Absence of complementary sex determination in *Trichogramma dendrolimi* Matsumura (Hymenoptera: Trichogrammatidae)

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Over 60 species in Hymenoptera have been reported to possess a complementary sex determination (CSD) system. Under CSD, sex is determined by allelic complementation at one or several sex loci. But this mechanism is still uninvestigated in parasitoid wasp *Trichogramma dendrolimi*, one of the most important biocontrol agents widely used against Lepidopteran pests. We tested CSD in this species by conducting ten consecutive generations of inbreeding, to monitor both direct evidence (diploid male production) and indirect evidence (brood size, sex ratio, mortality). In total 475 males detected from this inbreeding regime, only one was determined as diploidy. The observed proportions of diploid male offspring significantly differed from expected values under CSD model involving up to ten independent loci, allowing us to safely reject CSD in *T. dendrolimi*. Meanwhile, the possibility of unviable diploid males was excluded by the absence of significant differences in brood size, offspring sex ratio and offspring mortality among different generations. Our study of sex determination in *T. dendrolimi* provides useful information for the mass rearing conditions in a biofactory and the quality improvement of this biocontrol agent. It also brings necessary background to further study of the sex determination in *Trichogramma*.

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Short running title: Absence of CSD in *T. dendrolimi*

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Key words: multilocus complementary sex determination; maternal effect genomic imprinting sex determination; diploid male; inbreeding; biocontrol; mass rearing

Abstract

Over 60 species in Hymenoptera have been reported to possess a complementary sex determination (CSD) system. Under CSD, sex is determined by allelic complementation at one or several sex loci. But this mechanism is still uninvestigated in parasitoid wasp *Trichogramma dendrolimi*, one of the most important biocontrol agents widely used against Lepidopteran pests. We tested CSD in this species by conducting ten consecutive generations of inbreeding, to monitor both direct evidence (diploid male production) and indirect evidence (brood size, sex ratio, mortality). In total 475 males detected from this inbreeding regime, only one was determined as diploidy. The observed proportions of diploid male offspring significantly differed from expected values under CSD model involving up to ten independent loci, allowing us to safely reject CSD in *T. dendrolimi*. Meanwhile, the possibility of unviable diploid males was excluded by the absence of significant differences in brood size, offspring sex ratio and offspring mortality among different generations. Our study of sex determination in *T. dendrolimi* provides useful information for the mass rearing conditions in a biofactory and the quality improvement of this biocontrol agent. It also brings necessary background to further study of the sex determination in *Trichogramma*.

Introduction

Over million years of evolution, insects have established a wide diversity of sex determination mechanisms. Of which, the most well studied cases focus on the orders of Diptera, Lepidoptera and Hymenoptera. In Diptera, *Drosophila melanogaster* determines the sex through the dose of X chromosomes, i.e., double dose for females (XX) and single dose for males (XO), while in most other dipterans (Erickson and Quintero, 2007), the sex was decided by whether there is a Y chromosome to determine the females (XX) and males (XY) (Pane *et al*., 2002; Dubendorfer *et al*., 2003). In the meantime, an opposite mechanism to that of Diptera is applied in most Lepidopterans where females are heterogametic (ZW) and males are homogametic (ZZ) (Sahara *et al*., 2012). Haplodiploid reproducing hymenopterans, however, employ a completely different sex determination system which relies on the number of chromosome sets in embryos instead of the presence of sex chromosomes: females are diploid, developing from fertilized eggs, while males are haploid and arise from unfertilized eggs (Cook, 1993a; Heimpel and de Boer, 2008). Up till now, two genetic mechanisms of sex determination have been attested in the Hymenoptera: complementary sex determination (CSD) and maternal effect genomic imprinting sex determination (MEGISD) (Blackmon *et al*., 2016).

Compared to MEGISD only found in *Nasonia vitripennis* (Verhulst *et al*., 2010), CSD mechanism seems to have a much wider distribution. Thus far, it has been documented in over 60 species of Hymenoptera (Harpur *et al*.,2012). Under this mechanism, sex is determined by allelic complementation at sex (*csd*) locus, i.e., heterozygosity initiates the development of females, whereas homozygosity or hemizygosity leads to the diploid or haploid male

development respectively. And according to the number of sex loci, CSD can be divided into single-locus CSD (sl-CSD) and multilocus CSD (ml-CSD). The former was firstly proposed by Whiting (1933, 1943) and has now been documented in honeybee *Apis mellifera*, some bumble bees and ants (Beye *et al*., 2003; Hasselmann *et al*., 2008; Schmieder *et al*., 2012). However, diploid males are generally sterile or unviable with consequent serious fitness costs to a population (Cook and Crozier, 1995; Zayed, 2004; Zayed and Packer, 2005). Apparently, in this case, sl-CSD model is not reconciled with hymenopteran species of those with a life-history of frequent natural inbreeding, because this would sharply increase diploid males' production (Zayed, 2004; Zayed and Packer, 2005; Beukeboom *et al*., 2000; Niyibigira *et al*., 2004; Schrempf *et al*., 2006). Therefore, Snell (1935) and Crozier (1971) stated that regularly inbred species of Hymenoptera may reduce the production of diploid males by increasing the number of *csd* loci (i.e., ml-CSD), as under ml-CSD mechanism diploid males occur only when all *csd* loci are homozygous. In non-model systems it is exceedingly laborious to identify the number of *csd* loci using molecular methods, so multiple generations of inbreeding is usually taken as a more sensible alternative to detect the presence of ml-CSD (Ma *et al*., 2013). By this means successful identifications of ml-CSD have been reported in several species such as *Cotesia vestalis*, *C. rubecula* and *Diachasmimorpha longicaudata* (de Boer *et al*., 2008, 2012; Paladino *et al*., 2015). The aim here is to investigate the presence of CSD in an economically important egg parasitoid, *Trichogramma dendrolimi* Matsumura. This specie is one of the most important biocontrol agents in China widely used in agricultural and forestry production for its high parasitism rate, adaption to multiple lepidopteran pests, wide distribution, ability to be mass reared on big or

factitious eggs (Wang *et al*., 2014; Göttig & Herz, 2016; Li *et al*. 2016; Zhang *et al*. 2016; Hou *et al*. 2018). In order to optimize its production of good quality and field performance, the knowledge of its physiology, behavior and genetics are required. One of the important aspects is the identification of sex determination of *T. dendrolimi*, whereby improved measures to increase female production could be brought up, since only females are responsible for the damage to lepidopteran pests (i.e., only females make parasitization) and thus determine the productivity of a biofactory.

From the life-history it can be inferred that *T. dendrolimi* should be incompatible with sl-CSD, as frequent natural inbreeding was reported in this species (Tang *et al*., 1988). But whether it carries an ml-CSD mechanism still needs further investigation. In current study, an experiment of consecutive generations of inbreeding was carried out, to assess the presence of CSD by analyzing the primary indicator (proportion of diploid male offspring) and secondary indicator (brood size, offspring sex ratio and offspring mortality) among different generations. Our results bring useful information to the mass rearing conditions and quality improvement of this important biocontrol agent, and necessary background for further studying sex determination in *Trichogramma*.

Materials and Methods

Insects

All insects used in the study were supplied by the Pest Biological Control Laboratory, Shenyang Agricultural University, including one bisexual strain (Td-HR) of *T. dendrolimi* and its host *Corcyra cephalonica* (Lepidoptera: Pyralidae). The wasps were reared at controlled conditions:

(25 ± 1 °C, RH 70 ± 5%, 16 : 8 h light : dark photoperiod) on eggs of *C. cephalonica* (Lepidoptera: Pyralidae). The latter was reared on maize flour and wheat bran at similar conditions except that the temperature was 26 ± 1 °C (Yang *et al*., 1990). The eggs of *C. cephalonica* were collected daily and killed by ultraviolet (UV) lamps (30W; TUV30W; Philips, Amsterdam, the Netherlands) before they were used for parasitization. The parasitized eggs will turn black within four to five days when the *T. dendrolimi* pupates.

CSD Assay

The detection of CSD mechanisms with different *csd* loci referred to that of de Boer *et al*. (2008) and Ma *et al*. (2013). Briefly, ten generations of inbreeding was conducted. At each generation, the proportion of diploid male offspring, brood size, offspring sex ratio and offspring mortality were investigated. Of which, the first indicator was considered as the primary indicator which is the most direct evidence for the presence of CSD, while the rest three were secondary indicators that were monitored because: 1) the values of them could be affected by the occurrence of diploid males; 2) the diploid males could be unviable.

The offspring mortality was defined as:

$$\frac{\text{Shrivelled host eggs without vinsible wasps} + \text{Dead wasps unable to be sexed}}{\text{Total host eggs supplied for parasitization}}$$

The proportion of diploid male offspring was determined as:

$$\frac{\text{The number of diploid males}}{\text{The number of diploid males} + \text{The number of females}}$$

The inbreeding experiment started with one generation (I_0) of backcross between mother and son (M-S), followed by nine generations (I_1-I_9) of full-sib (brother-sister, B-S) matings. Under sl-

CSD, half of the offspring are expected to be diploid males in an M-S cross, since half of the fertilized eggs will be homozygous at *csd* locus. For the B-S cross, the production of diploid males depends on whether brother and sister carry an identical *csd* allele. Meanwhile, under ml-CSD, diploid males occur only when all *csd* loci become homozygous. Therefore, provided *T. dendrolimi* has a sl-CSD mechanism, the proportion of diploid males will remain 0.5 over the multiple generations of inbreeding. In contrast, under ml-CSD, proportion of diploid males from an M-S cross presents a function relation with the number of *csd* loci ($0.5^{\text{number of } csd \text{ loci}}$), and such proportion will increase rapidly during the subsequent B-S crosses (Ma *et al*., 2013).

Inbreeding Experiment

M-S Backcross

Two hundred parasitized black *C. cephalonica* eggs were collected and isolated individually in a single 220μL PCR tube to get the virgin females, since *T. dendrolimi* only deposit one offspring in a single *C. cephalonica* egg when the latter is sufficient. After emergence, 100 females (mother, I_0) were selected and each of them was allowed to oviposit on superfluous (~150) *C. cephalonica* eggs for 1h to produce haploid sons. The mothers were then supplied with 10% honey-water solution and kept under the conditions of 16 °C, 70 ± 5% RH, and no light, while their sons were developing. After the sons emerged, one of them was randomly picked and allowed to mate with its mother (if it was still alive). Totally 31 inbred lines were set up in this part. Hereafter surplus (150~400) *C. cephalonica* eggs were provided to each mother for 24h to produce I_1 offspring (diploid females). Before performance of the backcrosses, mothers were provided with honey-water solution again to get refreshed, and another extra 1h virgin

parasitization was taken to ensure the production of virgin haploid I_1 males.

B-S Cross

Similar to M-S backcross, for each inbred line ten black eggs containing I_1 wasps were isolated to get virgin I_1 female. After emergence, three females were randomly selected to produce haploid male offspring (I_2) on *C. cephalonica* eggs for 1h. Subsequently, they were individually matched with one of their haploid brothers (three repetitions). The mated I_1 females would parasitize the eggs of *C. cephalonica* for 24h. Such B-S crosses were continued for nine generations (I_1 to I_9).

For each generation, the brood size, offspring sex ratio (males / total emerged wasps), offspring mortality was counted. The ploidy of male offspring was detected only for generation 0-2 and 9 (I_0-I_2, I_9) due to the large sample size, by randomly selecting 5 males for each inbred line (total 475, averaging 37.7%). See below for the measurement of ploidy.

The control was conducted by performing a ten generations of continuous outcross (generation 0-9, O_0-O_9) between virgin females and non-related males. The process of outcross experiment was similar to that of inbreeding. At each generation, 15 outbred lines were established.

Detection of Diploid Males

Our previous application of flow cytometry failed to differentiate between haploid and diploid *T. dendrolimi*, thus a quantitative PCR (qPCR) method was applied in this study (Liu *et al*., unpublished data). The method is based on the mitochondrial content that is not or hardly affected by ploidy of *T. dendrolimi*, so that haploids have about twice mtDNA per nuclear copy as diploids (Tulgetske, 2010). Firstly, the genomic DNA was extracted from male heads (without

antennae) with a Chelex method (Sumer *et al*., 2009), because nervous tissue has been proved to provide accurate information of ploidy in Hymenoptera (Aron *et al*, 2005): the head of a single male wasp was dissected and ground in a 1.5 mL eppendorf tube containing 50μL 5% Chelex-100 and 2.5 μL proteinase K (20mg ml^{-1}), followed by 1h incubation at 55 °C and a final 10 min at 99 °C. To detect the ploidy, a mitochondrial gene cytochrome oxidase I (COI) and a nuclear gene *forkhead* were used in the qPCR operation. Primers for amplification of COI or *forkhead* region were newly designed in Primer 5.0 software (Primer-E LTd., Plymouth, UK) and listed in Table 1. The qPCR was performed in a Bio-Rad CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, California, USA) with following conditions: 95 °C for 5 min, then 40 cycles of 95 °C for 15 s and 55 °C for 45 s. Each qPCR reaction was done in a 20 μL total volume containing 10 μL 2 × GoTaq qPCR Master Mix (Promega, Madison, Wisconsin, USA), 0.5 μL of each respective primer (10μM), 1 μL DNA template and 8 μL ddH_2O. The specificity and efficiency of primers were checked and calculated respectively prior to estimating the ploidy. A single female per qPCR performance was used as diploid control. The haploids and diploids can be discriminated according to the different $2^{-\Delta Cq}$ value ranges or the $2^{-\Delta\Delta Cq}$ value which in a haploid is about twice as much as that of a diploid.

Statistical Analysis

The significant differences between observed proportions of diploid male offspring and values expected under CSD with one locus, two, three or ten loci were evaluated by one-sample Wilcoxon signed rank test.

The comparisons of brood size or offspring sex ratio among different generations were estimated

by a nonparametric method (Kruskal-Wallis test) followed by Dunn's multiple comparisons test. The offspring mortality was analyzed by generalized linear models (GLMs) based on a quasibinomial distribution, followed by Tukey post hoc test.

All statistical analyses were performed in R software (version 3.5.2) (R Core Team, 2017).

Results

Primary Indicator

Proportion of diploid male offspring was investigated for the first three (I_0-I_2) and the last (I_9) inbreeding generations. On average, five males per inbred line (resulting in a total of 475 samples) were randomly selected. Only one diploid male was found in the offspring of I_9. Results of Wilcoxon signed rank test showed that neither of the observed proportions of diploid male offspring from I_1 and I_9 fitted the CSD model with one, two, three, or ten independent loci (Table 2).

Secondary Indicator

The absence of diploid male offspring, though can be seen as strong evidence for the absence of CSD mechanism, diploid males could be unviable and hence undetected. The brood size, offspring sex ratio and offspring mortality were therefore monitored.

The comparison results for every secondary indicator were showed in Fig. 1-3 respectively. A significant difference in overall brood size was noted after the Kruskal-Wallis rank sum test (χ^2 =90.39, df = 19, $p < 0.001$; Fig. 1). The Dunn's multiple comparisons test further indicated a significantly smaller brood size of first generation in both inbreeding and outcross experiments than that of other inbreeding or outcross generations ($p < 0.001$ for all). However, there was no

198 significant differences between the two first generations ($z = -0.23$, $p = 0.992$). Also, no
199 significant differences were found in comparisons among other generations or between
200 inbreeding and outcross ($p > 0.05$ for all). The extra reproduction of females for 1h, which
201 supplied sons to be backcrossed with mothers, should be behind the significantly smaller brood
202 size of first generation.
203 Similarly, an overall significant difference appeared in offspring sex ratio ($\chi^2 = 40.60$, df = 19, p
204 = 0.003; Fig. 2). But further analyses showed that the differences were only between I6 and I1 (z
205 = -3.51, $p = 0.028$), I3 ($z = -3.88$, $p = 0.020$), or I5 ($z = -3.53$, $p = 0.039$). No other significant
206 sex ratio differences were found ($p > 0.05$ for all).
207 Offspring mortality was not significantly influenced by the type of cross (inbreeding or outcross)
208 ($\chi^2 = 3.17$, df = 1, $p = 0.075$), but generation ($\chi^2 = 25.65$, df = 9, $p = 0.002$) (Fig. 3). There was a
209 significant interaction between generation and cross type ($\chi^2 = 19.69$, df = 9, $p = 0.020$). Under
210 inbreeding, females of I_5 had a significantly higher offspring mortality than that of I_0 ($z = -3.6$, p
211 = 0.012), I_3 ($z = -3.38$, $p = 0.025$), I_9 ($z = -3.75$, $p = 0.007$). In generation 4 ($z = -2.10$, $p = 0.036$)
212 and 9 ($z = -2.54$, $p = 0.011$), the offspring mortality was significantly lower for inbreeding than
213 that of the corresponding outcross respectively. But an opposite scenario was exhibited in
214 generation 5 ($z = 2.11$, $p = 0.034$).

215 Discussion

216 This is the first attempt to explore the possibility of CSD mechanism in *T. dendrolimi*. Previous
217 study reported that *T. dendrolimi* had frequent natural inbreeding (Tang *et al*., 1988), thus
218 implies an ml-CSD or another mechanism underlying the sex determination of this species. But

the absence of diploid males and absence of differences in brood size, offspring sex ratio or offspring mortality excluded the first hypothesis.

The primary indicator, proportion of diploid male offspring, as the most direct evidence for presence of CSD was firstly investigated. In nearly 500 males, only one was determined as diploidy, significantly different from the theoretical value in the CSD involving up to ten loci. However, the absence of diploid males could be just because they are unviable (Beukeboom *et al*., 2000; Zayed and Packer, 2005; Petters and Mettus, 1980). Such unviability in this study can be split in two cases: invisible or visible (Ma *et al*., 2013; Paladino *et al*., 2015; Stouthamer and Kazmer, 1994). The former means that diploid males die when they are still hard to be seen by naked eyes. In this case, the brood size is expected to decrease while the offspring sex (male) ratio and offspring mortality would increase over the inbreeding generations, since invisible diploid males come at the expense of female production and host eggs. In contrast, the diploid males of latter case are alive until they develop into stages that are megascopic (e.g. pupal stage), where offspring sex ratio and offspring mortality are predicted to increase when the brood size keeps constant. However, our data fit neither of the cases, suggesting the absence of diploid male mortality.

With the confirmation of no unviable diploid males, the CSD involving up to ten loci can be got rid of in *T. dendrolimi*. Cook (1993b) pointed out that rejection of CSD mechanism with ten loci is a valid refutation of ml-CSD, because it will be hard for selection to maintain polymorphism at each sex locus (Crozier, 1971, 1977). Also, the author contended that ten generations of inbreeding is adequate to detection for CSD with up to 15 loci. Therefore, both sl-CSD and ml-

CSD mechanisms can be safely rejected in current study.

One question that arose in study is how come the single diploid male was produced when CSD mechanism was not behind the sex determination? Given the large sample size, it could be the result of sampling error. But the occasional diploid males have been also reported in other non-CSD hymenopterans (Schrempf *et al*., 2006; Trent *et al*., 2006). Ma *et al*. (2013) argued that such diploid males are likely the results of rare genetic mutation or errors of endoduplication. Though hymenopteran wasps were separated into haploid and diploid individuals, haploids, actually, also possess certain diploid tissue (Aron *et al*., 2005). Thus, if errors of endoduplication occur during early development, diploid males might be yielded.

Several alternative mechanisms have been proposed for non-CSD hymenopteran species (Beukeboom and van de Zande, 2010), but only MEGISD mechanism was experimentally demonstrated in *Nasonia vitripennis* (Verhulst *et al*., 2010). Under MEGISD, the female development requires an initiation of maternally silenced *transformer* gene by the input of paternal genome to autoregulate the female-specific transcript, whereas the silenced state of *transformer* in unfertilized eggs leads to the male development (van de Zande and Verhulst, 2014). Obviously, in contrast to CSD, the species with a MEGISD mechanism is not affected by the condition of inbreeding, and consequently do not produce diploid males that impose fitness costs on populations. Therefore, to hymenopterans like *T. dendrolimi* that have a life-history of frequent natural inbreeding, MEGISD could be a suitable mechanism underlying the sex determination. A recent study, however, showed that *N. vitripennis* could suppress the density of *Wolbachia*, the maternally inherited endosymbionts known for their widespread distribution and

ability to manipulate biological phenotype of their hosts (Werren *et al*., 2008; Zug and Hammerstein, 2012), by 100-fold through a maternal genetic effect (Funkhouser-Jones *et al*., 2018). The thelytokous manipulation of *Wolbachia* in species with a MEGISD mechanism might be constrained, since such thelytoky depends on the titer of *Wolbachia* (Ma *et al*., 2015). Previous studies have reported that *T. dendrolimi* can reproduce thelytokously under the control of *Wolbachia* (e.g., Liu *et al*., 2018). Following the reasoning, another mechanism than CSD and MEGISD may be responsible for the sex determination in *T. dendrolimi*. Interestingly and similarly, *Asobara* species of non-CSD also show frequent natural inbreeding and take thelytokous reproduction when infected with thelytoky-inducing *Wolbachia* (Ma *et al*., 2013, 2015). Furthermore, in an *Asobara* species, *A. tabida*, the maternally provided female-specific gene *transformer* is absent, making its sex determination system in some degree different from the MEGISD system of *N. vitripennis* (Geuverink *et al*., 2018). According to the information above, we can conjecture that *T. dendrolimi* and *Asobara* species have a similar mechanism of sex determination. However, further studies are needed to prove our assumption.

Diploid males are not a fitness cost for their absence to *T. dendrolimi*. Such is the case with inbreeding depression. Based on consecutive generations of inbreeding for a year, no evidence of inbreeding depression was showed in *T. dendrolimi* (Li and Zhang, 1980). In fact, the absence of inbreeding depression in genus *Trichogramma* seems generally (Li and Zhang, 1980; Sorati *et al*., 1996). Taken together, a potentially loose rearing condition of inbreeding can be expected in terms of *T. dendrolimi* mass-reared in biofactories for biocontrol strategies. And the key fitness costs involved in protocol of mass production should be the hosts of bad quality, superfluous

wasps to host eggs, confined and / or cluttered environment for parasitization, as stated by previous researchers (Li and Zhang, 1980; Lü *et al*., 2017). Moreover, given the absence of diploid males and inbreeding depression, selection and stabilization of beneficial biocontrol traits such as big size, long lifespan, high fecundity and vagility (Boivin, 2010), through progressive generations of inbreeding can be adopted (Bai *et al*.,2005; Anbesse *et al*., 2013), to ameliorate the efficiency and efficacy of *T. dendrolimi* in practical application.

Conclusions

The sex determination mechanism CSD was investigated in *T. dendrolimi*, an economically important biocontrol agent. Based on the absence of diploid male production and the absence of differences in brood size, offspring sex ratio and offspring mortality over successive generations of strict inbreeding, we were allowed to reject the CSD mechanism. This is reconciled with the specific life-history strategy of frequent natural inbreeding in *T. dendrolimi*. The absence of diploid males, together with the absence of inbreeding depression suggested a relatively loose rearing condition of inbreeding for *T. dendrolimi* in terms of mass-rearing for biocontrol. Such characteristics also provided us a possible measure, i.e., consecutive inbreeding, whereby we could obtain and stabilize important biocontrol traits. Lastly, since both *T. dendrolimi* and *Asobara* species have frequent inbreeding in nature and can be induced by *Wolbachia* to reproduce thelytokously, they might have a same or similar mechanism other than CSD and MEGISD underlying the sex determination. After elimination of CSD in *T. dendrolimi*, the next step should focus on the investigation of MEGISD or another unknown mechanism, which could be achieved by the identification of sex-determining gene and their roles in a molecular pathway.

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Figure 1(on next page)

Brood size in different generations.

I_0-I_9: represent generations of inbred lines; O_0-O_9: represent generations of outbred lines.

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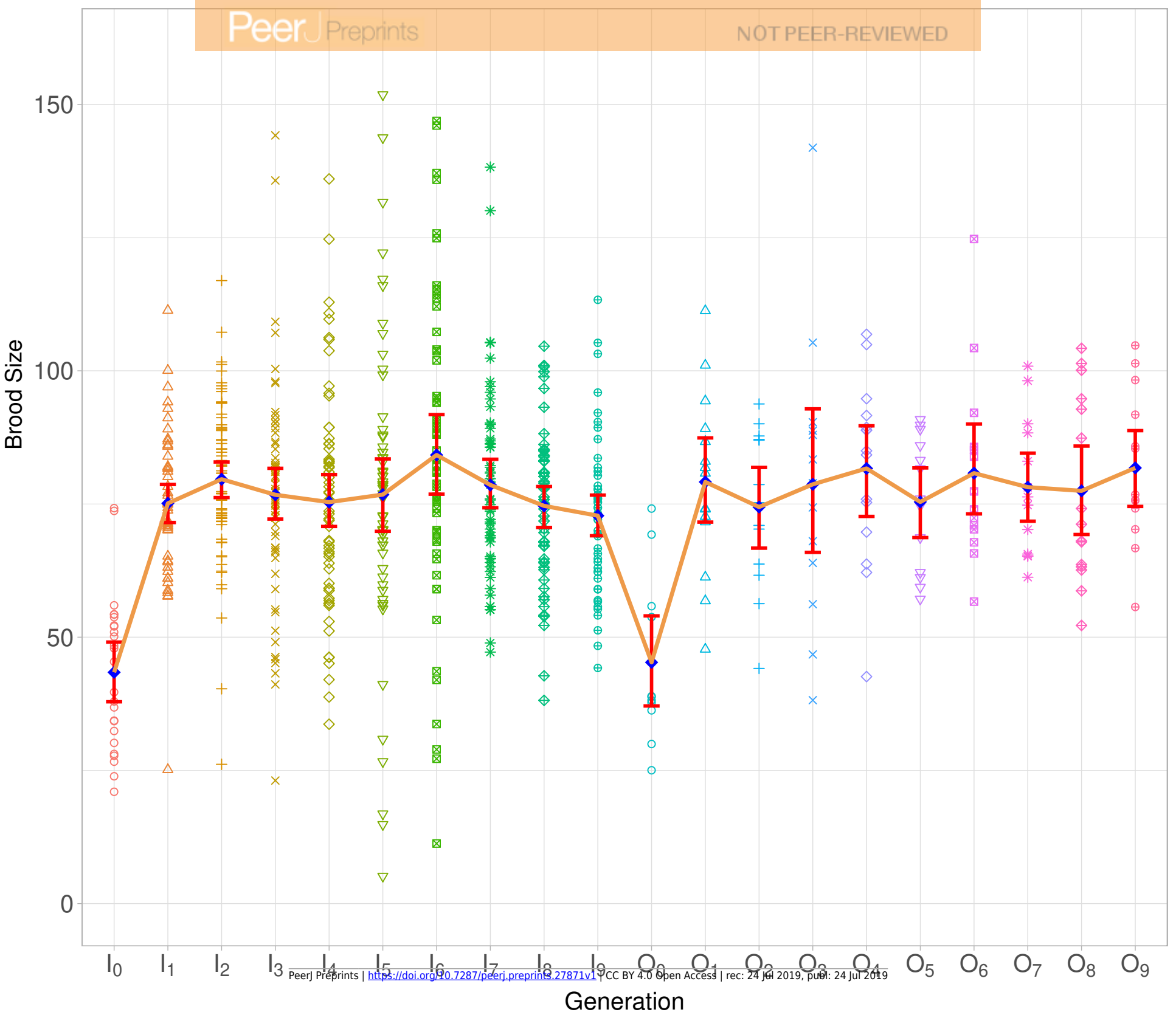

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Figure 2(on next page)

Offspring sex ratio in different generations.

I_0-I_9: represent generations of inbred lines; O_0-O_9: represent generations of outbred lines.

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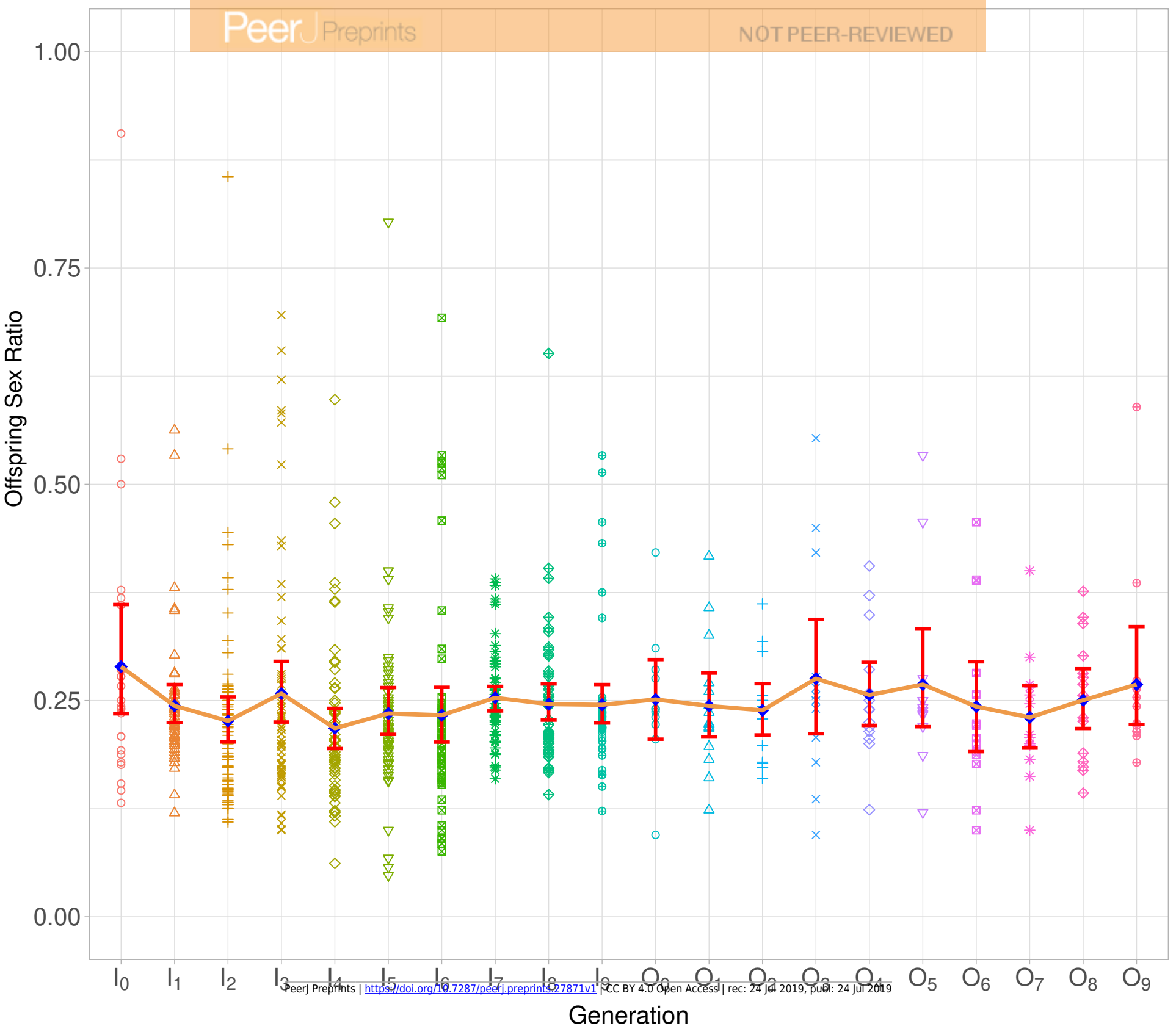

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Figure 3(on next page)

Offspring mortality rate in different generations.

I_0-I_9: represent generations of inbred lines; O_0-O_9: represent generations of outbred lines.

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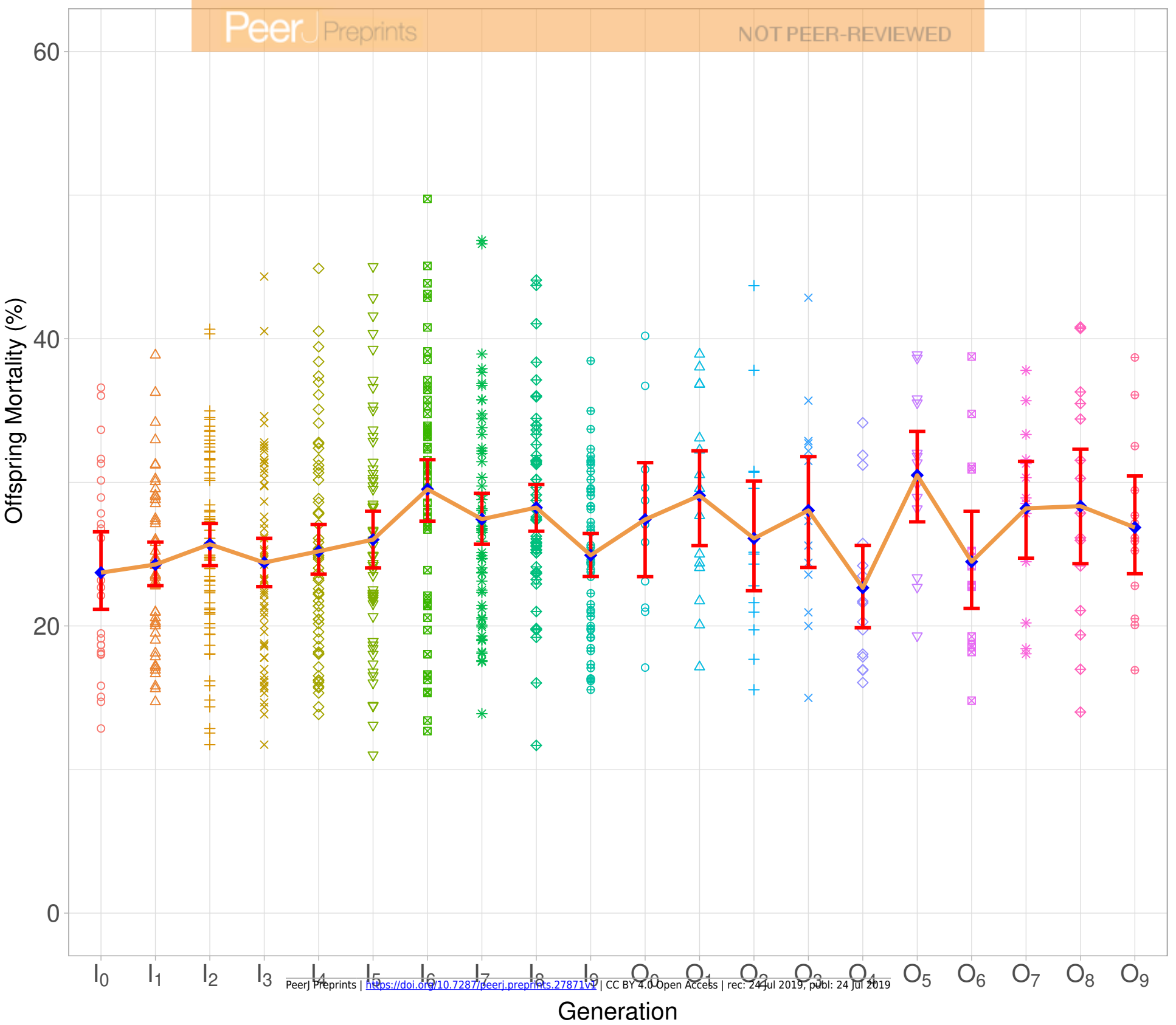

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Table 1(on next page)

Primer pairs for qPCR.

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Table 1:

Primer pairs for qPCR.

Target region	Gene	Primer name	Sequence	Product size (bp)
Mitochondrion	COI	dqCOIF	5'-TTGAACTGTTTATCCTCCTT-3'	96
		dqCOIR	5'-GATGAAACCCCAGCAATA-3'	
Nucleus	*forkhead*	dqFHF	5′-CTACGCCGATCTCATAACGC-3'	119
		dqFHR	5′-TGCTGTCGCCCTTGTCCT-3′	

Table 2(on next page)

Comparisons between observed proportion of diploid male offspring and expected values under CSD with different loci (Wilcoxon signed rank test).

Note: The expected values of generation I_9 should be higher than those of I_0 respectively under CSD with / or more than 2 loci (Ma et al., 2013). We here assume I_9 is equal of I_0 in expected values, to avoid the unnecessary complicated calculations.

Table 2:

Comparisons between observed proportion of diploid male offspring and expected values under CSD with different loci (Wilcoxon signed rank test).

Generation	Observed	Expected			
		One locus	Two loci	Three loci	Ten loci
I_0	0	0.5	0.25	0.125	0.0009765
		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
I_9	0.000806	0.5	0.25	0.125	0.0009765
		$P = 0.002$	$P = 0.002$	$P = 0.002$	$P = 0.006$

Note: The expected values of generation I_9 should be higher than those of I_0 respectively under CSD with / or more than 2 loci (Ma et al., 2013). We here assume I_9 is equal of I_0 in expected values, to avoid the unnecessary complicated calculations.