Gradual, temperature-induced change of secondary sexual characteristics in *Trichogramma pretiosum* infected with parthenogenesis-inducing *Wolbachia*

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Abstract

Intersex is an intermediate stage of sexual differentiation in insects. Determining intersex morphology and the cause of its production will aid in the understanding of the mechanism of sexual differentiation in insects. In this paper, *Wolbachia*-infected *Trichogramma pretiosum* (*T. preW+*) that shows thelytokous parthenogenesis were used as subjects. In order to determine the causes of the *T. preW+* intersex and the influence of parental generation temperature on gradual changes in secondary masculinization in intersex offspring, we examined the occurrence of

23   intersex offspring (F1 and F2 generation) after the parental generations were treated with high

24   temperature (27℃, 29℃, 31℃, and 33℃) and described the external morphology of the

25   intersexes. The results showed that the *T. preW*$^+$ parental generation temperature is positively

26   correlated with the probability of intersex offspring. The probability of F1 intersex is

27   significantly higher than that of F2 intersex in different high temperature. The degree of

28   secondary masculinization in *T. preW*$^+$ intersexes increases as parental generation temperature

29   increases. In addition, our study first identified 11 intersex types in *T. preW*$^+$ and found that the

30   primary and secondary sexual characteristics showed a regular distribution. And we found that

31   the D type and H type of intersex have the highest frequency of appearance. The external

32   genitalia of most intersexes were female, and only three intersex types have male external

33   genitalia. Conclusions were ultimately obtained: *Wolbachia* is a direct factor that causes the

34   occurrence of intersexes, while high temperature is an indirect factor that determines the external

35   morphology of intersexes. The effects of high temperature on *T. preW*$^+$ intersexes is passed

36   through the parental generation to offspring, and this maternal effect weakens as the number of

37   generations increases. In *T. preW*$^+$ intersex individuals, most exhibit female primary sexual

38   characteristics, and secondary sexual characteristics exhibit signs of masculinization.

39

40   **Introduction**

41   The difference between the sexes of the same species is called sexual dimorphism in nature.

42   Generally, two types of sexual characteristics are used to describe sexual dimorphism. Primary

43   sexual characteristics include male and female reproductive organs that are directly related to

44   reproduction (such as external genitalia and internal genitalia). Whereas the Secondary sexual

45   characteristics include body size, external morphology, antenna, etc., and are used to describe

46 characteristics that aid in reproduction (Schärer *et al.*, 2012; Tulgetske & Stouthamer *et al.*, 2012;

47 Bear *et al.*, 2013). Usually, sexually aberrant individuals with conflicting primary and secondary

48 sexual characteristics are known as intersexes, gynandromorphs and sexual mosaics (Bowen &

49 Monteiro, 1966; Pintureauand & Bolland, 2001; Pintureau *et al.*, 2002; Tulgetske *et al.*, 2012).

50 Of these terms, intersexes refer to individuals with uniform genetic constitution, but some tissues

51 show sexual phenotypes that conflict with the associated genotype (Goldschmidt *et al.*, 1949).

52 However, this concept is frequently confused or wrongly used with gynandromorphs (Tulgetske

53 *et al.*, 2012). In actual fact, the latter refers to individuals that are composed of genetically

54 distinct tissues that correspond to the sexual phenotypes observed (Brust *et al.*, 1966). An

55 example is the genus *Trichogramma* in the order Hymenoptera, sexually aberrant individuals

56 were often described as gynandromorphs and intersexes. However, Tulgetske et al.(2012)

57 showed that these individuals are intersexes and not gynandromorphs. The intersexes can be falls

58 into two categories: female intersex and male intersex in Hymenoptera (Whiting *et al.*, 1934;

59 Tulgetske *et al.*, 2012). Female intersexes begin their development as females but organs

60 developed later are masculinized, and male intersexes begin their development as males but

61 organs developed later are feminized (Whiting *et al.*, 1934). As intersex is an intermediate stage

62 of sexual differentiation in insects (Bowen *et al.*, 1966), in-depth research on these rare sexually

63 aberrant individuals is necessary and crucial. Therefore, determining intersex morphology and

64 the cause of its production will aid in the understanding of the mechanism of sexual

65 differentiation in insects.

66 Many studies showed that intersex formation may be affected by single or multiple regulatory

67 factors that participate in sex determination. These regulatory factors include mutations (Cline *et*

68 *al.*, 1984; Schutt & Nöthiger, 2000), chromosomal aberrations (Seiler *et al* 1958; Beukeboom &

69   Kamping, 2006), and epigenetic factors (eg. environment, sex hormones and symbionts) (Rigaud

70   & Juchault, 1998; Narita *et al.*, 2010; Tulgetske e*t al.*, 2012; Martin & Scholtz, 2012). In

71   *Trichogramma*, intersex formation is associated with *Wolbachia* regulation. The intracellular

72   symbiotic bacteria, *Wolbachia*, is a type of sex regulatory element that is ubiquitous in

73   arthropods. *Wolbachia* can induce insects to undergo parthenogenesis (PI), cytoplasmic

74   incompatibility(CI), male-killing(MK), and feminization (Werren & Windsor, 2000; Almeida &

75   Stouthamer, 2018). The sex determination mechanism for *Trichogramma* is haplodiploidy,

76   where fertilized eggs develop into diploid females and unfertilized eggs develop into haploid

77   males (Perlman *et al.*, 2008). However, PI *Wolbachia* can induce parthenogenesis in

78   *Trichogramma* by causing unfertilized eggs to undergo diploidization and feminization. Thus,

79   females could produce nearly 100% female offsprings even without mating (Ma *et al.*, 2015).

80   According to our knowledge of *Trichogramma*, sexually aberrant individuals are only present in

81   parthenogenetic species. (Tulgetske *et al.*, 2010). The *Wolbachia*-mediated phenotype are closely

82   related with *Wolbahcia* titers. Many studies revealed *Wolbachia* were sensitive to mutiple factors

83   (e.g. temperature and antibiotic treatment) (Tulgetske *et al.*, 2010; Tulgetske *et al.*, 2012;

84   Almeida & Stouthamer, 2018). The reduction of *Wolbachia* titers was often reported in high

85   temperature (Zchori-Fein *et al.*, 2000; Ma & Schwander, 2017). Almost no intersexes appear

86   under normal temperature. However, the number of intersexes significantly increases after high

87   temperature or antibiotic treatment (Pintureau & Bolland, 2001; Tulgetske *et al.*, 2010). The

88   specific reason for this may be that *Wolbachia* regulation of *Trichogramma* wasps dominate at

89   normal temperatures but high temperature and antibiotics eliminated the dominance of

90   *Wolbachia*, thereby decreasing *Wolbachia* titers in infected parthenogenetic insects (Schilthuizen

91   & Stouthamer, 1997; Kent *et al.*, 2011; Fukui *et al.*, 2015). This disrupts or inhibits *Wolbachia*-

92   induced feminization (Rigaud *et al.*, 1998), ultimately resulting in a higher proportion of intersex

93   offspring (Pintureau & Bolland, 2001; Pintureau *et al.*, 1999). Intersexes were reported at

94   frequencies of parental generation after continuous high temperature treatment (Pintureau *et al.*,

95   1999; Pintureau & Bolland, 2001). However, whether parental generation treatment will affect

96   intersex offspring and whether parental generation effects can be transmitted to the next

97   generation are still unknown. In addition, there is a lack of objective and accurate estimation of

98   gradual changes in secondary sexual characteristics as influenced by temperature in intersexes.

99   In view of the aforementioned phenomena, we carried out the following study.

100   *Trichogramma* is an extremely effective natural enemy of egg parasites and has an indispensable

101   role in biological pest control (Sigsgaard *et al.,* 2017). In this study, *T. preW*⁺ populations were

102   used as subjects to study the effects of different feeding temperature in the parental generation

103   (27℃, 29℃, 31℃, 33℃) on the probability of intersexes in the F1 and F2 generation. The levels

104   of masculinization, which were determined based on previous descriptions (Tulgetske *et al.*,

105   2012), were introduced to reveal the effector pattern of high temperature on gradual changes in

106   secondary sexual characteristics in *T. preW*⁺. Our study systematically described and identified

107   the external morphological characteristics of intersexes. The results of this study may aid in the

108   understanding of sex determination and sexual differentiation in *T. preW*⁺.

109

Materials & Methods

Insect breeding

112   *Trichogramma pretiosum* Riley (*T. preW*⁺) infected with *Wolbachia* were provided by the Pest

113   Biological Control Laboratory of Shenyang agricultural university. And the uninfected with

114   *Wolbachia* of *Trichogramma p*retiosum Riley (*T. preW*) were obtained by feeding starved one-

115   day-old wasps a 2mg/ml solution of the antibiotic, rifampicin, mixed with honey. The population

116   has been maintained in our laboratory for a long time over 20 generations (ca. 200 days) after

117   collection from the field. The presence of *Wolbachia* in the females was detected by using

118   forward   primer   5'-TGGTCCAATAAGTGATGAAGAAAC-3'   and   reverse   primer   5'-

119   AAAAATTAAACGCTACTCCA-3' for the *wsp* gene of *T. pretiosum* strains. The wasps were

120   reared on fresh eggs of *Corcyra cephalonica* Stainton (< 24h) that were scattered on paper cards

121   coated with Arabic gum and irradiated with ultraviolet (UV) lamp (30W, TUV30W; Philips;

122   Amsterdam, the Netherlands) for 30 min. A group of 30 females were introduced in a finger

123   tubes (1.8cm×5cm) containing a host egg card with ca. 600 *C. cephalonica* eggs for 2 h.

124   Thereafter, the females were removed and the host eggs were reared for ca.10 days until the

125   emergence of wasps. The *T. preW*$^+$ have been cultured in laboratory conditions (25 ± 1 °C, 70 ±

126   5% relative humidity (RH) with a 16 : 8 h light : dark photoperiod) in growth chamber (MLR-

127   352H-PC, Panasonic, Osaka, Japan) before use.

128   **Maternal temperature treatment**

129   Four elevated temperatures: 27℃, 29℃, 31℃, 33℃, were used in this study. And 3 repetitions

130   were performed for each temperature treatment. In addition, no intersex were found at 25℃ in *T.*

131   *preW*, so they are not described in this paper. Take *T. preW* as the control group. *T. preW*$^+$ is the

132   experimental group.

133   We firstly selected 25 newly emerged females for each temperature and evenly separated them

134   into 5 finger tubes (1.8cm×5cm) with surplus *C. cephalonica* eggs glued on paper cards. These

135   wasps were allowed to lay eggs within 2 hours at room temperature. After that, the finger tubes

136   were placed at 27℃, 29℃, 31℃, and 33℃ in the growth chamber (MLR-352H-PC, Panasonic,

137   Osaka, Japan). The subsequently emerged wasps (F0 generation) were maintained under

138   laboratory conditions and supplied with superfluous *C. cephalonica* eggs and 50% honey-water.

139   After 3 days, the wasps were gently removed. Then the emerged F1 generation wasps were

140   allowed to reproduce to get F2 generation. The 1-day-old wasps of F1 or F2 were killed under

141   -80℃ conditions for observation. The intersexes were discriminated under the stereomicroscope

142   lens (Motic SMZ-161; Motic; China). The external characteristics of intersex individuals from

143   F1 and F2 generations were observed and described, and the intersex number in each generation

144   was counted. The intersex individuals were determined based on external characteristics with

145   Tulgetske 's method (Tulgetske *et al.*, 2012). Based on the characteristics of antennae and

146   external genitals, we were able to classify the intersexes into 11 morphological categories (from

147   level 1 to level 11 which was labeled by A to K respectively) with increasing masculinization

148   (Fig. 1). The number of intersex in each level was counted as well.

149   **Sample preparation and photograph**

150   In order to obtain the pictures of all masculinization levels of intersex and make the size of the

151   sample remains unchanged for a long time, the intersex samples were fixed 2 hours in Carnoy's

152   solution (methanol : glacial acetic acid =3 : 1). A step by step dehydration was performed with

153   different gradient ethanol, 10-20 min each. Subsequently, the solution in different proportions of

154   ethanol and glycerol was treated step by step for only 1 time with a span of 30 min. Finally, the

155   samples were treated with pure glycerol twice for 30 min each time. Then, samples were stored

156   in the refrigerator (HYC-940; Haier; Qingdao, China) at 4℃.

157   The intersex samples were softened at room temperature. To observe the characteristics of

158   antennae and external genitals, we first removed the wings and feet from the body and then the

159   head and abdomen were cut off by a scalpel under the stereomicroscope lens (Motic SMZ-161;

160   Motic; China). Thereafter, the samples were photographed with the camera (Nikon SMZ25;

161   Nikon; Japan).

162   **Data analysis**

163   The probability of the intersex (binomial distribution) and the level of masculinization of the

164   intersex (poisson distribution) as influenced by generations and temperature were analyzed by

165   the logistic model and generalized log-linear model respectively. The multiple comparisions of

166   all treatment pairs were carried by Tukey-Kramer tests. The homogeneity of all models was

167   tested by studentized Breusch-Pagan test and Shapiro test respectively (Hall, 1992; Koenker,

168   1981). All analyses were carried out with R ver. 3.5.1 (R Core Team, 2017).

169

170   # Results

171   **General characterization of intersex individuals produced by *T. preW*$^+$**

172   In this study, we found 235 intersexes from 83699 offspring wasps produced by female of *T.*

173   *preW*$^+$ infected *Wolbachia* under different temperatures (Supplemental Information S1). Based

174   on external characters, we were able to classify the intersex into 11 morphological categories (A-

175   K) with increasing masculinization (Fig. 1). The external genitalia of most intersexes were

176   female, and only I, J, K intersex types have male external genitalia(Supplemental Information

177   S2). In addition, the intersexes were not found in the population of *T. preW* (Supplemental

178   Information S1).

179

180   **The probablility of intersex as influenced by temperature**

181   The probability of intersex was significantly influenced by generations ($\chi^2 = 17.16$, $P < 0.001$) or

182   temperature ($\chi^2 = 143.13$, $P < 0.001$), but it was not influenced by the interaction of generations

183   and temperature (χ^2 = 0.68, P = 0.41) (Fig. 2). The probability of intersex increased with

184   temperature (Coefficient $\pm$ SE = 0.40 $\pm$ 0.036, z = 11.10, P < 0.001). The average probability of

185   intersex in F1 (27°C: 0.17 $\pm$ 0.12 %(Mean $\pm$ SE); 29°C: 0.51 $\pm$ 0.25 %; 31°C: 1.43 $\pm$ 0.40 %;

186   33°C: 1.41 $\pm$ 0.52 %) was significantly higher (z = 4.70, P < 0.001) than it was in F2 (27°C: 0.07

187    $\pm$ 0.016 %; 29°C: 0.26 $\pm$ 0.040%; 31°C: 0.25 $\pm$ 0.029 %; 33°C: 1.06 $\pm$ 0.12 %).

188

189   **The degree of secondary masculinization level of intersex individuals in *T. preW*⁺**

190   The masculinization level of intersex individuals were significantly influenced by temperature

191   (χ^2 = 8.25, P < 0.01), but they were not influenced by generations (χ^2 = 0.37, P = 0.54) and the

192   interaction of generations and temperature (χ^2 = 0.56, P = 0.45) (Fig. 3). The masculinization

193   level of intersex individuals significant increased with temperature (Coefficient $\pm$ SE = 0.039 $\pm$

194   0.014, z = 2.85, P < 0.01).

195

196   **Discussion**

197   In order to determine the causes of intersex formation in *T. preW*⁺ and the effects of parental

198   generation temperature of gradual changes in the masculinization of secondary sexual

199   characteristics in intersex offspring, we focused on sexually aberrant intersexes and found that

200   intersex offspring occurs after the *T. preW*⁺ parental generation experiences high temperature.

201   The parental generation temperature affects the probability of occurrence of intersex offspring

202   and the degree of masculinization in secondary sexual characteristics in intersexes. A large

203   volume of studies has showed that high temperature will decrease *Wolbachia* titers inside

204   *Trichogramma* wasps and produce males and intersexes, thereby changing the original

205   reproduction method and offspring sex ratio in *Trichogramma* wasps (Pintureau *et al.*, 1999;

206 Zchori-Fein *et al.*, 2000; Ma & Schwander, 2017). In addition, it is found that there is no intersex

207 in the offspring in *Wolbachia*-uninfected *T. pretiosum* in this paper. The following conclusions

208 can be obtained: *Wolbachia* is a direct factor that causes the occurrence of intersexes, while high

209 temperature is an indirect factor that determines the external morphology of intersexes.

210 All intersexes are caused by sex determination and aberrant sexual differentiation. Therefore, a

211 strict classification of intersex types has low significance (Gusmao & McKinnon, 2009; Narita *et*

212 *al.,* 2010). Hence, our study did not compare the external morphology of different types of

213 intersexes but examined the relationship between parental generation temperature and different

214 offspring with intersexes through calculating the probability of intersex occurrence. Results

215 showed that *T. preW*$^+$ parental generation temperature is positively correlated with the

216 probability of intersex occurrence. The probability of intersex occurrence in the F1 generation is

217 significantly higher than that of the F2 generation. This shows that the effects of high

218 temperature on *T. preW*$^+$ intersexes is passed through the parental generation to offspring, and

219 this maternal effect weakens as the number of generations increases. A previous study found that

220 high temperature can affect *Wolbachia* titers to affect the sex of offspring (Sakamoto *et al.*,

221 2008; Narita *et al.*, 2007; Sugimoto *et al.*, 2015). If this is true, it is not difficult to imagine that

222 the effect of high temperature on the first generation is stronger, causing lower *Wolbachia* titers.

223 At this point, the effect of sex determination mechanisms of the host is relatively stronger than

224 feminization induced by *Wolbachia*, and it can causes stronger genetic conflict between

225 *Wolbachia* and host genes, and the number of intersex individuals increases. In contrast, the

226 weakening of the effects of high temperature on the second generation combined with self-

227 recovery of *Wolbachia* increases *Wolbachia* titers. This causes *Wolbachia* to gradually restore

228 the ability that control host reproductive patterns, and the number of intersex individuals

229   decreases. However, another potential explanation is that intersexes may be induced in some

230   Hymenoptera species with complementary sex determination (CSD) mechanism (Tulgetske &

231   Stouthamer, 2012). CSD is a sex-determining mechanism of alleles at sex locus determine the

232   sex of offspring (Vorburger C, 2014). However, our unpublished results has showed that the sex

233   determination mechanisms for *Trichogramma* wasps does not conform to complementary sex

234   determination (CSD) but may conform to a form of genome imprinting (QQ Liu, 2018-2019,

235   personal observations). The genome imprinting model has been described in *Nasonia vitripennis*

236   (Heimpel & de Boer, 2008). In this model, a maternally active sex-determination gene imprints a

237   sex-determination gene (potentially the same gene) of fertilized eggs. Haploid eggs contain only

238   a maternally derived, and thus imprinted, sex-determination gene and develop into males.

239   Diploid eggs contain a paternally derived nonimprinted copy of the sex determination gene in

240   addition and develop as diploid females. Genomic imprinting is often caused by DNA methy-

241   lation in organisms, but not yet been found in hymenopteran insects(Heimpel & de Boer, 2008).

242   With regards to *T. preW$^+$* in this study, if we assume that genome imprinting is retained in the

243   offspring by the parental generation and the presence of *Wolbachia* inhibits the effects of

244   genome imprinting, when temperature reduces *Wolbachia* titers, the effects of parental

245   generation genome imprinting will become stronger. This may be the cause of intersex

246   formation.

247   In this study, the degree of masculinization in primary and secondary sexual characteristics in

248   intersexes was used to identify 11 intersex types. In addition, we found that the frequency of the

249   D (one male and one female antenna, male antennas contain long setae, female antennas do not

250   contain bristles, external genitalia present in females) and H types (two male antennas that

251   possess long setae, female external genitalia present) of intersex was the highest. The external

252   genitalia of most intersex types were female, and only 3 intersex types have male external

253   genitalia. In *T. preW*+ intersex individuals, most exhibit female primary sexual characteristics,

254   and secondary sexual characteristics exhibit signs of masculinization. Tulgetske et al. (2012)

255   found that the degree of masculinization in secondary sexual characteristics of intersexes is

256   randomly distributed and not associated with high temperature. Another paper has showed that

257   high temperature will cause feminization of secondary sexual characteristics in *Culex stimulans*

258   intersexes with a male genotype (Anderson & Horsfall, 1965). In contrast, high temperature can

259   also induce masculinization of secondary sexual characteristics in *Trichogramma deion* (Bowen

260   & Stern, 1966), *S. triquetrella* and *Carausius morosus* intersexes (Narita *et al.*, 2010). In our

261   study, we found that the degree of masculinization of secondary sexual characteristics in *T.*

262   *preW*+ intersexes increases as parental generation temperature increases. The differences in

263   results may be due to the differences in sex determination mechanisms in different hosts and the

264   presence of *Wolbachia.* The genetic conflict between host genes and *Wolbachia* may also be the

265   cause of intersex development. (Rigaud & Juchault, 1993; Rigaud & Juchault, 1998; Stouthamer

266   *et al.*, 2010).

267   In summary, high temperature affects *Wolbachia* in the parental generation to affect sex changes

268   in the host offspring, and the maternal effect that causes this phenomenon decreases as the

269   number of generations increases. Many papers on insect intersexes in the order Hymenoptera

270   have focused on external morphology and behavior and less on sex-determining genes. As the

271   frequency of intersex occurrence in *T. preW*+ is low, and *T. preW*+ individuals are small, it is

272   relatively difficult to collect or dissect these intersexes and determine whether all intersex can

273   reproduce. More studies should be carried in future. Therefore, we only studied the external

274   genitalia of intersexes in this paper. In the future, in-depth research on internal genitalia and the

275 fertility of *T. preW⁺* can be carried out to facilitate the understanding of intersex formation

276 mechanisms and a deeper understanding of sex determination and differentiation in the order

277 Hymenoptera.

278

Conclusions

280 we focused on sexually aberrant intersexes and found that intersex offspring occurs after the *T.*

281 *preW⁺* parental generation experiences high temperature. The following conclusions can be

282 obtained: *Wolbachia* is a direct factor that causes the occurrence of intersexes, while high

283 temperature is an indirect factor that determines the external morphology of intersexes. This

284 change of sex may be associated with the changes in the expression levels of sex-determining

285 genes. The changes in the expression levels of sex-determining genes in *T. preW⁺* that are jointly

286 caused by temperature-*Wolbachia* is the root cause for intersex formation. In view of the

287 aforementioned situation, we recommend that more in-depth research on relevant sex-

288 determining gene regulation mechanisms for intersexes be carried out in the future to determine

289 whether changes in the expression levels of sex-determining genes results in intersex formation

290 to elucidate the mechanisms for intersex formation.

291

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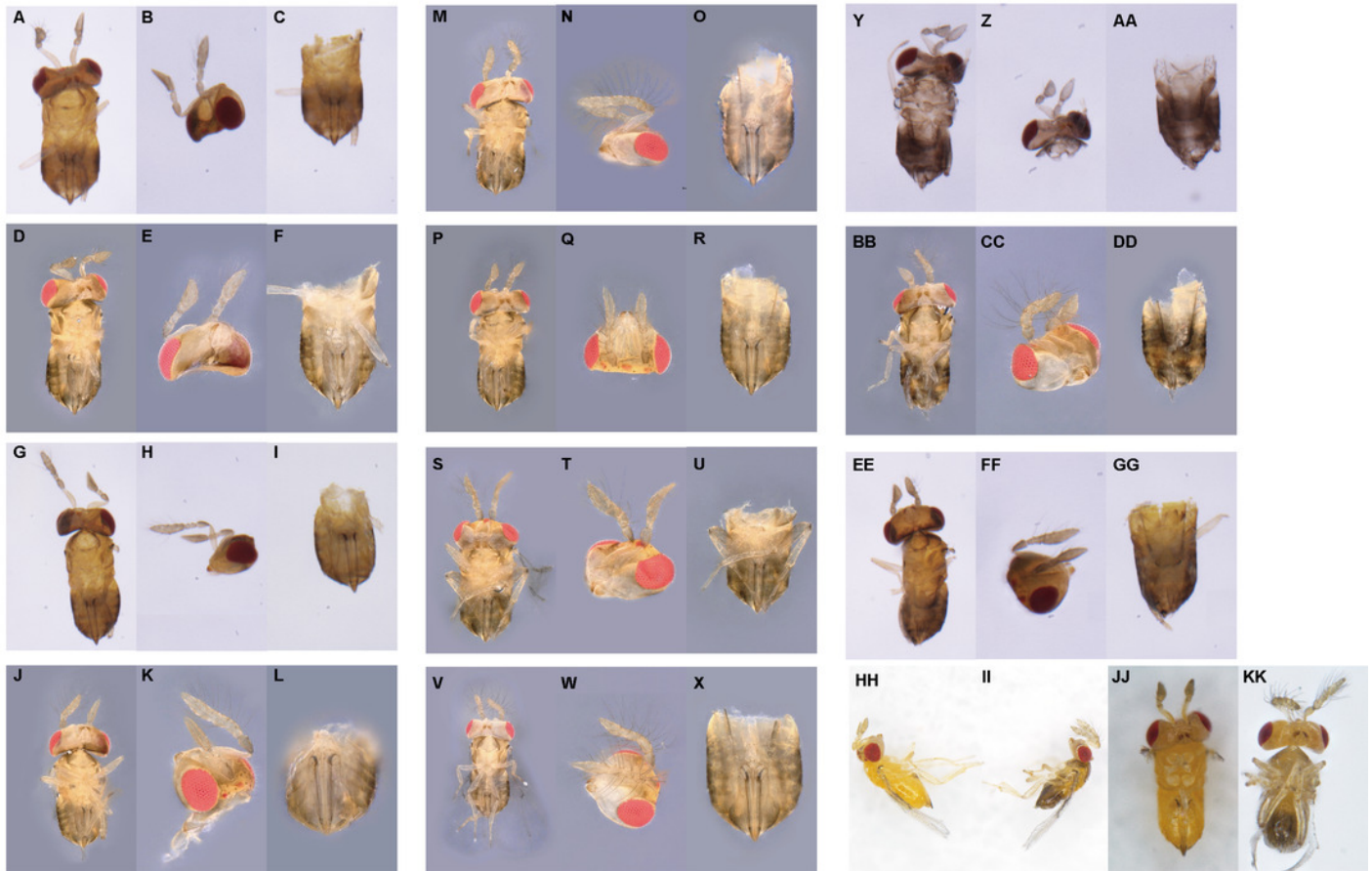
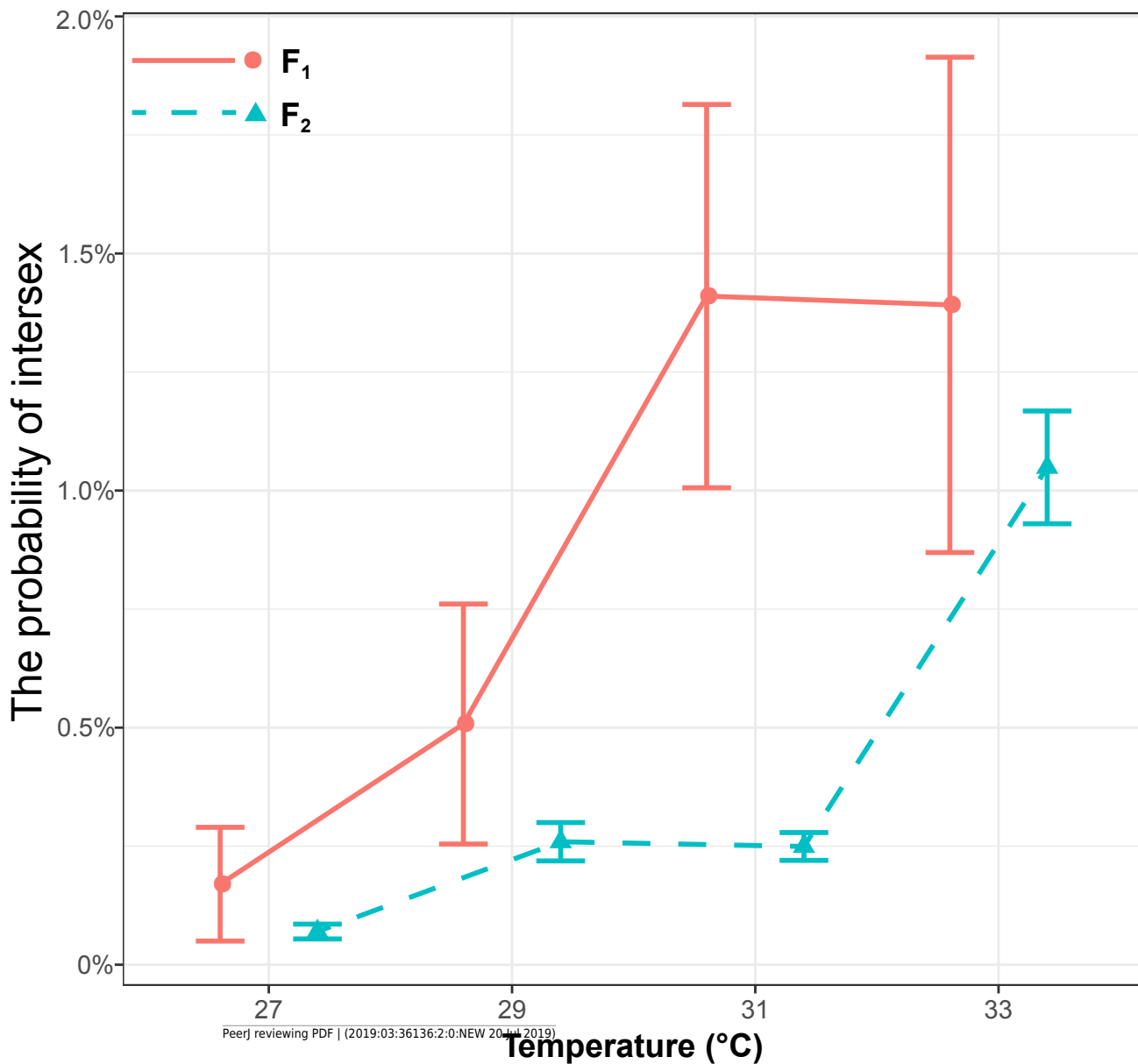
Figure 1

Description of the external morphology of 11 types of intersexes in *T. preW*[+].

(1) Picture A to X: Female intersex, the external genitalia are female and the antennae tend to masculinized. The picture A, D, G, J, M, P, S, V represents the ventral (Secondary sexual characteristics), the picture B, E, H, K, N, Q, T, W represents the antennae (Secondary sexual characteristics) and C, F, I, L, O, R, U, X represents the external genitalia (Primary sexual characteristics) respectively. Type A of intersex: Picture A to C (A: the ventral of intersex; B: 2 female antennae, 1with few short setae; C: female genitalia).Type B of intersex: Picture D to F (D: the ventral of intersex; E: 2 female antennae, both with few short setae; F: female genitalia). Type C of intersex: Picture G to I (G: the ventral of intersex; H: 1 male antenna with few short setae, 1 female antenna; I: female genitalia). Type D of intersex: Picture J to L (J: the ventral of intersex; K: 1 male antenna with few long setae, 1 female antenna; L: female genitalia). Type E of intersex: Picture M to O (M: the ventral of intersex; N: 1 male antenna with few long setae, 1 female antenna with few short setae; O: female genitalia). Type F of intersex: Picture P to R (P: the ventral of intersex; Q: 2 male antennae, both with short setae; R: female genitalia). Type G of intersex: Picture S to U (S: the ventral of intersex; T: 2 male antennae, 1 with few long setae, 1 with few short setae; U: female genitalia). Type H of intersex: Picture V to X (V: the ventral of intersex; W: 2 male antennae, both with long setae; X: female genitalia).

(2) Picture Y to GG: Male intersex, the external genitalia are male and the antennae tend to feminized. The picture Y, BB, EE represents the ventral (Secondary sexual characteristics), the picture Z, CC, FF represents the antennae (Secondary sexual characteristics) and AA, DD, GG represents the external genitalia (Primary sexual characteristics) respectively. Type I of intersex: Picture Y to AA (Y: the ventral of intersex; Z: 2 female antennae; AA: male genitalia). Type J of intersex: Picture BB to DD (BB: the ventral of intersex; CC: 1 male antenna with long setae, 1 female antenna; DD: male genitalia). Type K of intersex: Picture EE to GG (EE: the ventral of intersex; FF: 1 male antenna with long setae, 1 female antenna with few short setae; GG: male genitalia).

(3) Picture HH to KK: As control group, is the normal female in *T. preW* and *T. preW*[+] , normal male in *T. preW*. The picture HH is the lateral of female and II is the lateral of male. The picture JJ and KK represents ventral of female and male respectively. The external morphology of female is 2 female antennae, none of setae, female genitalia. The external morphology of male is 2 male antennae, both with long setae, male genitalia.

The effects of different temperature in the parental generation (27℃, 29℃, 31℃, and 33℃) on the probability of intersex in the F1 and F2 generation in *T. preW*⁺.

The blue line indicates the F2 generation and the red line indicates the F1 generation.

The degree of masculinization level of intersexes in *T. preW*[+].

The left ordinate of the graph represents the masculinization level of intersex individuals. The right ordinate of the graph represents the types of intersexes. The shaded area said confidence interval.

The masculinization level of intersex individuals vs Temperature (°C) scatter plot showing F₁ and F₂ data points.