

# Effects of Polyploidization on Locomotor and Flight Activity, and Body Size of Males in Japanese Bumblebee, *Bombus ignitus*

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## ABSTRACT

Polyploid animals are seen in the wild in only a few groups such as planaria, weevils, ants, wasps, and bees. The sex of bumblebees is determined by the single-locus complementary sex determination (sl-CSD) system resulting in females emerging from fertilized eggs and males (normal males, haploid) emerging from unfertilized eggs. However, diploid males (polyploids) emerged from fertilized eggs when the sex-determining locus become homozygous caused by inbreeding due to population contraction. To examine the effects of polyploidization on behavioral characteristics in bumblebee males, *Bombus ignitus*, haploid and diploid males were compared in locomotor activity, ease of staying inside the hive, and flight activity in a laboratory experiment. The results showed that diploid males had higher locomotor activity levels, were less likely to stay inside the hive, and had lower flight activity levels than haploid males. The reduced flight activity levels may lead to lower mating rates in the field. Furthermore, the body size of diploid males was smaller than that of haploid males. Diploid males of bumblebees have a low reproductive and produce diploid sperms to emerge sterile triploids, thus creating a considerable genetic load on the population. The behavioral and morphological characteristics of diploid males may unintentionally serve as a mechanism to prevent the emergence of sterile triploids.

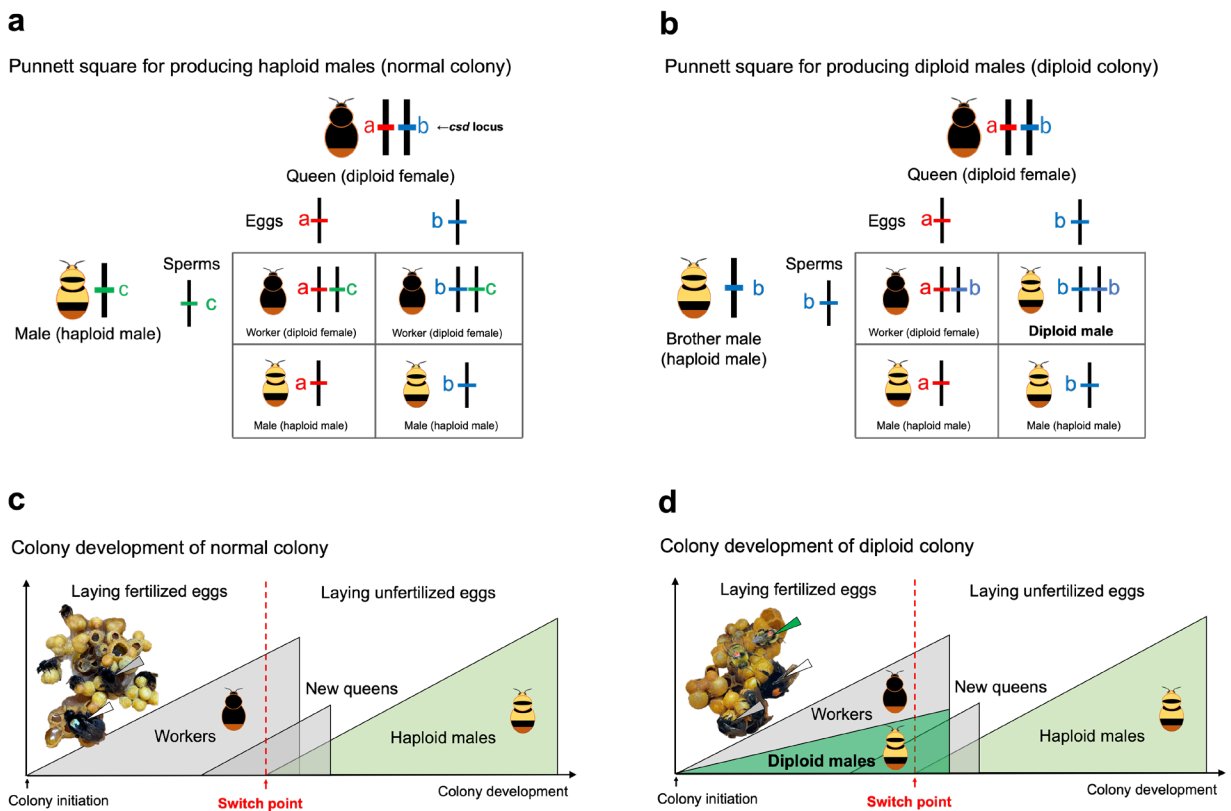
## Introduction

Polyploidization is a phenomenon in which the number of chromosomes increases by an integer multiple. Polyploids in the natural world are found in a few groups, such as planaria (Platyhelminthes), weevils (Coleoptera), and ants, wasps, and bees (Hymenoptera) (Takeuchi 1979; Tamura et al. 1995; Knakievicz et al. 2007; Brock 2013).

Bumblebees are social insects that are headed by a single queen and are an annual life cycle. The queens mate with a male in autumn and hibernate underground during winter. In spring, the queens leave their hibernation and search for nesting space such as an abandoned field mouse nest or some other similarly shaped cavity. The foundation queen first lay fertilized eggs to produce female workers. In the late stages of colony development, the queen switches from fertilized eggs laying to unfertilized eggs laying (switch point; SP) and produces males from unfertilized eggs (Duchateau and Velthuis 1988). At this time, the female larvae that already hatched from the fertilized eggs have developed into new queens due to a lot of food provided by workers. The newly emerged queens and males leave the nest for mating flights. Thus, females are invariably developed from fertilized eggs, and males are developed from unfertilized eggs (Haplodiploidy). This genetic mechanism is known as single-locus complementary sex determination (sl-CSD; Whiting 1943). It has been shown that females are produced when the sex-determining locus (complementary sex determiner; *csd*) is heterozygous, while haploid males are developed when hemizygous. For example, as shown in Fig. 1a, if the genotype of the *csd* locus in the queen is a/b, and that of the haploid male (father) is c, the fertilized eggs will be heterozygous a/c or b/c, and will be female because has two different alleles. The unfertilized eggs laid by the queen will be hemizygous a or b and will be male because it has only one allele (Fig. 1a). However,

diploid males can be developed from fertilized eggs when the *csd* locus becomes homozygous due to inbreeding. As shown in Fig 1b, if the genotype of the *csd* locus in the queen is *a/b*, and that of the brother male is *b*, the fertilized eggs will be heterozygous *a/b* or homozygous *b/b*. The fertilized eggs with genotype *b/b* will develop into diploid males because it has only one allele. In hymenopteran insects, diploid males are usually sterile or slightly fertile and have low viability (Whiting 1943; Bostian 1935; Naito and Suzuki, 1991; van Wilgenburg et al. 2006), thereby representing a substantial genetic load in wild populations and acceleration of the extinction vortex (Zayed and Packer 2005). In fact, diploid males in bumblebees have been found in wild populations due to reduced genetic diversity of *csd* locus caused by population fragmentation (Darvill et al. 2006; Ellis et al. 2006; Takahashi et al. 2008; Darvill et al. 2012).

Thus, the effects of polyploidization on behavioral characteristics have not been examined although there have been several studies of diploid males in bumblebees under the sl-CSD system. Since diploid males are expected to emerge through inbreeding and population fragmentation, it is important to understand the behavioral characteristics of diploid males in terms of providing a measure of the health of wild populations. Therefore, to examine the effects of diploidization on the three behavioral characteristics, we compared locomotor activity level, the ease of staying inside the nest, and flight activity level between haploid and diploid males in native Japanese bumblebees, *B. ignitus*. We also examined the effects of polyploidization on morphological parameters by measuring body size.



**Figure 1.** Punnett squares for producing haploid and diploid males of *Bombus ignitus* under the sl-CSD model. **a** Normal haploid male producing colony. **b** Diploid male-producing colony. **c** Colony developmental stages of a normal colony. **d** Colony developmental stages of a diploid colony. The switch point indicates when the queen switches from fertilized to unfertilized egg laying. Arrows in photographs indicate that the white ones are queens, the gray ones are workers (diploid females) and the green one is a diploid male.

## Methods

### Bumblebees

Commercial colonies of bumblebees, *B. ignitus* were purchased from Agrisect Inc. (Ibaraki, Japan) and transferred to wooden rearing boxes (200mm × 280mm × high 140mm). Colonies were reared in a darkroom in Yasuda Gakuen Junior and Senior High School (Tokyo, Japan) maintained at a temperature of 28±2°C and humidity of 40%-60%, and were supplied ad libitum with a 60% sugar solution and fresh pollen collected by honeybees. To produce diploid males (polyploids), new virgin queens hatching from these colonies were mated with brother males in the same colony under the sl-CSD model as shown in Fig. 1b. Mated queens hibernated in a freezer at 5°C for at least two months, and were then placed in a flight cage (400mm × 400mm × high 550mm). After three days they were transferred to wooden rearing boxes and allowed to colony initiation. The bumblebee queens first lay fertilized eggs to produce diploid females (workers) and then lay unfertilized eggs to produce haploid males during the reproductive season (usually from summer to autumn). The switching period of laying from fertilized eggs to unfertilized eggs is called the switch point (Fig. 1c; Duchateau and Velthuis 1988). Because a matched mating at the sl-CSD locus will produce diploid offspring in a 1:1 sex ratio (Fig. 1d), we carefully determined diploid male colonies that produced females and males in similar numbers of first, second, and third broods and collected their males as diploid males. Haploid males were obtained from other post-switch point colonies where no males had hatched by the third broods. We identified the days after hatching by placing stickers with numbers on their thorax after haploid and diploid males emerged from each colony.

### Quantification of locomotor activity level

#### *Experimental setup*

To measure locomotor activity levels, we introduced individual haploid or diploid males at 1-, 3-, 6-, and 12 days after hatching in activity test boxes (90 mm × 90 mm × high 60 mm). Test boxes were divided into 9 subdivisions, with each subdivision being 30 mm square. The introduced male movements between subdivisions were counted for 5 minutes as an indicator of locomotor activity level. Counts were made at 5 and 30 minutes after the male introduction. The locomotor activities were measured at the same time of the day (14:00 to 17:00) to prevent differences in circadian rhythm from affecting results. Measured males were returned to their native colonies and repeatedly measured for 12 days old. Data sets were collected in each colony from different ploidy groups (i.e., ten haploid males from colony-A, six haploid males from colony B, ten diploid males from colony C, and five diploid males from colony D).

#### *Statistics*

The locomotor activity levels were analyzed by using a generalized linear mixed model (GLMMs) with a negative binomial distribution error and log-link function fitted by R version 3.6.3. To the effect of days after hatching, a model was constructed by using locomotor activity level as a response variable, days after hatching as a fixed effect, and colony, ploidy level, and individual as a random effect. To effect of ploidy level, a model was constructed by using locomotor activity level as a response variable, ploidy level as a fixed effect, and colony, days after hatched and individual as a random effect.

### Number of males outside and inside the hive

#### *Experimental setup*

To examine the ease of exiting or staying in the hive for males of different ploidy, we measured the number of haploid and diploid males outside and inside the hive. The observation hives (90mm × 180mm × high 60mm) were prepared

with two rooms separated by an acrylic panel with a small hole for bees to pass through. One room was made of red translucent acrylic panels and the other of translucent acrylic panels. Since bumblebees are not receptive to red light, the nest was placed in the red translucent room with sugar water and pollen in the other room to reproduce the inside and outside of a natural nest. We introduced one queen, three diploid males, and three workers from the diploid colony into the observation hive. Similarly, one queen, three haploid males, and three workers were introduced from the haploid colony into another observation hive. Since bumblebee males are sexually mature  $12.3 \pm 1.3$  days after (Tasei et al. 1998), haploid and diploid males between 6 to 12 days old were collected for the experiments. The two experimental colonies were set up in a dark room ( $28 \pm 2$  °C and RH 40%-60%) and reared for two days with light 12 h / dark 12 h conditions to synchronize their circadian rhythms. Starting on the third day, the entire two hives were photographed with an infrared camera (ELP-USB100W; ELP) every 3 minutes for 24 hours (20 pictures per hour). The total number of male bees per hour outside the hive, inside the hive, and in contact with the nest were counted. The experiment with different nest combinations was conducted twice (October 2021 and September 2022).

### Statistics

The number of males outside the nest, inside the nest, and in contact with the nest in each experiment was analyzed by using a generalized linear model (GLMs) with a negative binomial distribution error and log-link function fitted by R version 3.6.3. A model was constructed by using the number of males as a response variable, and ploidy level as a fixed effect.

### Quantification of flight activity

#### Experimental setup

To examine flight activity levels, we measured whether haploid and diploid males fly or not per day old. We transferred individual haploid or diploid males at 1-, 3-, 6-, and 12 days after hatching in a dish covered with a red transparent lid. Then, the dish was placed in the center of a flight activity test cage (400 mm × 400 mm × high 550 mm) in an illuminated room (light intensity 820–940 lx, temperature  $24 \pm 2$ °C). After 60 seconds, we slowly removed the red transparent lid and observed whether the male flew or not for 1 min. Flew males were judged as “motivated for flying”. The flight activity level of each group was measured at the same time of the day (14:00 to 17:00) to prevent differences in circadian rhythm from affecting results. Measured males were returned to their native colonies and repeatedly measured for 12 days old. Data sets were collected from each colony from different ploidy groups (i.e., ten haploid males from colony E, six haploid males from colony F, ten diploid males from colony G, and nine diploid males from colony H). After completing the 12-day-old measurements, all males were stored in a freezer and measured for head width and forewing length with a digital caliper.

### Statistics

Data sets of head width and forewing length were analyzed by a principal component analysis to obtain a first principal component (PC1) as an indicator of body size. We analyzed the effects of days after hatching, ploidy level, body size on the percentage of flying, and the relationship between ploidy level and body size using generalized linear mixed models (GLMMs) fitted by R version 3.6.3. A list of these analytical models was presented in Table 1.

**Table 1. A list of analytical models using generalized linear mixed models (GLMMs).**

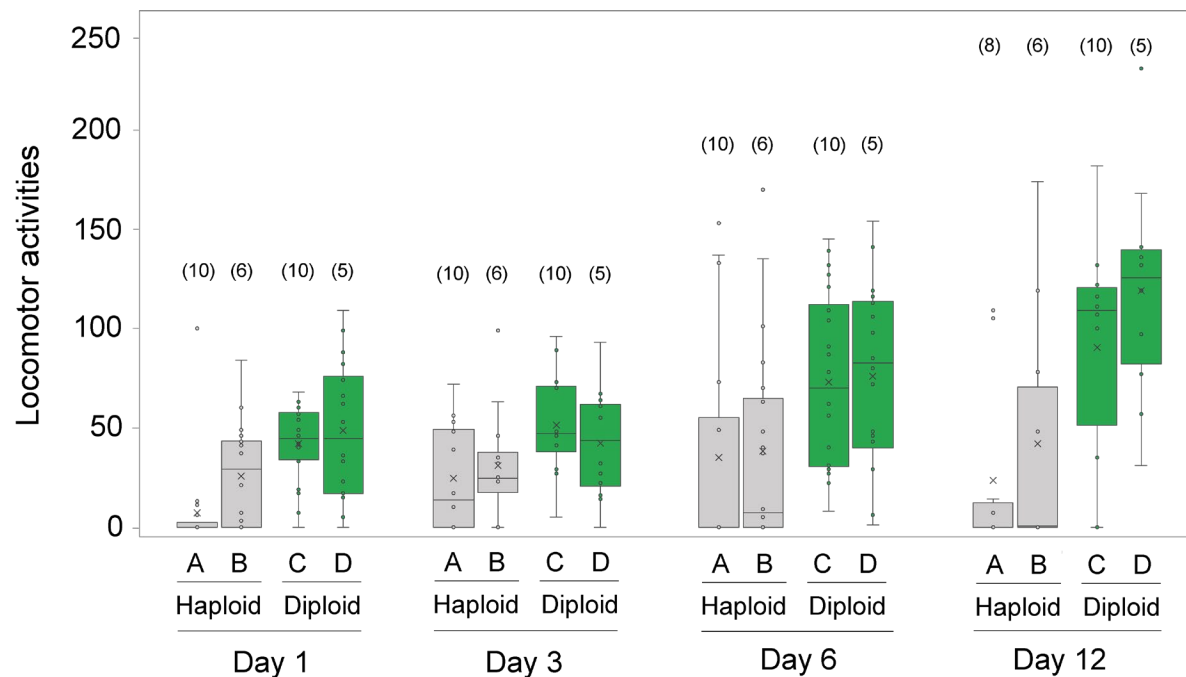
Analysis items	Response variable	Fixed effect	Random effect	Error distribution and link function
Effects of days after hatching on the flight percentage	Fly or not	Days after hatched	Colony, ploidy level, and PC1	Binomial distribution error and link-logit

<b>Effect of the ploidy level on the flight percentage</b>	Fly or not	Ploidy level	Colony, days after hatched, and PC1	Binomial distribution error and link-logit
<b>Effect of the body size on the flight percentage</b>	Fly or not	PC1	Colony, ploidy level, and days after hatching	Binomial distribution error and link-logit
<b>Relationship between ploidy level and body size</b>	PC1 variable transformed to a continuous variable greater than zero	Ploidy level	Colony	Gamma distribution error and link-inverse

## Results

### Comparison of locomotor activity level between haploid and diploid males

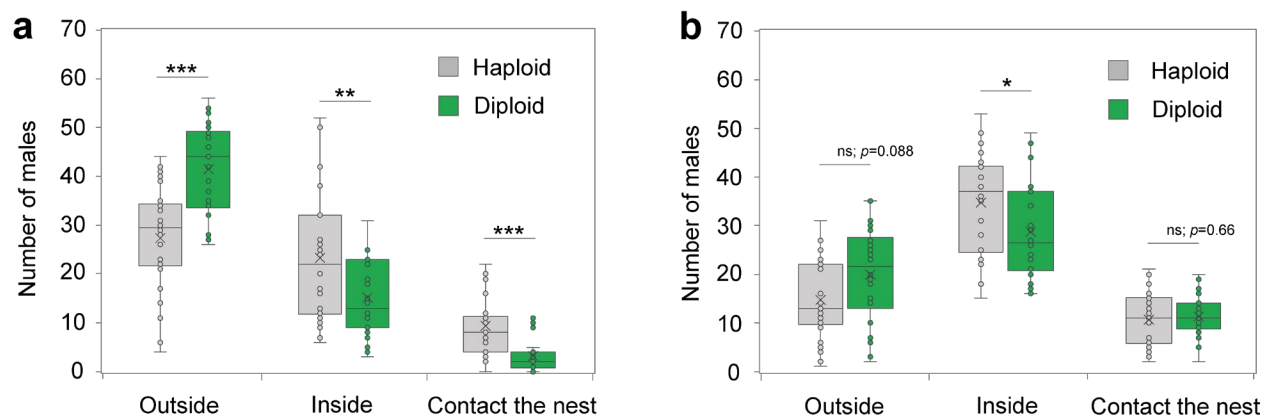
The locomotor activity level of both haploid and diploid males was significantly increased days after hatching (Fig. 2; GLMM with a negative binomial distribution error and link-log,  $z=38.4$ ,  $p<0.001$ ). Male bees increased their locomotor activity as days passed after hatching regardless of different ploidy levels. A comparison of the amount of locomotor activity in males showed that diploid males were significantly higher activity levels than haploid males (GLMM with a negative binomial distribution error and link-log,  $z=2.88$ ,  $p<0.01$ ).



**Figure 2.** Locomotor activity levels in haploid and diploid males. Alphabets denote colony code. Boxplots indicate median, first and third quartiles, and maximum and minimum values. Sample sizes are in parentheses.

### Comparison of the number of males outside and inside the hive

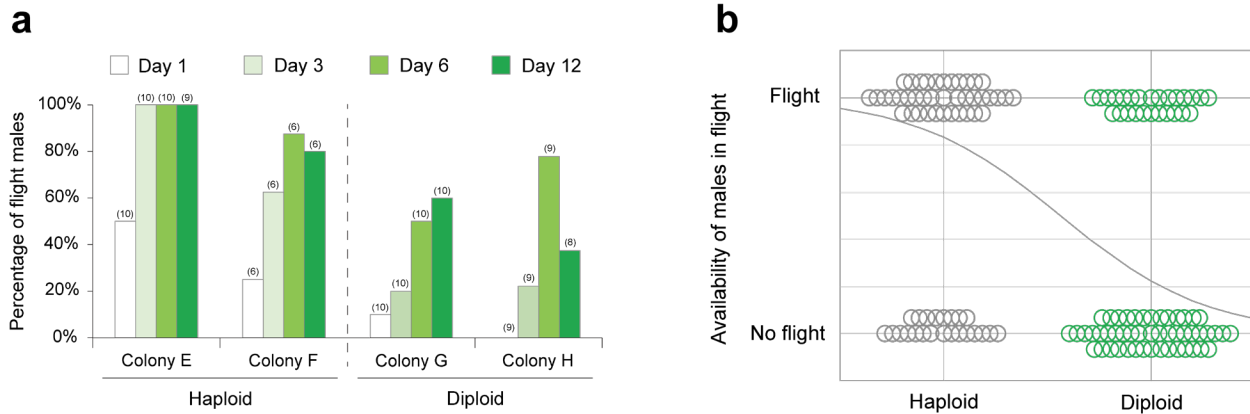
In the first experiment, the number of diploid males outside the hive was significantly higher than that of haploid males (GLM with a negative binomial distribution error and log-link,  $z = 4.14$ ,  $p < 0.001$ ). In contrast, the number of diploid males inside the hive and contact the nest was significantly lower than that of haploid males (GLMs with a negative binomial distribution error and log-link, inside the nest;  $z = -2.65$ ,  $p < 0.01$ , contact the nest;  $z = -4.34$ ,  $p < 0.001$ ). In the second experiment, the number of diploid males inside the hive was significantly lower than that of haploid males (GLM with a negative binomial distribution error and log-link,  $z = -1.99$ ,  $p < 0.05$ ). The number of diploid males outside and in contact with the nest was no significant differences from that of haploid males (GLMs with a negative binomial distribution error and log-link, outside the hive;  $z = 1.71$ ,  $p = 0.088$ , contact the nest;  $z = 0.419$ ,  $p = 0.66$ ). Therefore, diploid males were more likely to remain inside the hive than haploid males.



**Figure 3.** Comparison of the number of males outside and inside of the hive in first (a) and second (b) experiment dates. Boxplots indicate median, first and third quartiles, and maximum and minimum values. Asterisks indicate a statistically significant difference (GLM; \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

### Effects of days after hatching and ploidy levels on flight activity

Percentages of flight males were shown in Fig. 4a. For both haploid and diploid males, the flight percentage was increased with days after hatching (Fig. 4a; GLMM with a binomial distribution error and link-logit,  $z = 4.01$ ,  $p < 0.001$ ). Results of the effect of different ploidy levels on flight activity, the flight percentage of diploid males was significantly lower than that of haploid males (Fig. 4b; GLMM with a binomial distribution error and link-logit,  $z = -1.97$ ,  $p < 0.05$ ). Therefore, we found that the increased ploidy level reduced the ability of males to fly.



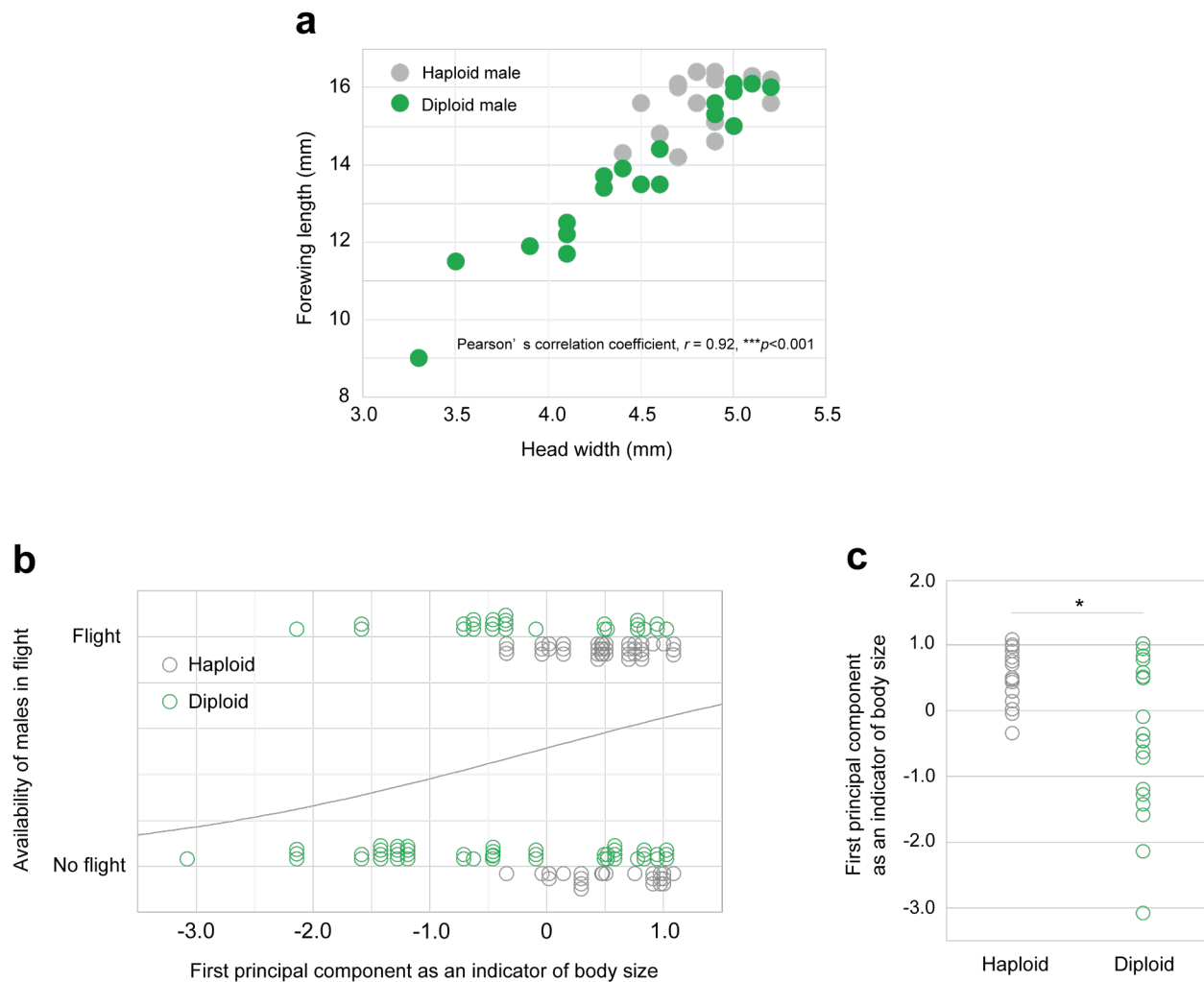
**Figure 4. a** Percentage of flight males of haploid and diploid males. Sample sizes are in parentheses. One male died in each of colony E and H, resulting in a smaller sample size at 12 days. **b** Flight activity levels in haploid and diploid males. The curve represents the estimates of GLMM analysis with binomial distribution error for the response variable.

### Effects of body size on flight activity

The relationship between head width and forewing length in males was shown in Fig. 5a. There was a significantly positive correlation between head width and forewing length (Pearson’s correlation coefficient,  $r = 0.92$ ,  $p < 0.001$ ). The contribution of the first principal component score (PC1) as an indicator of body size obtained by principal component analysis was 95.9%. Results of the effect of body size on the flight activity, a positive regression between body size and flight activity was found, but was not statistically significant (Fig. 5b; GLMM with a binomial distribution error and link-logit,  $z = 1.89$ , ns,  $p = 0.059$ ). In this experiment, we were not able to show that the increase in body size of males affected their flight activity.

### Effects of ploidy levels on body sizes

A comparison of the body size of haploid and diploid males showed that the body size of diploid males was significantly smaller than that of haploid males (Fig. 5c; GLMM with a Gamma distribution error and link-inverse,  $z = -2.42$ ,  $p < 0.05$ ). Thus, the increase in ploidy level caused a dwarfing of male body size.



**Figure 5.** **a** Relationship between head width and forewing length in haploid and diploid males. **b** Relationship between the body size and flight activity in haploid and diploid males. The curve represents the estimates of GLM analysis with binomial error distribution for the response variable. **c** Comparison of body size between haploid and diploid males. Asterisks indicate a statistically significant difference (GLM; \*  $p < 0.05$ ).

## Discussion

### Effects of polyploidization on the behavioral traits of males

The effects of polyploidization on the behavioral traits and body size of *B. ignitus* males are shown in Fig. 6. Our study showed that diploid males had higher locomotor activity levels, were less likely to stay inside the hive, and had lower flight activity levels than haploid males (Fig. 6). Normal haploid males are sexually mature  $12.3 \pm 1.3$  days after hatching and fly outside the nest for mating flight in the field (Tasei et al. 1998). The increase of locomotor activity due to polyploidization can be expected to accelerate the diploid males leave the nest for mating flights and thus lead it more difficult for them to remain in the nest. If diploid males leave the nest in a difficult-to-fly state where sexual



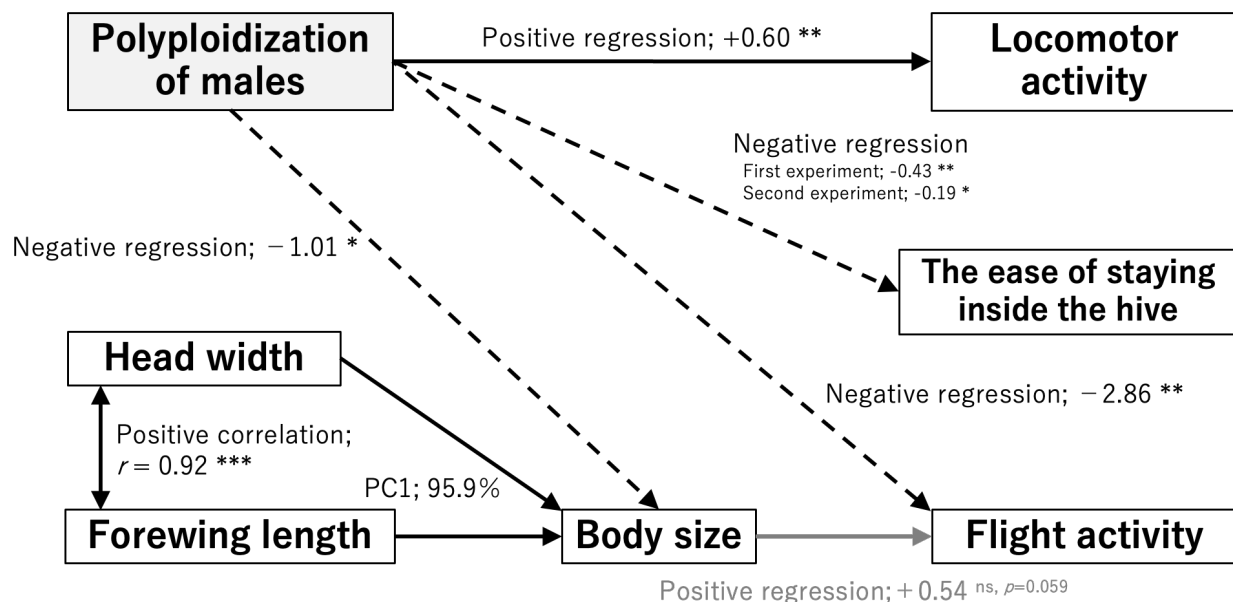
maturity is not completed due to polyploidization, the mating rate of diploid males will be reduced. Thus, the polyploidization that occurs in males *B. ignitus* negatively affects their behavioral traits.

Diploid males of bumblebees produce exceptionally diploid sperms (Duchateau and Marien 1995; van Wilgenburg et al. 2006). Therefore, a queen that mates with a diploid male produces sterile triploid females and males (Ayabe et al. 2004; Mori et al. 2018). Perhaps, the negative behavioral traits of diploid males shown in this study contribute to lower mating rates in the field with queens and may avoid the emergence of sterile individuals.

### Effects of polyploidization on the body size of males

Results of the present study, it was found that the body size of diploid males was smaller than that of haploid males. The body size of diploid and triploid males in *B. terrestris* has also been found to be smaller than that of haploid males (Ayabe et al. 2004). It has also been shown that the wet weight and reproductive organs of diploid drones of honeybees, *Apis cerana* are significantly lighter than those of haploid drones (Yan et al. 2016). In general, the body size of polyploids is often bigger than that of normal individuals. For example, the pupae of diploid and triploid males and triploid females in turnip sawfly, *Athalia rosae ruficornis* have a longer head width and heavier body weight than those of haploid males (Naito and Suzuki 1991). Thus, the effect of polyploidization on morphological characteristics of males varies among species, and social bees such as bumblebees and honeybees tend to dwarf in body size.

Since diploid males of bumblebees are born from fertilized eggs in the early stage of colony development when the number of workers is low, it predicts that the amount of food provided to them in the larval stage is lower than that of haploid males born from unfertilized eggs in the late stage of the colony development. Further verification is needed to determine whether the factor causing the smaller body size is due to polyploidization or the nutritional conditions (amount of food provided by workers).



**Figure 6.** Effects of polyploidization on the behavioral traits and the body size in *Bombus ignitus* males. The arrows indicate regression coefficients obtained by regression analysis and correlation coefficients obtained by correlation analysis and their statistical significance (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

## Conclusion

In this study, we showed that the polyploidization occurring in male bumblebees, *B. ignitus* affects their behavioral traits and morphological parameters. In particular, increased locomotor activity, reduced flight ability and smaller body size would be expected to cause reduced mating rates of diploid males in the field. As a result, the emerged sterile triploids may be unintentionally avoided. These findings contribute to a further understanding of the characteristics of diploid males that emerged under the sl-CSD system.

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## References

- Ayabe T., Hoshiba H., Ono M. (2004). Cytological evidence for triploid males and females in the bumblebee, *Bombus terrestris*. *Chromosome Research*, 12: 215–223. <https://doi.org/10.1023/B:CHRO.0000021880.83639.4b>
- Bostian C.H. (1935). Biparental males and hatchability of eggs in *Habrobracon*. *Genetics*, 20: 280–285. [10.1093/genetics/20.3.280](https://doi.org/10.1093/genetics/20.3.280)
- Brock A. H. (2013). A review of the consequences of complementary sex determination and diploid male production on mating failures in the Hymenoptera. *Entomologia Experimentalis et Applicata.*, 146: 156–164. <https://doi.org/10.1111/j.1570-7458.2012.01306.x>
- Darvill B., Lepais O., Woodall L.C., Goulson D. (2012). Triploid bumblebees indicate a direct cost of inbreeding in fragmented populations. *Molecular ecology*, 21: 3988–3995. <https://doi.org/10.1111/j.1365-294X.2012.05679.x>
- Darvill B., Ellis J.S., Lye C., Goulson D. (2006). Population structure and inbreeding in a rare and declining bumblebee, *Bombus muscorum* (Hymenoptera: Apidae). *Molecular Ecology*, 15: 601–611. <https://doi.org/10.1111/j.1365-294X.2006.02797.x>
- Duchateau M.J., Marien J. (1995). Sexual biology of haploid and diploid males in the bumblebee *Bombus terrestris*. *Insectes Soc.*, 42: 255–266. <https://doi.org/10.1007/BF01240420>
- Duchateau M.J., Velthuis H.H.W. (1988). Development and reproductive strategies in *Bombus terrestris* colonies. *Behaviour*, 107, 186–207. <https://doi.org/10.1163/156853988X00340>
- Ellis J.S., Knight M.E., Darvill B., Goulson D. (2006). Extremely low effective population sizes, genetic structuring and reduced genetic diversity in a threatened bumblebee species, *Bombus sylvarum* (Hymenoptera: Apidae). *Molecular Ecology*, 15: 4375–4386. <https://doi.org/10.1111/j.1365-294X.2006.03121.x>
- Knakievicz T., Lau A.H., Prá D., Erdtmann B. (2007). Biogeography and karyotypes of freshwater Planarians (Platyhelminthes, Tricladida, Paludicola) in Southern Brazil. *Zoological Science*, 24: 123–129. <https://doi.org/10.2108/zsj.24.123>

- Mori R., Kobayashi T., Yoshida A. (2018). Adaptive significance of the experimentally obtained diploid male fertility in the Japanese bumblebee *Bombus ignitus* with complementary sex determination. <https://abstracts.societyforscience.org/Home/FullAbstract?ISEFYears=2019%2C&Category=Any%20Category&AllAbstracts=True&FairCountry=Any%20Country&FairState=Any%20State&Keywords=bumblebee&ProjectId=17376>
- Naito T. and Suzuki H. (1991). Sex determination in the Sawfly, *Athalia rosae ruficornis* (Hymenoptera): occurrence of triploid males. *The Journal of Heredity*, 1991:82. 101–104. <https://doi.org/10.1093/oxfordjournals.jhered.a111042>
- Takahashi J., Ayabe T., Mitsuhashi M., Shimizu I., Ono M. (2008). Diploid male production in a rare and locally distributed bumblebee, *Bombus florilegus* (Hymenoptera, Apidae). *Insect. Soc.*, 55: 43–50. <https://doi.org/10.1007/s00040-007-0976-z>
- Takeuchi K. (1979). On the chromosomes of polyploid races of parthenogenetic weevils (Coleoptera: Curculionidae) *Zoological magazine*, 88: 602. (in Japanese)
- Tamura S., Oki I., Kawakatsu M. (1995). A review of chromosomal variation in *Dugesia japonica* and *D. ryukyensis* in the Far East. *Hydrobiologia*, 30579–84. <https://doi.org/10.1007/BF00036366>
- Tasei J.N., Moinard C., Moreau L., Himpens B., Guyonnaud S. (1998). Relationship between aging, mating and sperm production in captive *Bombus terrestris*. *J. Apic. Res.*, 37:107–113 <https://doi.org/10.1080/00218839.1998.11100962>
- van Wilgenburg E., Driessen G., Beukeboom L. (2006). Single locus complementary sex determination in Hymenoptera: an "unintelligent" design? *Frontiers in Zoology*, 3: 1. <https://doi.org/10.1186/1742-9994-3-1>
- Whiting P.W. (1943). Multiple alleles in complementary sex determination of *Habrobracon* *Genetics*, 28: 365–382. <https://doi.org/10.1093%2Fgenetics%2F28.5.365>
- Yan W.Y., Gan H.Y., Li S.Y., Hu J.H., Wang Z.L., Wu X.B., Zen Z.J. (2016). Morphology and transcriptome differences between the haploid and diploid drones of *Apis cerana*. *Journal of Asia-Pacific Entomology*, 19: 1167–1173. <https://doi.org/10.1016/j.aspen.2016.10.010>
- Zayed A., Packer L. (2005). Complementary sex determination substantially increases extinction proneness of haplodiploid populations. *PNAS*, 226: 10742–10746. <https://doi.org/10.1073/pnas.0502271102>