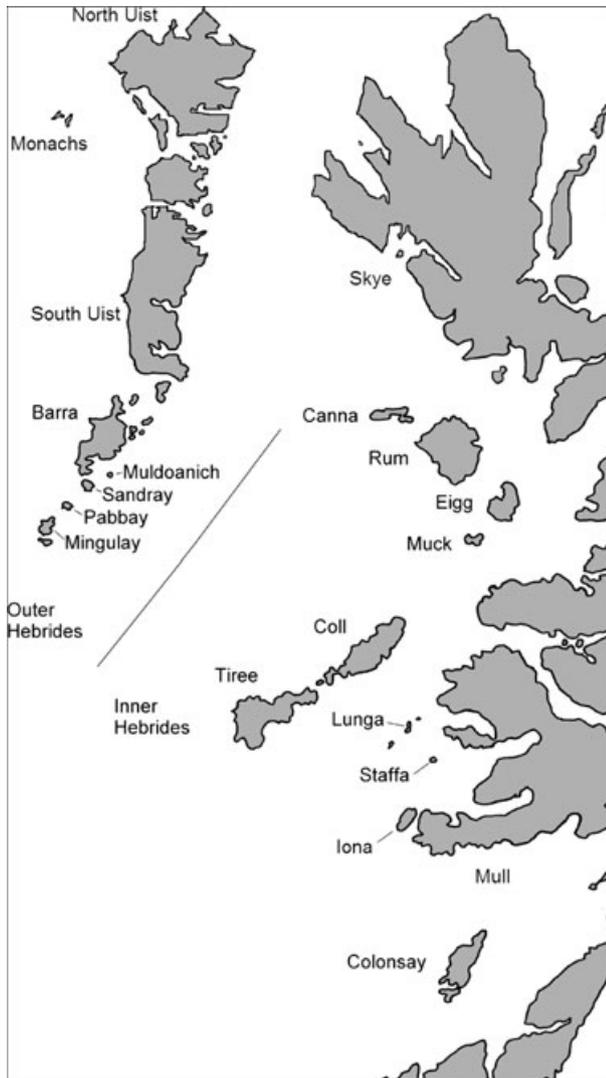


Fig. 1 A map of the study area (Hebrides, NW Scotland) showing the locations of the islands from which samples were collected.

correctly identified, depends on the number of alleles present at each locus within the population. Therefore, in small (genetically depauperate) populations where triploids are likely to be more frequent, they are also more difficult to detect. Similarly, where two species differ in overall genetic diversity, as was the case here, the power with which triploids can be resolved differs accordingly. For each species, the total frequency of triploids in each population was estimated from the detected frequency following the iterative method of Krieger & Keller (1998).

#### Population size and area of suitable island habitat

Bumblebee population sizes are difficult to determine because their nests are hard to find. The limited genetic

variation observed in this study and the widespread sampling method employed precluded the use of established methods that estimate nesting density indirectly (Darvill *et al.* 2004). Instead, analyses were performed based on area of suitable habitat, estimated using 1:50 000 Ordnance Survey maps. During fieldwork, each island was surveyed extensively on foot or by car. The estimated areas represent the total extent of the habitats in which each species was found foraging (moorland, species-rich grassland, etc). Clearly, this is an inexact estimate of population size, but it should be noted that results are qualitatively the same if analysed according to island size rather than habitat area, and island size and habitat area are strongly correlated ( $r^2 = 0.982$  for *B. muscorum*,  $r^2 = 0.999$  for *B. jonellus*).

#### Effect of available habitat area on triploid frequency

We used a generalized linear model to assess the effect of the area of suitable habitat (predictor) on triploid frequency (response variable). We used a quasi binomial distribution with a logit link function for the error structure to account for the overdispersion because of the low occurrence of triploids individuals. Habitat area figures were log-transformed to reduce scale effect. The prediction of the model and the 95% confidence intervals were estimated using the effects package for R (Fox 2003).

## Results

#### Observed triploid frequencies

From the original samples collected in 2003–2005, 12 (1.2%) *B. muscorum* and 6 (0.8%) *B. jonellus* workers were found to be triploid, out of a total of 976 and 764, respectively (Table 2). The frequencies of observed triploids did not differ significantly between species (Fisher's exact test, two-tailed,  $P = 0.48$ ). No triploid males were detected from either species. Triploids were detected in 6 of 17 sampled populations for *B. muscorum*, and in 4 of 17 *B. jonellus* populations. With one exception (2nd September 2004), all triploids were caught during the month of August (range 6th–30th August). Triploids were most frequent on Staffa and the Monachs, both of which are small and relatively isolated islands. None of the bees collected from Staffa on the 8th September 2007 were triploid.

#### Estimated total triploid frequencies

Predicted total triploid frequencies are given in Table 3. The four *B. muscorum* triploids detected on the Monachs represent 8% of the total worker sample.

**Table 3** Observed and estimated total triploid frequencies in the subset of populations in which triploids were found. Total frequencies were estimated using the multi-locus procedure of Krieger & Keller (1998). The triploid percentages that are estimated to remain undetected are also shown

	Population	Obs. triploid frequency	Estimated total frequency	Percentage of undetected
<i>Bombus muscorum</i>	Staffa	0.0714	0.0981	27.2
	Monachs	0.0800	0.2000	60.0
	Muck	0.0189	0.0256	26.3
	Rum	0.0233	0.0334	30.4
	Pabbay	0.0263	0.0372	29.3
	Sandray	0.0169	0.0279	39.3
<i>Bombus jonellus</i>	Staffa	0.0417	0.0421	1.0
	Mingulay	0.0588	0.0604	2.6
	Canna	0.0222	0.0223	0.4
	Coll	0.0222	0.0223	0.4

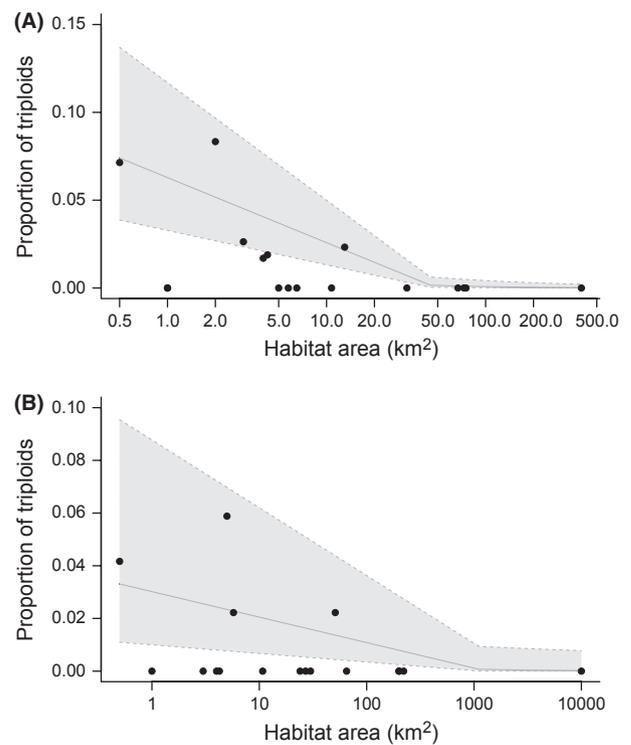
However, there were (on average) just 2.22 alleles per locus in this population, so a high proportion of triploids (estimated at 60%) will have remained undetected. It is not possible to calculate the true frequency of triploids in populations where none were detected, but it seems likely that in many cases, zero frequencies are underestimates. The greater genetic diversity of *B. jonellus* populations led to a larger proportion (>97%) of triploids being detected. On the basis of these figures, it follows that, in addition to the 12 triploids that were detected in *B. muscorum*, there are likely to have been approximately 11 additional triploid individuals (23 in total). In contrast, it is probable that there were no undetected triploids in *B. jonellus*, so that the six detected reflect the likely total. If these estimates accurately reflect triploid frequencies, then *B. muscorum* triploids occurred significantly more frequently than those of *B. jonellus* (Fisher's exact test, two-tailed,  $P = 0.01$ ).

#### Population size and triploid frequency

For both species, the available area of suitable habitat had a significant effect on the frequency of triploids (GLM with quasi binomial error distribution, *B. muscorum*,  $P < 0.001$  and *B. jonellus*,  $P = 0.022$ ; Fig. 2).

#### Diploid male frequencies

From the original samples collected in 2003–2005, two of the 98 *B. muscorum* males (2.04%) were diploid (found on Pabbay and Tiree). None of the four *B. jonellus* caught and genotyped from the Hebrides were diploid. A greater number of males were collected from the small isolated island of Staffa in September of 2007.



**Fig. 2** The relationship between the available habitat area and observed triploid frequencies for (A) *B. muscorum* and (B) *B. jonellus*. The solid grey line represents the relationship estimated by the generalized linear model with a quasi binomial error function. The grey area delimited by dashed lines illustrates the 95% confidence interval of the relationship. Note that data points illustrate the average proportion for each population and are shown for illustrative purposes. The GLM was fitted on individual data points.

More diploid males were found in this sample [six of 63 *B. muscorum* (9.52%) and two of 58 *B. jonellus* (3.45%)]. This difference between years approached significance for *B. muscorum* (Fisher's exact test, two-tailed  $P = 0.06$ ). Given the genetic variability observed among diploid individuals (females), we expect that 0.3% and  $2.2 \times 10^{-4}$ % of diploid males are homozygote at all loci for *B. muscorum* and *B. jonellus* respectively so that the error because of nondetection of diploid males is negligible in comparison with our estimates.

#### Discussion

Triploid workers were found in wild bumblebee populations, indirectly indicating the presence of diploid males. Estimated triploid frequencies varied between populations and in one case may have comprised up to 20% of sampled workers, suggesting that a substantial genetic load may be being carried by the smallest populations. The estimated figure of 20% (*B. muscorum* on the Monachs) should be viewed with a degree of

caution, as in arriving at this estimate, we assumed that sampled triploids were from independent colonies. Because of low genetic diversity, it was not possible to reliably remove sisters from the *B. muscorum* data set, so it is not known whether this assumption was valid. Triploid colonies are significantly smaller than normal colonies (Ayabe *et al.* 2004), so it could be argued that these relative worker frequencies underestimate the proportion of triploid nests. Furthermore, in *B. terrestris*, only a small percentage of diploid males are fertile (Duchateau & Marien 1995; Ayabe *et al.* 2004); if this applies to *B. muscorum* and *B. jonellus*, then our data indicate frequencies of diploid males far in excess of previous estimates, with associated costs and consequences for population fitness (Zayed & Packer 2005).

Our data demonstrate a direct link between a surrogate of population size and triploid frequency. Under equilibrium conditions, the number of sex-determining alleles in a population should depend on the effective population size, reflecting a balance between mutation and drift (Yokoyama & Nei 1979). It follows that diploid male frequencies, and therefore triploid frequencies, should be negatively correlated with population size; a prediction confirmed here. The production of diploid males in inbred hymenopteran populations theoretically increases local extinction rates (Zayed & Packer 2005), and diploid male producing colonies of *B. terrestris* have low fitness (Whitehorn *et al.* 2009). This study therefore provides clear evidence of inbreeding depression in small bumblebee populations. Estimated total triploid frequencies were significantly higher in *B. muscorum* than in *B. jonellus*, in accordance with our estimates of smaller effective population sizes in *B. muscorum* (Darvill *et al.* 2010).

The discovery of triploid workers indirectly confirms that diploid males were present in the previous generation. In the original samples from 2003 to 2005, a small proportion of males were diploids (2.0% *B. muscorum* and 0% *B. jonellus*), with higher values from our 2007 sampling on Staffa (9.5% *B. muscorum* and 3.4% *B. jonellus*). Samples collected in 2007 were from late in the season. The difference in frequency for *B. muscorum* approached significance and raises interesting questions that warrant further investigation. Across all populations, triploid frequencies were 1.2% and 0.8% for *B. muscorum* and *B. jonellus*, respectively (with frequencies from Staffa of 7.1% and 4.2%). It has previously been shown that only a small percentage of diploid males are capable of mating successfully [estimated at 5% in *B. terrestris* (Ayabe *et al.* 2004), and 2.4% in *Solenopsis invicta* (Krieger *et al.* 1999)], so a higher frequency of diploid males than triploids would be expected. This has been shown in other Hymenoptera (Krieger *et al.* 1999). Our data suggest that diploid

males of *B. muscorum* and *B. jonellus* do not suffer a major handicap in mating, because frequencies of diploid males and triploid females are broadly similar.

It is likely that our estimates of diploid male frequencies are a poor estimator of the proportion of nests that produce such males. Such nests are presumed to produce equal numbers of diploid males and worker females from the beginning of the colony cycle (June–July in the Hebrides). Thus in early season, we might predict that the frequency of diploid male producing colonies should be twice the ratio of diploid males to workers. However, in *B. terrestris*, such colonies fare poorly and tend to die out swiftly (Whitehorn *et al.* 2009). It is thus surprising that there are any diploid males still available to mate with gynes when they are produced in August–September. The longevity of these males under field conditions is unknown, but the presence of diploid males in our late-season sample from Staffa suggests that they probably survive for a month or more.

#### Implications for conservation

It seems that populations limited to less than around 15 km<sup>2</sup> of suitable habitat are particularly likely to suffer from diploid male production, in addition to other problems associated with small population size. To maintain populations without evidence of inbreeding, in the form of triploid females, conservationists and policy makers should ensure that habitats are managed on an appropriate scale.

#### Acknowledgements

We are grateful to the Leverhulme Trust (Grant no. F/00180/M) for providing funding to support this work. Thanks also to RSPB, SNH, the Scottish Wildlife Trust, the National Trust for Scotland and the Hebridean Trust for access to field sites. Thank you to Helen Darvill for assistance with field work and to Malcolm and Lynn Teed for logistical support. Finally, we are grateful to Mike Edwards, Murdo MacDonald and Stuart Roberts for advice and information.

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### Data accessibility

The complete data set for this MS is available as Supporting information.

### Supporting information

Additional supporting information may be found in the online version of this article.

**Appendix S1** The genotypes of all of the sampled individuals.

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