

Article

Meet Me Halfway: Will Photoperiodic Responses of Interpopulation Hybrids of the Brown Marmorated Stink Bug *Halyomorpha halys* (Hemiptera: Heteroptera: Pentatomidae) Promote or Constrain Subsequent Invasions?

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Abstract: Biological invasions often result from multiple invasion events. In the case of several subsequent invasive episodes, 'newcomers' are usually poorly adapted to local environmental conditions in contrast to the representatives of the already-established invasive population of the same species. Therefore, the mode of inheritance of life-history features determining the survival and performance of interpopulation hybrids is an important component of invasiveness. We investigated the mode of inheritance of the photoperiodic response in the brown marmorated stink bug, *Halyomorpha halys*, by crossing between the native population of South Korea and the invasive population of the Black Sea coast of Russia. The aim of this study was to predict the overwintering potential of the progeny from possible crosses between representatives of the native and invasive populations. The pre-adult development time and the incidence of winter adult diapause in the progeny of the interpopulation crosses were close to the average of the values recorded in 'pure' (unmixed) crosses. Female and male genotypes were equally important in the determination of these characters. Such a mode of inheritance is most likely determined by a polygenic control and would only partially promote subsequent invasion events from the native South Asian range of *H. halys* into Europe.

Keywords: adaptation; diapause; development; inheritance; interpopulation crosses; pest insect; photoperiod; invasion; the brown marmorated stink bug; *Halyomorpha halys*

1. Introduction

Biological invasions are complex multi-step processes, starting with an intentional or accidental introduction; each invader has to somehow leave its native range, reach a new territory, and survive there. However, even the survival of the first unfavorable season (usually winter) does not complete the process of invasion. Very often, invasive episodes can repeat again and again, and the invasive individuals might arrive from the same or different regions. Thus, many (if not most) cases of invasions result from not a single invasion event, but multiple invasion events [1–3]. This process is generally referred to as multiple invasions. Although 'newcomers' are usually much less numerous than the

already-established and successfully reproducing invasive populations and thus, their initial impacts on overall population dynamics and as pests are limited, the ‘newcomers’, nevertheless, might be able to increase genetic diversity, thereby affecting the adaptability of the invader [4–10]. However, this potentially continuous enrichment of genetic diversity is hampered by the fact that ‘newcomers’ are usually not well adapted to local environmental conditions (in contrast to the ‘old-timers’, i.e., the representatives of the already-established invasive populations of the same species). Moreover, even the progeny of crosses between ‘newcomers’ and ‘old-timers’ can also be poorly adapted to local environmental conditions. Therefore, the mode of inheritance of various adaptive life-history traits determining the survival and performance of the interpopulation hybrids is an important but poorly studied component of invasiveness.

The induction of facultative diapause is a pivotal element in the control of insect seasonal cycles. It is one of the adaptations that can vary markedly among populations of widely distributed insect species. Facultative diapause is crucial not only for survival in harsh seasons, but also for synchronizing activity with favorable periods of the year [11–18]. True facultative diapause is an anticipatory response induced by environmental cues (most commonly day length) before the actual arrival of an unfavorable season [18–22]. However, the pattern of the natural correlation between the seasonal dynamics of day length and vital environmental factors (such as temperature, precipitation, food availability, etc.) evidently depends on the geographic location. Therefore, photoperiodic, thermal, and other diapause-controlling responses may differ among geographically distant populations of the same species, including native and invasive insect populations [15,23–25].

Substantial differences between the photoperiodic responses of native and invasive populations have been found in the model species of this study, the brown marmorated stink bug, *Halyomorpha halys* (Stål, 1855) (Hemiptera: Pentatomidae). This polyphagous agricultural and forest pest originated in eastern Asia (China, Korea, Japan, Myanmar, Vietnam, and Taiwan) and is now considered one of the most harmful invasive insects in North America and Europe [26–31]. It was recently recorded in Georgia, Abkhazia, and Russia (Krasnodar Territory) [31–34]. A recent comparative experimental study demonstrated that the critical day lengths of the photoperiodic response of the winter adult diapause induction (i.e., day length which induces diapause in 50% of the population) of *H. halys* males and females falls between 14.5 and 15.0 h in individuals of the native population from Andong (South Korea) and between 15.0 and 15.5 h in individuals of the invasive populations from Basel (Switzerland), Torino (Italy), and Sochi (Russia) [35,36]. Observations recently conducted under the natural conditions of the Black Sea coast agreed with the predictions based on the experimental laboratory results: the photoperiodic response with a critical day length between 15.0 and 15.5 h ensured the proper timing of the beginning of the winter adult diapause [34].

Both the Western European and Caucasian invasive populations of the brown marmorated stink bug were most likely initially established due to multiple invasion events [27,30,33,37,38]. This suggests that subsequent accidental introductions of the same species in the Caucasus region are still quite possible. Thus, a question arises about the fitness of the progeny from the crosses between ‘newcomers’ and representatives of the earlier-established and currently breeding invasive population.

Experiments have demonstrated that the differences in the photoperiodic responses of adult winter diapause induction between the Western European and Caucasian invasive populations were not significant [36]. Therefore, it can be expected that the progeny from the crosses between adults of the Western European and the Caucasian populations are well adapted to the local Caucasian climate. On the contrary, the critical day length of the studied native Korean population of *H. halys* is 1.0 h shorter than that of the invasive Caucasian population [36]. This difference suggests that, if accidentally introduced to the Caucasus, bugs from more distant (e.g., Korean or other Asian) populations would not be able to timely enter winter adult diapause before the autumn temperature decreases, and this would substantially reduce the probability of successful overwintering and general fitness.

The question about ability of *H. halys* hybrids to overwinter successfully has never been addressed. The aim of the present study is to understand the mode of inheritance of *H. halys* photoperiodic response, and thereby estimate the overwintering potential of the progeny from the possible crosses between native Asian and invasive Caucasian populations of this species. The earlier studies on *H. halys* demonstrated that day length influence not only the induction of adult diapause, but also the duration of pre-adult development [35,36]. Therefore, we investigated the mode of inheritance of both parameters.

2. Materials and Methods

2.1. Insects

The study was conducted with two laboratory populations of *H. halys*. The native population (hereafter referred to as the ‘Andong population’) originated from 30 individuals collected in the environs of Andong, South Korea (ca. 36°41' N, 128°44' E; 140 m a.s.l.) in July 2019. The invasive population (hereafter referred to as the ‘Sochi population’) originated from more than 100 individuals collected in Sochi, Krasnodar Territory, Russia (ca. 43°36' N, 39°35' E; 50 m a.s.l.) in July–August 2019. Afterwards, the Sochi population was renewed annually by adding about 50 individuals collected from the same locations. It should be emphasized that South Korea is a part of the natural geographic range of *H. halys*, whereas in Sochi, this invader was recorded for the first time quite recently (in 2014) [26,27,30–36].

Before the beginning of the experiment, both populations were reared in a laboratory at a temperature of 25–28 °C and a photoperiod of L:D 16:8 h (hereafter, the duration of the light and dark periods is given in hours). Under these optimal long-day conditions, diapause was never observed in *H. halys*. In the culture, insects were kept in ventilated transparent plastic containers (22 cm × 13 cm × 12 cm) and fed with peanuts, sunflower seeds, and broad-bean seedlings. In addition, nymphs were provided with wet cotton balls as a water source. During the experiments, all nymphs and adults were fed the same diet.

2.2. Experimental Design

2.2.1. Parental Generation

To start the experiment, egg masses laid by females from the two laboratory populations were collected and subsequently kept at 25 °C and L:D 16:8 h. The hatched nymphs were reared under the same conditions. Upon emergence, the adults were randomly selected and paired up, according to the design of the experiment: four cross types, i.e., four combinations of males and females, from the two populations were tested. Hereafter, the cross types are indicated by the initials of the population origin of the parental female and male, respectively: two ‘pure’ crosses (within-population, unmixed, or pure lines) (AA and SS) and two hybrid (reciprocal, or mixed) crosses (AS and SA), where A stands for Andong and S stands for Sochi. When necessary, the emerged adults were kept individually isolated for up to 7 days waiting for the planned mate. These ‘parental pairs’ were kept in ventilated transparent plastic containers (height 12, diameter 10–12 cm) under the same optimal long-day conditions (25 °C and L:D 16:8 h) to ensure fast maturation and oviposition. Eggs laid by a female of each pair were collected 3 times a week, i.e., every 2 or 3 days.

2.2.2. Progeny Generation

The progeny that emerged from each egg mass (first 10–30 individuals) was reared separately. Nymphs were kept in the same size of ventilated transparent plastic containers under moderately diapause-inducing conditions: 25 °C and a near-threshold photoperiod of L:D 15:9 h. The progeny pre-adult development time (from the deposition of an egg mass to the emergence of adults) was recorded and the data for males and females were pooled, as our earlier study [35] demonstrated that there is no difference in the time of development between two sexes. The emerged progeny adults were collected every 2 or 3 days and kept in groups of up to 15 individuals per container under the same laboratory conditions. Our earlier studies demonstrated that, under these photoperiodic conditions, nearly all *H. halys*

males and females from the Andong population matured, whereas most individuals from the Sochi population entered diapause [36].

Twenty days after emergence, all progeny adults were frozen and dissected. This age was also chosen based on our earlier studies [35,36] and constituted approximately 1.2 times the mean period from female emergence to the deposition of its' first egg mass under the optimal long-day conditions [39,40]. No less than 5 male and 5 female (usually more than 10 male and 10 female) progeny individuals of each parental pair were dissected. Data on the parental pairs with less than 5 dissected progeny individuals of each sex were discarded. Upon dissection, the reproductive state of males and females was evaluated by the criteria commonly used for *H. halys* and other pentatomids [15,35,36,39–44] with a three-point scale used in our previous study [34]. Based on the state of their ovaries, females were separated into the following categories: (1) diapausing (no visible signs of development of ovarioles); (2) maturing or intermediate (initial stages of development of ovarioles); and (3) mature (ovaries fully developed, mature eggs or vitellogenic oocytes in ovarioles). Based on the state of their ectodermal sacs of accessory glands, males were separated into the following categories: (1) diapausing (ectodermal sacs were not filled with secretory fluids and shrunk); (2) maturing or intermediate (ectodermal sacs were only partly filled with secretory fluids); and (3) mature (ectodermal sacs were fully filled with secretory fluids).

2.3. Statistical Analysis

In total, the pre-adult development time of 2549 individuals was recorded (the progeny of 65 parental pairs, 13–20 pairs per each of the four cross types, 13–76 progeny individuals per parental pair), and then 1045 male and 1131 female progeny individuals were dissected. The data on the pre-adult development time were subjected to a two-stage statistical treatment: first, the mean time of the pre-adult development was calculated separately for each parental pair of each cross type; and second, these mean data were averaged again over all parental pairs of each cross type and used for further analysis. The proportions of the diapausing and mature progeny individuals were also calculated separately for each parental pair of each cross type. Thus, in order to avoid pseudoreplications, heterogeneity (the difference among parental pairs of the same cross type) was evaluated, using each progeny individual as an independent data point, whereas the differences between the cross types were estimated based on the means for parental pairs. For the final analysis, ANOVA, followed by the Tukey's HSD test, was used; nonparametric data (incidence of diapause) were ranked before this treatment. The proportions of diapausing and mature progeny were compared by chi-square test. All calculations were performed with SYSTAT 10.2. (Systat Software Inc., Richmond, CA, USA).

3. Results

3.1. Pre-Adult Development Time

The mean pre-adult development time of individuals from the Sochi population (SS) was 9.0 days (i.e., more than 20%) longer than that of individuals from the Andong population (AA); the interpopulation hybrids had intermediate values (Figure 1). The difference between the AS and SA hybrids was definitely not significant ($p = 0.619$), whereas the difference between the hybrids and within-population crosses was either statistically significant (Figure 1) or only marginally not significant ($p = 0.075$ for the difference between the SA and AA crosses, and $p = 0.126$ for the difference between the AS and SS crosses).

The variation in the mean progeny pre-adult development time among parental pairs of the same cross type (heterogeneity) estimated by Tukey's HSD test was highly statistically significant ($p < 0.001$) for all cross types. When the means for the parental pairs were compared, the coefficients of variation calculated for pure crosses AA and SS, and for hybrids AS and SA, constituted 0.135, 0.082, 0.090, and 0.89, correspondingly (not shown), i.e., the variability of hybrids was not higher than that of pure crosses (see also in Figure 1).

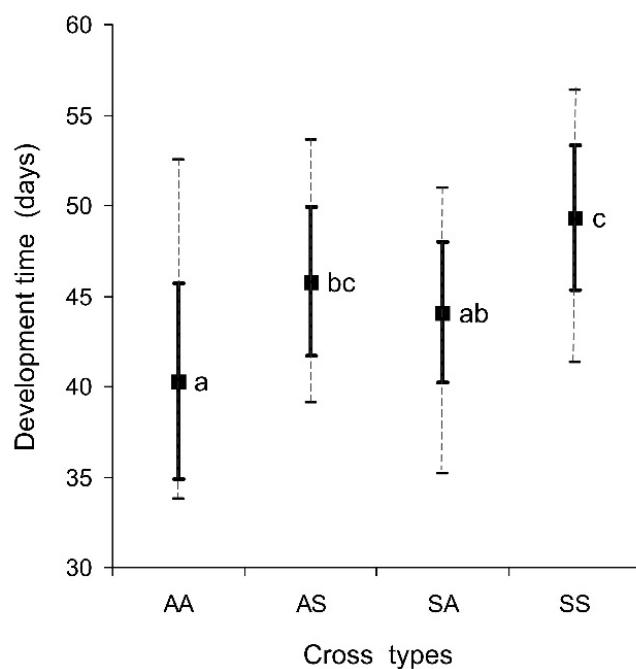


Figure 1. Pre-adult development time of the progeny of four *Halyomorpha halys* cross types. The cross types are indicated by the initials of population origin of female and male: A—Andong, S—Sochi. Means, SD (solid bold lines), and ranges (broken lines) for the mean values separately calculated for each parent pair of each cross type ($n = 13\text{--}20$) are presented. Symbols with different letters correspond to significantly different means ($p < 0.05$ by the Tukey's HSD test). Progeny of all pairs of all cross types developed under the same conditions (25 °C, L:D 15:9 h).

3.2. Diapause

A two-way ANOVA of the ranked data on the incidence of diapause in male and female progeny of all parental pairs ($n = 130$, that is 65 parental pairs \times 2 sexes) showed that the proportion of diapausing progeny strongly depended on the cross type ($F = 142.3$, $df = 3$, $p < 0.001$): the difference between sexes ($F = 18.1$, $df = 1$, $p < 0.001$), as well as the interactions of these two factors ($F = 7.5$, $df = 3$, $p < 0.001$), were also significant. Tukey's HSD test, conducted separately for males and females, demonstrated that not only interpopulation difference, but also differences between the progeny of pure crosses and hybrids were highly significant ($p < 0.001$), whereas the difference between the two hybrid cross types was not significant ($p = 0.224$) for females, and only marginally significant ($p = 0.042$) for males.

The variation in the incidence of diapause among the parental pairs of the same cross type (heterogeneity) estimated by the chi-square test was highly significant for the AS pairs ($n = 307$, $\chi^2 = 38.4$, $df = 16$, $p = 0.001$ and $n = 355$, $\chi^2 = 119.5$, $df = 16$, $p > 0.001$ for male and female progeny, correspondingly) and for the female progeny ($n = 330$, $\chi^2 = 48.1$, $df = 19$, $p < 0.001$), but not the male progeny ($n = 309$, $\chi^2 = 29.6$, $df = 19$, $p = 0.057$), of the SA pairs. Moreover, the heterogeneity of the parental pairs of pure (within-population) SS crosses was also highly significant for females ($n = 223$, $\chi^2 = 43.5$, $df = 14$, $p < 0.001$) but not for males ($n = 229$, $\chi^2 = 23.2$, $df = 14$, $p < 0.057$). The heterogeneity of AA crosses was not significant both for males ($n = 200$, $\chi^2 = 8.7$, $df = 12$, $p = 0.724$) and females ($n = 223$, $\chi^2 = 13.9$, $df = 12$, $p = 0.309$).

Indeed, most of the AA parental pairs produced only non-diapausing progeny, although up to 20% of the progeny of some pairs of this type entered diapause (Figure 2). Most of the parental pairs of the SS crosses, on the contrary, produced only diapausing progeny, although the progeny of some atypical pairs of this type included up to 40% of non-diapausing individuals. The progeny hybrids (AS and SA) demonstrated intermediate values compared to both pure crosses (AA and SS). Their variability was much higher,

and the difference between the AS and SA hybrids, as noted above, was not statistically significant. It is noteworthy that in all graphs (Figure 2), the range of interpair variation in female progeny was wider than that in male progeny; this difference agrees well with the above shown differences in the significance of heterogeneity. Thus, although the mean diapause incidence was about 40% in the AS and SA hybrids, more than the half of the progeny of some parental pairs entered diapause, whereas in the progeny of some other pairs, diapause incidence was less than 20%.

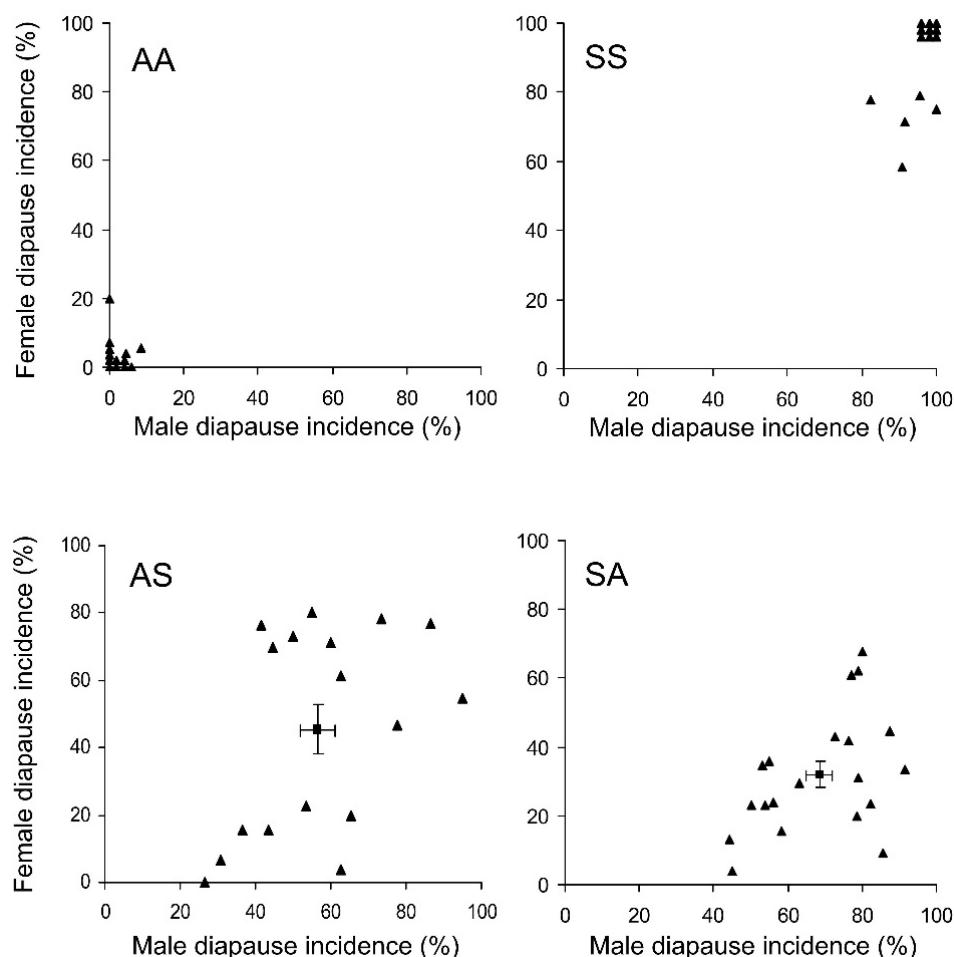


Figure 2. Diapause incidence in male and female progeny of different parental pairs of the four *Halyomorpha halys* cross types. The cross types are indicated by the initials of population origin of female and male: A—Andong, S—Sochi. Each symbol (triangle) corresponds to one parental pair; the percentages of diapause in male (X-axis) and female (Y-axis) progeny are presented. For the two hybrid crosses (AS and SA), mean and SEM are also presented (for pure crosses, means are not presented as they would overlap data points). Some symbols are slightly shifted to avoid overlap. Progeny of all pairs of all cross types developed under the same conditions (25°C , L:D 15:9 h).

3.3. Adult Maturation

The non-diapausing fraction of the progeny included two categories of adults: mature and intermediate (maturing) (see the Section 2). The ratio between adults from these two categories also differed among cross types. A two-way ANOVA demonstrated that the proportion of mature males and females among non-diapause individuals ($n = 110$) depended on the cross type ($F = 18.5$, $df = 3$, $p < 0.001$) and sex ($F = 28.7$, $df = 1$, $p < 0.001$), whereas the interactions of these two factors were not significant ($F = 1.0$, $df = 3$, $p = 0.405$). A one-way ANOVA, followed by Tukey's HSD test, demonstrated that the proportions of mature males and females in both hybrid crosses (SA and AS) were highly significantly

($p < 0.005$), different from those in the AA pure crosses, and not significantly different from each other. Indeed, on the day of dissection, most of the non-diapausing males and females in the progeny of AA pairs were already mature, whereas about one-third of the progeny of the mixed pairs were still maturing (more precisely, they were in the intermediate state; Figure 3). The data on SS pure crosses are somewhat unclear because, as noted above, most of the parental pairs of this type produced only diapausing progeny.

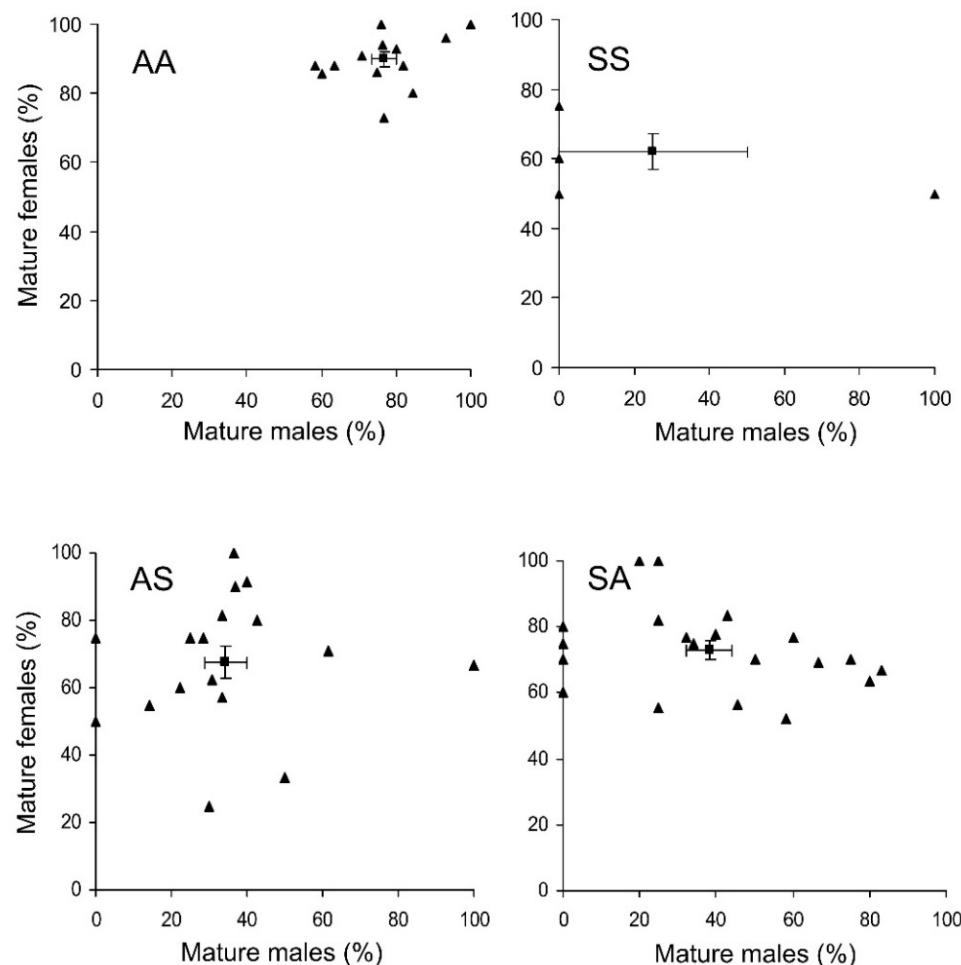


Figure 3. The proportion of mature individuals among non-diapausing progeny of different parental pairs of *Halyomorpha halys* cross types. The cross types are indicated by the initials of population origin of female and male: A—Andong, S—Sochi. Each symbol (triangle) corresponds to one parental pair; the percentages of mature male (X-axis) and female (Y-axis) progeny are shown, mean and SEM are also presented. Some symbols are slightly shifted to avoid overlap. Progeny of all pairs of all cross types developed under the same conditions (25°C , L:D 15:9 h).

The chi-square tests demonstrated that the heterogeneity of the percentage of mature individuals among the non-diapausing progeny was not statistically significant for both AS ($n = 127$, $\chi^2 = 17.9$, $df = 16$, $p = 0.332$ and $n = 187$, $\chi^2 = 23.9$, $df = 16$, $p = 0.093$ for male and female progeny, correspondingly) and SA ($n = 100$, $\chi^2 = 19.3$, $df = 19$, $p = 0.437$ and $n = 224$, $\chi^2 = 15.0$, $df = 19$, $p = 0.720$ for male and female progeny, correspondingly) hybrids, as well as for AA ($n = 197$, $\chi^2 = 9.8$, $df = 12$, $p = 0.634$ and $n = 218$, $\chi^2 = 9.0$, $df = 12$, $p = 0.700$ for male and female progeny, correspondingly) and for SS pure crosses ($n = 6$, $\chi^2 = 6.0$, $df = 3$, $p = 0.112$ and $n = 24$, $\chi^2 = 0.9$, $df = 5$, $p = 0.966$ for male and female progeny, correspondingly). However, the range of interpair variation (at least for the mixed pairs) was rather large (Figure 3). It is possible that the insignificance of heterogeneity was caused by low sample sizes (as only non-diapausing individuals were considered).

4. Discussion

Our experiments clearly demonstrate the following: (1) both pre-adult development time and incidence of adult diapause in interpopulation hybrids (AS and SA) of the brown marmorated stink bug are close to the average corresponding values of the two parental populations (AA and SS); and (2) in both parameters, the differences between the two reciprocal interpopulation crosses (AS and SA) are not significant. It should be emphasized that both the induction of adult diapause and the pre-adult development time are largely determined by the threshold of the photoperiodic response of the adult diapause induction: the proportion of diapausing individuals increases sharply when the day length decreases below the threshold level, whereas the pre-adult development time is longest when nymphs are reared under the pre-threshold day length [35,36]. Thus, the mode of inheritance of both studied parameters is determined by the mode of inheritance of the photoperiodic response.

The intra-specific variability and inheritance of diapause control has been studied in different species from different insect orders: Hemiptera-Heteroptera [45], Coleoptera [46–52], Lepidoptera [53–60], Hymenoptera [61], and Diptera [62–68]. These studies revealed different modes of inheritance of various (and particularly photoperiodic) diapause-inducing responses. In some insect species, the tendency to diapause is dominant [45,52,62,69] or partly dominant [64,67]. In some other species, on the contrary, diapause is a recessive character [46,49,58,66]. Finally, in some of the studied insects (similarly to *H. halys*), the proportion of diapausing individuals in first-generation hybrids is close to the average between that of their parents [47,48,54,61]. Interestingly, different results were obtained in different studies of the Asian corn borer, *Ostrinia furnacalis* (Guenée). Xia et al. [55] and Huang et al. [57] demonstrated that diapause in this species is incompletely dominant over non-diapause, whereas Fu et al. [59] demonstrated that diapause is completely dominant. Finally, Xiao et al. [56] proved that the degree of dominance depended on photoperiodic conditions. Therefore, it should be kept in mind that the results of the dominance analysis can depend on the photothermal regimes used in a particular experiment. Similar environmental effects on dominance were found in other insects [70].

The ‘intermediate’ mode of inheritance observed in *H. halys* in the current study can be most probably explained by the polygenic control of diapause induction, which has been demonstrated in a number of insect species, such as the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) [50], the ragweed leaf beetle, *Ophraella communa* LeSage [51], the comma butterfly, *Polygona c-album* (L.) [54], the spotted stem borer, *Chilo partellus* (Swinhoe) [71], the face fly, *Musca autumnalis* De Geer [64], and the fruit fly, *Drosophila melanogaster* Meigen [72]. Generally, most studied insect species demonstrate polygenic patterns of diapause inheritance [18,73] although, in some cases, ‘classical’ monogenic Mendelian inheritance was found [45,62,66,74,75].

We did not find any significant difference between reciprocal interpopulation hybrids (AS and SA). This means that male and female genotypes have an equal impact on both the duration of the pre-adult development and induction of adult diapause in *H. halys*. In certain other insect species (for example, in several lepidopterans), males play a leading role in the determination of diapause in progeny [53,55–60], whereas in other cases, females have the same [61], or an even stronger [47–49], influence on progeny diapause than the males. Moreover, the relative importance of males and females might depend on the studied parameter of diapause: in the blow fly, *Calliphora vicina* R.-D., diapause induction is mainly determined by female genotype, whereas the duration of diapause depends on both parents [65]. In the Asian corn borer, *O. furnacalis*, diapause duration is also equally influenced by each parent, but the induction of diapause is mainly determined by male genes [56].

Regarding the main aim of our study, we conclude that whatever the mechanism of the observed mode of inheritance, it can only partially promote subsequent multiple invasions from the native range of the brown marmorated stink bug. If newcomers from Asia cross with the local conspecifics, the probability of the timely induction of winter adult diapause in the progeny will be half of that in the progeny of the local pairs from the established

invasive population. However, it will be still much higher than could be expected in the case of an initial invasion, i.e., in the progeny of parents from the native (South Asian) populations of *H. halys*. At the same time, further potential newcomers from Europe are expected to be more pre-adapted to timely enter diapause than their conspecifics from Asia.

5. Conclusions

- (1) The pre-adult development time and the incidence of winter adult diapause in the progeny of the interpopulation crosses between the native Korean and the invasive Caucasian populations of *H. halys* are close to the average of the values recorded in their parents;
- (2) Male and female genotypes are equally important in the determination of the pre-adult development time and the incidence of winter adult diapause;
- (3) The modes of inheritance of these characters are most probably determined by polygenic control;
- (4) The observed modes of inheritance would only partially promote subsequent multiple invasion events from the native South Asian range of *H. halys*.

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