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# Can things get worse when an invasive species hybridizes? The harlequin ladybird *Harmonia axyridis* in France as a case study

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## Abstract

So far, only a few studies have explicitly investigated the consequences of admixture for the adaptative potential of invasive populations. We addressed this question in the invasive ladybird *Harmonia axyridis*. After decades of use as a biological control agent against aphids in Europe and North America, *H. axyridis* recently became invasive in four continents and has now spread widely in Europe. Despite this invasion, a flightless strain is still sold as a biological control agent in Europe. However, crosses between flightless and invasive individuals yield individuals able to fly, as the flightless phenotype is caused by a single recessive mutation. We investigated the potential consequences of admixture between invasive and flightless biological control individuals on the invasion in France. We used three complementary approaches: (i) population genetics, (ii) a mate-choice experiment, and (iii) a quantitative genetics experiment. The invasive French population and the biological control strain showed substantial genetic differentiation, but there are no reproductive barriers between the two. Hybrids displayed a shorter development time, a larger size and a higher genetic variance for survival in starvation conditions than invasive individuals. We discuss the potential consequences of our results with respect to the invasion of *H. axyridis* in Europe.

## Introduction

Hybridization (interbreeding between genetically differentiated lineages) takes place in a very wide range of organisms (Barton and Hewitt 1985, Dowling & Secor 1997, Mallet 2005) and may play an active role in a variety of evolutionary processes ranging from local adaptation to speciation (Stebbins 1959; Arnold 1992; Barton 2001; Rieseberg et al. 2003). In the field of invasion biology, hybridization is now seen as a potential stimulus for the evolution of invasiveness (Ellstrand and Schierenbeck 2000; Lavergne and Molofsky 2007; Ryan et al. 2009; Blair and Hufbauer 2010).

Traditionally, hybridization involves interspecific or intergeneric crosses as exemplified by the invasive plant *Spartina anglica* that mixes with native and other alien *Spartina* species (Gray et al. 1991; Baumel et al. 2002). However, crosses between individuals from genetically differentiated populations of the same species (i.e. admixture, Ellstrand and Schierenbeck 2000; Culley and Hardiman 2009) are also considered hybridization (Wolfe et al. 2007; Culley and Hardiman 2009). Admixture seems to be frequent in biological invasions. An increasing number of studies document biological invasions resulting from multiple introductions from distinct populations that bring together genetically differentiated individuals into a

 

common introduced area (Facon et al. 2003; Kolbe et al. 2004; Bossdorf et al. 2005, Wares et al. 2005; Lavergne and Molofsky 2007). To date, most studies dealing with admixture have aimed at detecting multiple source populations in biological invasions from selectively neutral markers (e.g. Kolbe et al. 2004). Only a few studies have explicitly investigated the consequences of intraspecific hybridization for the evolution of life-history traits and thus for the adaptative potential of introduced populations (Lavergne and Molofsky 2007; Wolfe et al. 2007; Facon et al. 2008).

Hybridization may lead to very different outcomes ranging from detrimental to beneficial (Arnold and Hodges 1995; Burke and Arnold 2001). On the one hand, hybridization may reduce the fitness of parental individuals either due to incipient reproductive isolation in the form of genetic incompatibilities that reduce the mating success of parents (prezygotic isolation) or through a decrease in the fitness of offspring due to the loss of local adaptation and/or breakdown of co-adapted gene complexes (outbreeding depression, as exemplified in tension zones; Barton and Hewitt 1985). On the other hand, hybridization has the potential to boost invasiveness through two nonexclusive mechanisms: heterosis and generation of new genotypes. Heterosis (or hybrid vigor) occurs when hybridization masks deleterious alleles (Keller and Waller 2002) or in case of overdominance and/or synergistic epistasis between alleles inherited from the parental taxa. Allopolyploidy, which sometimes accompanies hybridization, may also contribute to the heterotic effect (Ainouche et al. 2009). The generation of new genotypes occurs through recombination (Arnold et al. 1999; Ellstrand and Schierenbeck 2000; Facon et al. 2005; Schierenbeck and Ellstrand 2009), and alleviates the loss of genetic variance after founder events and hence restores or even increases the efficiency of selection (Lee 2002).

Given its invasion history, the invasive harlequin ladybird *Harmonia axyridis* provides an opportunity to examine whether individuals from genetically distinct populations interbreed freely and how admixture affects life-history traits. Native to Asia, *H. axyridis* has been introduced repeatedly as a biological control agent against aphids since 1982 in Europe (Ongagna et al. 1993). Despite recurrent intentional releases of beetles for acclimation attempts, the species did not establish for 20 years. For unknown reasons it recently and suddenly became invasive on four different continents (Poutsma et al. 2008). The species is known to be a harmful predator of nontarget arthropods, a household invader, and a pest of fruit production (Koch 2003); In Europe, invasive populations were first recorded in Belgium in 2001 (Adriaens et al. 2003). It has now spread widely in Europe with a current distribution that extends from Southern France to Denmark (Brown et al. 2008). Up to now, whether the European invasive populations result from intentional introductions, accidental migrants or both remains unknown.

In France, a flightless strain of *H. axyridis* is sold commercially for biological control (Tourniaire et al. 2000). This flightless strain, called Coccibelle® (BIOTOP, Valbonne, France) was selected in the late 1990s for its inability to fly and disperse from a traditional flying biological control stock. The flightless phenotype is caused by a single recessive mutation in a gene involved in flight muscles (Tourniaire et al. 2000); thus only individuals homozygous for the mutant allele cannot fly. The Coccibelle® strain was developed with the goal of obtaining a more localized and hence effective control of aphids by both larvae and adults. As with most coccinellids, *H. axyridis* diapauses during cooler periods. It congregates into large groups (up to thousands individuals) to overwinter and is attracted to light colored dwellings and other man-made objects as overwintering sites (Labrie et al. 2008). Thus, an additional advantage of the Coccibelle® strain is the inability of flightless individuals to reach wintering sites which minimizes both its impact as a household pest, and its ability to establish populations in the wild. However, the continued use of Coccibelle® for biological control raises the possibility that it will cross with invasive individuals in Europe, especially in France. If such crosses occurred, they would yield individuals able to fly and hence could potentially impact the invasive process.

The purpose of this study was to investigate the potential role of intraspecific hybridization (i.e. admixture) between Coccibelle® and invasive individuals on the invasion of *H. axyridis* in France. Wolfe et al. (2007) outlined three criteria that must be met for intraspecific crosses to play a role in biological invasions. First, the populations involved in the admixture process should be genetically differentiated. Second, crosses should be possible between individuals from the different populations. Third, the admixed individuals should differ from parental ones in some of their life-history traits to impact the invasion process. This last criterion may involve direct heterosis, an increase in genetic variance, or both (Ellstrand and Schierenbeck 2000; Burke and Arnold 2001; Lee 2002; Facon et al. 2005; Culley and Hardiman 2009). Here, we assessed the three above criteria for crosses between the Coccibelle® biological control strain and the invasive French population of *H. axyridis*. First, we determined the level of differentiation between Coccibelle® and the invasive French populations at 18 microsatellite markers. Second, we evaluated whether there are reproductive barriers that could prevent interbreeding between biological control and invasive populations using a mate

  

choice experiment. Third, we used a quantitative genetics experiment to estimate the phenotypic means and variances for several key life-history traits of offspring produced by crossing Coccibelle® with the French invasive population.

## Material and methods

### Population sampling and rearing conditions

Invasive individuals (hereafter referred to as INV) were collected in the wild from an invasive population in Croix, Northern France (50°40′35″N, 3°08′33″E) where *H. axyridis* has been observed since 2004 (Brown et al. 2008). It is worth stressing that we previously genotyped seven French populations covering the French repartition area (in 2007–2008) and found no genetic structure between them at 18 microsatellite loci (average $F_{ST}$ = 0.052; Arnaud Estoup, unpublished data). This absence of genetic structure at neutral loci made it reasonable to base our quantitative genetics study on a single invasive French population sample. The corresponding experimental design, while large (2400 larvae, as described below), was feasible, while additional crosses would not have been. Individuals from the Coccibelle® biological control strain (hereafter referred to as BIO) were obtained from the firm BIOTOP (Valbonne, France), which originally commercialized it.

Approximately 70 mature individuals of both INV and BIO were obtained in September 2007. These first generation individuals ($G_0$) were used to initiate both INV and BIO populations in the laboratory for two generations, under strictly controlled conditions, to avoid potential biases due to maternal effects. During these two generations, populations were fed with ionized *Ephestia kuehniella* (Lepidoptera: Pyralidae) eggs and reared at constant environmental conditions (23°C; 65% RH; L:D 14:10). At generation $G_2$, males and females were separated immediately after emergence to prevent mating. They were then maintained in the same environmental conditions for 2 weeks to ensure that all individuals had reached reproductive maturity at the beginning of the experiments.

### Are INV and BIO genetically distinct at microsatellite loci?

To answer this question, we genotyped 28 $G_0$ individuals per population (both INV and BIO) at 18 microsatellite loci following Loiseau et al. (2009). We estimated the genetic diversity within-population by computing both the allelic richness ($R_S$; ElMousadik and Petit 1996) and the expected heterozygosity ($H_E$; Nei 1987). The level of genetic differentiation between INV and BIO populations was estimated by computing $F_{ST}$ (Weir and Cockerham 1984). All computations were processed using the software FSTAT (Goudet 1995). Differences in $R_S$ and $H_E$ values were tested using a Wilcoxon Sign Rank test and the $F_{ST}$ value was tested for significant deviation from zero using the permutation test implemented in FSTAT (Goudet 1995).

### Are there reproductive barriers between the INV and BIO populations?

We addressed this question by performing mate choice trials involving three individuals (one female and two males) in cylindrical boxes (height = 3 cm; diameter = 8.5 cm). We used virgin $G_2$ adults 2 weeks after emergence and created trios of one female from the focal population for an individual trial (either INV or BIO) and one male from each of the two populations (INV and BIO). We set up 23 such trios with BIO females and 26 with INV females. We left the three partners together until the female laid her first clutch. We then collected the males and preserved them in ethanol for genetic analysis. We isolated the first clutch and counted the eggs. After 5 days, we counted the number of living larvae and preserved them in ethanol. We repeated the procedure for another clutch 4 weeks later. We then preserved all females in ethanol for genetic analysis.

We extracted individual genomic DNA using the Chelex® method (Estoup et al. 1996) for each mother and the two putative fathers as well as for eight larvae from each clutch ($N$ = 49, 98 and 784 respectively for females, males and larvae). All these individuals were genotyped following Loiseau et al. (2009) for a subset of seven microsatellite loci (Ha 005, Ha 201, Ha 215, Ha 244, Ha 267, Ha 281, Ha 605). These seven loci were selected among a total of 18 loci available, as they can unambiguously discriminate the genetic origin (INV or BIO) of individuals, using the program WHICHRUN (Banks and Eichert 2000). We assigned each offspring to their parents based on their multilocus genotypes using the program PROBMAX version 1.3 (Danzmann 1997). This program assigns progeny to a set of possible contributing parents given that the genotypes are known for both the progeny and the possible parents.

We used SAS version 9.1 (SAS Institute 2003) to analyze these data. We tested the null hypothesis that the male reproductive success is equal (1:1 ratio) for the two types of males (INV and BIO) separately for each female type (INV or BIO) using a chi-square test for proportions. We also tested the effect of the female type on the male reproductive success with an analysis of independence in two way table. Finally, we tested whether the hatching rate differed significantly according to the parents using a generalized linear model with a binomial

probability distribution and a logit link function; with female and male and the interaction as factors.

### Do life-history traits differ between hybrids and their parents?

We addressed this question by creating four types of crosses (female × male) from the two parent samples BIO and INV: BIO × BIO, BIO × INV, INV × BIO and INV × INV. For each cross, we randomly set up 10 couples (all the larvae produced by a couple will be thereafter referred to as a family) by putting one male and one female in a cylindrical box (height = 3 cm; diameter = 10 cm). As a consequence of this experimental design, the factor family was actually nested within the factor cross as it was not possible to produce the four crosses from a given pair of male and female (whose offspring formed a given family). At the beginning of the experiment, we collected and isolated four clutches (more than 20 eggs per clutch) of each couple. At the day of hatching (the fourth day), 15 larvae per clutch were randomly chosen and placed in a small cylindrical box (height = 2 cm; diameter = 5 cm) with a damp piece of cotton wool. For this experiment, we thus used of 2400 larvae (4 boxes × 15 larvae × 10 couples × 4 crosses). Larvae were fed *ad libitum* every 2 days until adulthood with freeze-dried aphids (*Acyrthosiphon pisum*) for 30 larvae per family and with eggs of *Artemia salina* for the 30 remaining larvae. Individuals were maintained at constant environmental conditions (23°C; 65% HR; L:D 14:10) during the experiment. Larvae were checked every day and we recorded the number of individuals reaching adulthood (i.e. the larval survival) and the total development time from egg laying to adult emergence of each individual.

A subset of individuals reaching adulthood was used to estimate four additional traits: reproductive investment of females, the lifespan of starving adults, the survival rate in quiescent conditions and the body length. To estimate reproductive investment, two adult females from each family were dissected and the number of ovarioles was counted using a binocular microscope (Ware et al. 2008).

To estimate the lifespan of starving adults from one to three females and one to three males (depending on the size of the family) were randomly collected and placed individually in a small cylindrical box (height = 2 cm; diameter 5 cm) with no food and thereafter checked every day for 45 days.

To estimate the survival rate in quiescent conditions, from one to three females and one to three males (again, depending on the size of the family) were randomly collected and placed in a cylindrical box (height = 3 cm; diameter 10 cm) with no food in constant abiotic conditions that corresponded to conditions for diapause (5°C; 60% HR; L:D 12:12). After 5 weeks, we measured the number of individuals still alive in each box to estimate the survival rate. Finally, the body length of all the adults used to estimate survival rate in quiescent conditions was measured with a binocular stereomicroscope micrometer using the software ImageJ© (http://rsbweb.nih.gov/ij/index.html).

We analyzed data on the two juvenile traits (larval survival and development time) and the four adult traits (reproductive investment, lifespan of starving adults, survival rate in quiescent conditions and body length) using SAS version 9.1 (SAS Institute 2003). For the response variables known to deviate markedly from a normal distribution (i.e. counts and proportions), we used the traditional transformations (square root for reproductive investment and arcsin for larval survival and survival rate in quiescent conditions; Sokal and Rohlf 1995). For the remaining variables, which followed approximately normal distributions, we used the original data. This choice is justified by the fact that (i) there was no obvious transformation that improved the normality of residuals and (ii) the experimental design was almost perfectly balanced and included large sample sizes, two features known to mitigate the effects caused by a non-normal distribution and/or the heterocedasticity of variances (Ananda and Weerahandi 1997).

We used model selection following Burnham and Anderson (1998) and Shoukri and Chaudhary (2007) to determine the appropriate models on which to test the significance of effects of interest. First, including all main fixed effects (cross and food for the response variables reproductive investment, larval survival, development time, and cross, food, and sex for body length, survival rate in quiescent conditions, and survival in starvation) and their interactions, we compared models with different random effects. Models for all response variables included family nested within cross and family (cross) × food as random effects. For the variables that included sex as a fixed effect, we also considered the interactions family (cross) × food × sex, family (cross) × sex as random effects. Note that with the random effect of family (cross), we can either estimate one variance component (assuming the same variance in families over the four crosses) or four variance components (each one specific to each cross, assuming that the variances were heterogeneous).

We compared the full models with simpler nested models by removing a different variance component each time, using Restricted Maximum Likelihood (REML) to assess the significance of random effects. If this removal worsened the fit of the model significantly as evidenced by likelihood ratio tests, the variance component was kept in the model; otherwise, the variance component was

  

removed from the model and the model selection pursued from this simpler model (Shoukri and Chaudhary 2007; Goldman and Whelan 2000; Shapiro 1988; see Appendix A for details).

Once a covariance structure was selected, we used Maximum Likelihood (ML) to select which fixed effects improved the fit of the model. Model selection was carried out based on the Information Criterion of Akaike corrected for small sample sizes (hereafter $AIC_c$) following Burnham and Anderson (1998). As suggested by the same authors, we considered models with a delta $AIC_c$ of 2 or less as undistinguishable on statistical grounds; and on the basis of parsimony, we selected the model with the lower number of parameters for inferences. Results of the models selection procedures are detailed for each variable in Appendix A.

To compare the genetic variance of the life-history traits between hybrid individuals and their parents, we used the variance components estimated for the family effect within each cross ($V_G$). The genetic variances of the measured traits were compared among crosses using the genetic coefficient of variation ($CV_G$), which is the square root of the genetic variance ($V_G$) divided by the trait mean (see Houle 1992). For each trait, we tested the hypothesis that admixture increases the genetic variance by comparing the $CV_G$ of the four crosses using Likelihood Ratio Tests.

## Results

### Are INV and BIO genetically distinct at microsatellite loci?

The within-population variability was significantly higher in the INV sample ($R_S = 6.08$, $H_E = 0.60$) than in the BIO sample ($R_S = 2.44$, $H_E = 0.40$; $P < 0.0001$ for $R_S$ and $P = 0.0005$ for $H_E$). We also found that the BIO and INV populations were genetically substantially differentiated with $F_{ST} = 0.13$ ($P < 0.0001$).

### Are there reproductive barriers between the INV and BIO populations?

We observed mating and egg clutches production in all mate choice trials. All genotyped larvae could be unambiguously assigned to a male. Within clutch, eggs were sired by one or two males in variable proportion. For a given female fertilized by two males, the proportion of eggs sired by a given father could change drastically among successive clutches.

Interestingly, we found that for both type of females (BIO and INV), the BIO males sired a higher proportion of offspring than INV males (Fig. 1). BIO males sired 80.3% of BIO female offspring, and 71.8% of INV females. Both proportions are significantly higher than the expected 50% fertilization by each male type ($\chi^2 = 132.01$, $P < 0.0001$ and $\chi^2 = 81.70$, $P < 0.0001$ for BIO and INV females, respectively). A similar result was obtained when using the clutch as an independent statistical unit, (excluding in this case the clutches sired by two males): for BIO females, 81% of clutches are sired only by BIO male and 19% only by INV male; for INV females, 78% of clutches are sired only by BIO male and 22% only by INV male. In both cases, BIO males sired significantly more offspring than INV males ($P < 0.05$). It is worth noting that we rejected the null hypothesis of independence between the two variables (Female type and Male type; $P = 0.0135$, Fig. 1). This result could be interpreted as the BIO males siring more offspring when mated with BIO females than with INV females.

To test whether the hatching rate differed significantly according to the parents, we split up the male status in three categories: BIO, INV or a mixture of both types. The mean hatching rate across all the observed clutches was 73%. We did no detect any significant effect of male parent ($P = 0.58$), female parent ($P = 0.52$), or the interaction ($P = 0.96$) (see Fig. 2).

### Do life-history traits differ between hybrids and their parents?

Results for models selection are detailed in the Appendix A. The results of the best models for the six studied traits are summarized in Table 1 and results of the full models in Appendix B.

We first focused our analysis on the comparison between the hybrids and their parents. We found that the type of cross had a significant effect on development time ($P = 0.0009$) and length ($P = 0.0006$). INV individuals had a significantly longer development time than the BIO individuals and both hybrid types (INV-INV vs. BIO-BIO: $P = 0.0011$, INV-INV vs. BIO-INV: $P = 0.0055$,

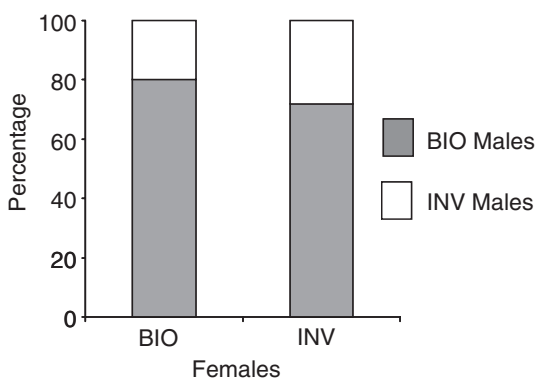


**Figure 1** INV and BIO male reproductive success mated to each type of female (INV or BIO).

INV-INV vs. INV-BIO: $P = 0.0361$; Fig. 3B). BIO-INV and INV-BIO hybrids did not differ for this trait ($P = 0.98$). Individuals of both hybrid types were marginally longer than those from pure parental crosses (BIO-INV vs. INV-INV: $P = 0.09$, BIO-INV vs. BIO-BIO: $P = 0.09$, INV-BIO vs. INV-INV: $P = 0.09$, INV-BIO vs. BIO-BIO: $P = 0.08$; Fig. 3F). Individuals from pure parental crosses did not differ between each other ($P = 0.97$). The type of cross did not have any significant effect for the four remaining traits (larval survival, reproductive investment, survival in starvation, and survival in quiescent conditions; respectively Fig. 3A,C,D,E). However, for reproductive investment, Fig. 3C shows that INV females tend to invest more in reproductive structures. Although the cross effect had not been retained in the best model for reproductive investment (see Appendix A), this effect was marginally significant in the full model ($P = 0.094$). In pairwise comparisons, the only significant comparison is between pure invasive females and pure biological control females.

Regarding random effects, we found a significant family effect for all traits except for length and a significant interaction between food and family for development time, survival in starvation and length. This result means that variation for all the studied traits was, at least partly, genetically based (Table 1). Genetic coefficients of variation ranged widely among traits (Table 2). $CV_G$ was low for development time, reproductive investment and length (less than 5%) but high for larval survival, survival in starvation and survival in quiescent conditions (between 10% and 68%; Table 2). For development time, survival in quiescent conditions and length, there was no obvious difference between the four crosses. For reproductive investment, the two crosses involving an INV mother (i.e. INV-INV and INV-BIO) had a higher $CV_G$ than the two crosses involving a BIO mother (i.e. BIO-BIO and BIO-INV), although this trend was not significant (Table 2). For the two other traits (larval survival and survival in starvation), the observed pattern was an increase of $CV_G$ in the hybrid crosses relative to the invasive cross. This trend was significant, however, only for survival in starvation ($P = 0.017$; Table 2). Accordingly, survival in starvation is the only trait for which taking into account four specific variance components for the family effect improves the model (Table 1). For larval survival, the $CV_G$ of INV-INV was lower than that of the three other crosses. For survival in starvation, the two hybrid crosses had a higher $CV_G$ than the two parental crosses. Moreover, if we consider the family mean for this later trait as an average genotype within a family, we can observe some 'genotypes' in admixed individuals (INV-BIO or BIO-INV) that consistently outperformed both parental genotypes (Fig. 3D).

We now deal with two factors, the type of food and sex, which are worth mentioning although they do not directly relate to the comparisons between hybrids and their parents. The type of food had a significant influence on development time, larval survival, survival in quiescent conditions and length (Table 1). Larvae fed with aphids had a greater larval survival and a shorter development time than larvae fed with *Artemia* eggs (SurvLarv = 80% and 65%, DvptTime = 22.01 and 24.02 days for individuals fed with aphids and *Artemia* eggs respectively). Individuals fed with *Artemia* eggs survived better in quiescent conditions than individuals fed with aphids (60% and 39% respectively), but had a smaller adult body size (6.27 and 6.56 mm for individuals fed with Artemia eggs and aphids respectively). Sex had a significant effect on survival in starvation and length (Table 1) with females having a greater survival in starvation (10.1 days) than males (8.4 days) and a larger body size (6.7 and 6.1 mm for females and males respectively). The interaction between food source and sex was only significant for length, and that no other interaction between fixed effects was significant. Finally, we did not find any significant interaction between cross and food or sex.

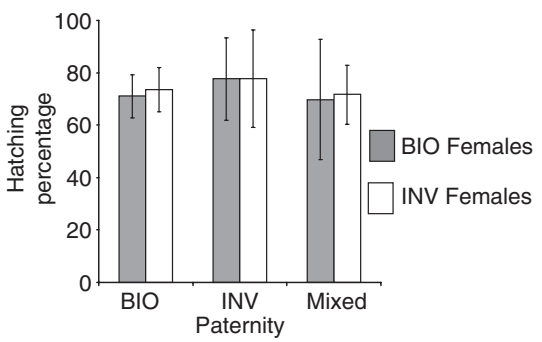


**Figure 2** Mean hatching rate (±SE) according to the involved parents. We split male status into three categories: BIO, INV or a mixture of both types.

## Discussion

Our study clearly demonstrates that admixture between individuals from the French invasive population and from the flightless biological control strain of the harlequin ladybird could potentially alter the invasion process.

The first criterion proposed by Wolfe et al. (2007) to evaluate the potential role of intraspecific hybridization in invasion was that populations involved in admixture should be genetically differentiated. Using 18 microsatellites, we found that the two studied populations showed substantial genetic differentiation ($F_{ST} = 0.13$). This differentiation could at least partly result from the loss of allelic diversity in the biological control population.


 

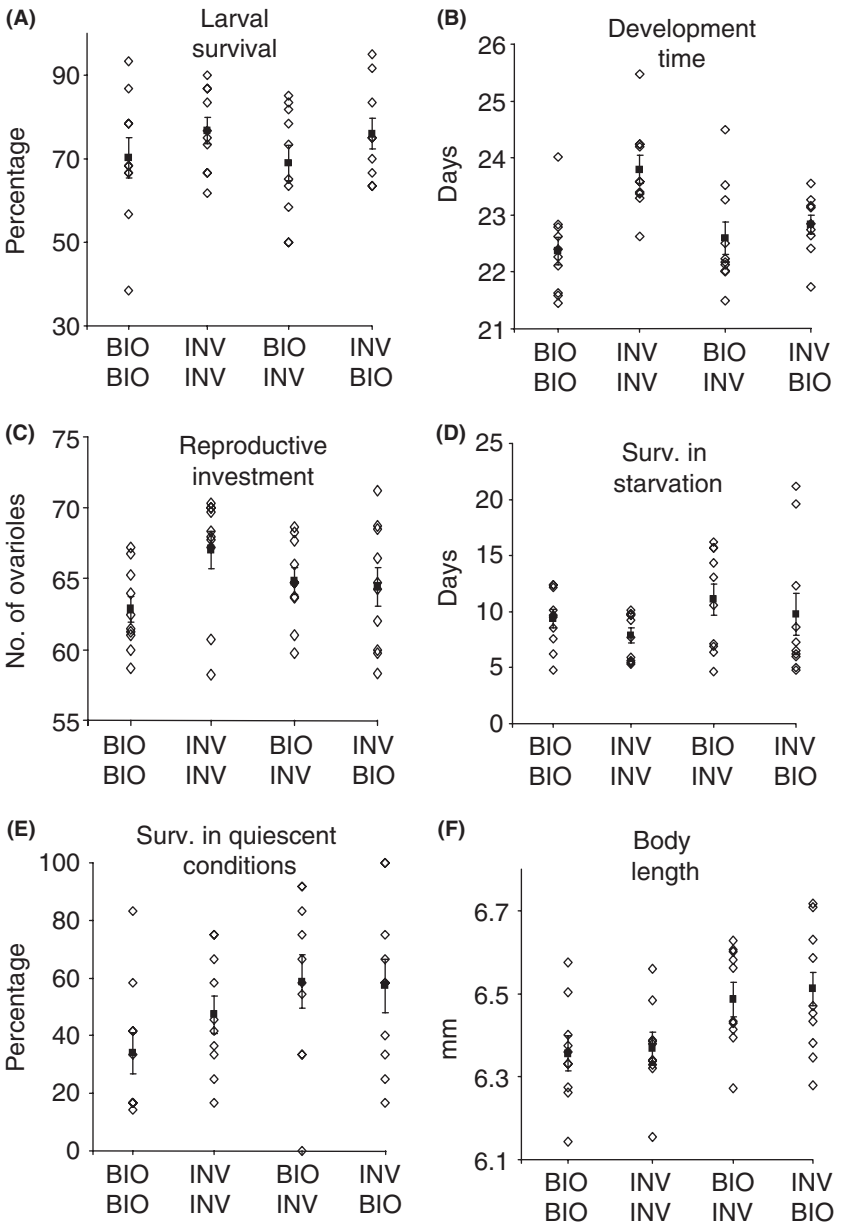


**Figure 3** Life-history trait values for each cross. Black squares stand for the means for the four different crosses with associated standard errors. Diamonds represent family mean values within each cross. The six panels correspond to the six life-history traits studied: larval survival, development time, reproductive investment, lifespan of starving adults, survival rate in quiescent conditions, and body length. In each type of cross, female is indicated first and male in second. For instance, the cross named INV-BIO involved an invasive female and a biological control male.

This result can be explained by the fact that captive populations usually experience strong genetic drift due to a small number of initial founders and small effective population size during subsequent generations (Fiumera et al. 2000). With regards to the flightless biological control strain, it is worth noting that low effective size probably also occurred during selection for the flightless phenotype.

The second criterion of Wolfe et al. (2007) is that there must not be substantial barriers to crossing. Indeed, for *H. axyridis*, crosses turned out to be possible between the involved populations, at least in laboratory conditions. Our mating experiment, based on trios of one female and two males (one of each population), clearly illustrates that no reproductive barrier has evolved between these two distinct *H. axyridis* populations as every cross yielded viable offspring in similar proportions. Moreover, we found that males from the flightless biological control strain sired more offspring whatever the type of female.

**Table 1.** Results from the best model after model selection among the different linear mixed models run for the six traits studied.

| Source | Degrees of freedom | Test statistic | *P* |
|---|---|---|---|
| *(A) Larval survival* | | | |
| Fixed effects | | Type III *F* | |
| Food | 1 | 27.27 | <0.0001 |
| Random effect | | Wald test | |
| Fam (cross) | | 1.97 | 0.0246 |
| *(B) Development time* | | | |
| Fixed effects | | Type III *F* | |
| Cross | 3 | 6.74 | 0.0009 |
| Food | 1 | 161.68 | <0.0001 |
| Random effect | | Wald test | |
| Fam (cross) | | 2.31 | 0.0105 |
| Food × Fam (cross) | | 3.81 | <0.0001 |
| *(C) Reproductive investment* | | | |
| Random effect | | Wald test | |
| Fam (cross) | | 2.14 | 0.0162 |
| *(D) Survival in starvation* | | | |
| Fixed effects | | Type III *F* | |
| Sex | 1 | 14.94 | 0.0001 |
| Random effect | | Wald test | |
| Fam (BIOBIO) | | 0.81 | 0.2089 |
| Fam (BIOINV) | | 1.8 | 0.0361 |
| Fam (INVBIO) | | 1.69 | 0.0457 |
| Fam (INVINV) | | 0.24 | 0.4039 |
| Food × Fam (cross) | | 2.14 | 0.0161 |
| *(E) Survival in cold conditions* | | | |
| Fixed effects | | Type III *F* | |
| Food | 1 | 17.97 | 0.0001 |
| Random effect | | Wald test | |
| Fam (cross) | | 2.57 | 0.0051 |
| *(F) Body length* | | | |
| Fixed effects | | Type III *F* | |
| Cross | 3 | 6.42 | 0.0006 |
| Food | 1 | 70.68 | <0.0001 |
| Sex | 1 | 932.57 | <0.0001 |
| Food × Sex | 1 | 10.49 | 0.0013 |
| Random effect | | Wald test | |
| Food × Fam (cross) | | 4.07 | <0.0001 |

This result suggests that the cross between wild females and males from the flightless biological control strain might even be favored in nature. The advantage that males of the flightless biological control strain exhibited might be explained by selection on traits that increase male fitness in captive conditions, a feature already demonstrated in captive populations of several other invertebrates (Sgro & Partridge 2000, Lewis and Thomas 2001).

The third criterion of Wolfe et al. (2007) is that the admixed individuals should differ from the parental ones in life-history traits in a direction likely to enhance invasion. In the case of *H. axyridis*, the relevant comparison is between pure invasive individuals and admixed individuals, because individuals of the flightless biological control strain are unlikely to be able to overwinter and thus to durably settle a sustainable population *in natura* due to their flightless phenotype.

A first important point is that invasive individuals never significantly outperformed the admixed ones. This result highlights that the use of flightless individuals as biological control agents in the field could potentially enhance invasion by decreasing the Allee effect typical of dispersing individuals founding new populations (Tobin et al. 2007). Indeed, in the invasion front, population sizes are expected to be low. If recurrent releases of flightless individuals are made near the invasion front, Allee effects would be reduced. A comparison of invasive females directly with pure biological control females reveals that they tend to invest more in reproductive structures. Additional experiments should be performed to understand whether this difference translates into effective fecundity.

A second important point is that we found that admixture led to both heterosis and increased genetic variance. Admixed individuals developed more quickly and grew larger. These shifts indicate heterosis. Admixture increased genetic variance for survival in starvation, with $CV_G$ of hybrids significantly exceeding parental ones for this trait. While there was no significant shift in the mean value for survival in starvation some hybrid genotypes consistently outperformed parental ones. Thus, admixture could boost the efficiency of selection in direction of higher survival under stressful conditions of starvation (Ellstrand and Schierenbeck 2000; Lee 2002; Facon et al. 2005).

We will now consider how changes in development time, body length and increased variability for survival in

**Table 2.** Genetic coefficients of variation within each cross for the six traits studied and the associated likelihood ratio tests.

| | BIO-BIO | BIO-INV | INV-BIO | INV-INV | Test |
|---|---|---|---|---|---|
| Larval survival | 0.140 | 0.103 | 0.113 | 0.044 | LRT = 1.4; *P* = 0.474 |
| Development time | 0.035 | 0.037 | 0.024 | 0.033 | LRT = 1; *P* = 0.447 |
| Reproductive investment | 0.010 | 0.011 | 0.026 | 0.026 | LRT = 2.5; *P* = 0.295 |
| Survival in starvation | 0.227 | 0.384 | 0.684 | 0.174 | LRT = 7.7; *P* = 0.017 |
| Survival in quiescence | 0.344 | 0.456 | 0.376 | 0.310 | LRT = 2.5; *P* = 0.295 |
| Body length | 0.015 | 0.017 | 0.024 | 0.016 | LRT = 1.3; *P* = 0.382 |


 

starvation could affect the ongoing invasion of *H. axyridis*. Shifts in life-history traits due to hybridization/admixture events and associated with higher invasiveness have already been reported (e.g. Facon et al. 2005; Lavergne and Molofsky 2007). Several studies have also highlighted that such recombination events often produce an increase in cell volume, body size or seed/juvenile size (see for instance Vila and D'Antonio 1998). In the case of *H. axyridis*, the observed increase of body size in admixed individuals has the potential to impact the interactions between this species and the native coccinellid species by enhancing the dominance of *H. axyridis* in interspecific competition and intraguild predation (Polis et al. 1989; Lucas et al. 1998). It is worth noting that this increase in adult size does not occur at the expense of a longer development time. On the contrary, admixed individuals grow faster than invasive ones. This shorter development time should enhance population growth rate and hence impact the invasive potential of the species. As mentioned above, *H. axyridis* diapauses during cooler periods. During the rest of the year, it can complete between two and five generations (Koch 2003), and a shorter generation could shift that range up. The third trait impacted by admixture is linked to survival in stress conditions (absence of food). Several studies have pointed out that invasiveness may be associated with a higher stress-tolerance (see for instance Milne and Abbott 2000). For *H. axyridis*, increased ability to survive periods of famine may be especially advantageous when prey populations fluctuate or in areas where preys are at low density.

The three traits for which admixture had an effect are hence likely to be advantageous in the context of invasion. Therefore, if crosses do occur in nature, selection should promote the introgression of genes from the flightless biological control strain into the invasive populations and enhance the invasive potential of *H. axyridis*.

As noted, changes in these traits fall into two different categories: (i) for development time and body length, the shift in trait means provides evidence for heterosis and (ii) for survival in starvation, the difference between hybrids and parents stems from an increase in the genetic variance in hybrids. Predicting the long-term consequences of hybridization/admixture is not an easy task as they are strongly influenced by the genetic basis of hybrid fitness (Fitzpatrick and Shaffer 2007). Indeed, heterosis effects could be transitory due for instance to increasing homozygosity in later generations. Hybrids are also known to often express phenotypic breakdown in the $F_2$ generation as a result of recombination disrupting co-adapted gene complexes or meiotic problems (Barton and Hewitt 1985; Burke and Arnold 2001). It is hence possible that outbreeding depression might be expressed in future generations of admixed *H. axyridis* individuals. Our results are only based on a $F_1$-hybrid generation. Additional studies over further generations are hence needed to forecast the long-term consequences of a possible hybridization event.

To better apprehend the evolutionary consequences of admixture between *H. axyridis* invasive and biological control individuals, both empirical and theoretical studies should be performed. For instance, it would be fruitful to simulate the introgression process through experimental evolution in the lab or in semi-natural conditions during several generations. The impact of the 'flightless' allele on the flying ability of heterozygous individuals should also be tested in experimental wind tunnel or flight mills. Moreover, it would be interesting to test how the higher male reproductive success of the flightless males translates into the admixed individuals. Another direction for future research would be to include into theoretical models the fitness consequences of admixture (with both the changes in traits we measured and the presence of the recessive 'flightless' allele), to better predict the impact of admixture with flightless biological control individuals on the invasion dynamics.

We are still at an early stage in understanding how admixture between invasive individuals and biological control ones could affect invasion. Our ongoing study of *H. axyridis* supports the view that intraspecific hybridization (admixture) potentially alters the evolutionary process by contributing novel genetic advantages to admixed individuals (Facon et al. 2005; Lavergne and Molofsky 2007, Schierenbeck and Ellstrand 2009). Finally, our study illustrates a new situation where such admixture can occur, i.e. between invasive and biological control individuals, whereas situations documented so far corresponds to biological invasions resulting from multiple introductions from distinct native range populations bringing together genetically differentiated individuals into a common introduced area (Facon et al. 2003; Wares et al. 2005; Lavergne and Molofsky 2007; Wolfe et al. 2007).

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## Appendix A: Procedures of models selection

Regarding model selection in the context of mixed models, Shoukri and Chaudhary (2007) recommend (i) to select only the variance components that improve significantly the fit of the model (with all fixed effects kept in the model) and (ii) to carry out the tests of significance of fixed effects (with all variance components deemed significant at the first step). The first step allows the user to carry out a decomposition of the variance, by identifying the factors contributing much to the variance, keeping them into the model and discarding the other, less important, variance components. In the present study, we therefore started from a model with all variance components (and all fixed effects) and then built simpler nested models, by removing each time a different variance component. If this removal worsened significantly the fit of the model (as assessed by a Likelihood Ratio Test), the variance component was kept in the model; otherwise, if the removal of the variance component under investigation did not worsen significantly the fit of the model, the variance component was removed from the model and the model selection pursued from this simpler model. The same procedure was followed for the fixed effect once a reasonable covariance structure has been selected (see main text for additional details). For variables reproductive investment, larval survival, development time and survival in quiescent conditions fixed effects were cross, food and the interaction cross × food; we thus compared five models in the model selection. For variables Length and survival in starvation, we incorporated sex as a fixed effect into the models. The fixed effects were then cross, food, sex and the interactions cross × food, cross × sex, food × sex and cross × food × sex. All models run with an interaction included the main effects involved in that interaction for fixed effects. All models run with an interaction as a fixed effect included the main effects involved in that interaction for fixed effects. A total of 19 models were hence run.

In the tables presented below, we used the '+' to indicate additive relationships between effects, the '.' to indicate an interaction and the '*' to indicate the two main effects and the interaction (notations as in Lebreton et al. 1992). To spare space we used the following code for each variable in the tables (c for cross, f for food, s for sex and fam for family). The notation 'fam (4 VCs)' means that a different variance component is estimated for each cross while the notation 'fam' means that a single variance component is estimated for all the four crosses in the model (assuming thus the same variance for each cross). For all tables, the column entitled 'Description' displays the list of effects present in the model under concern, the column 'effect removed' the list of effects removed from the reference model and the column 'Ref.' the model from which is derived the model under concern (i.e. the reference model).

**(1) Length**

*Random effects*

| | Variable length: random effects | | | | |
|---|---|---|---|---|---|
| Model | Description | LRT | *P*-value | Effect removed | Ref. |
| M1 | fam (4 VCs)<br>s.fam<br>f.fam<br>s.f.fam | . | . | None | . |
| M2 | fam (4 VCs)<br>s.fam<br>f.fam | 0.1 | 0.9999 | s.f.fam | M1 |
| M21 | fam (4 VCs)<br>s.fam | 18.4 | 0.0078 | f.fam<br>s.fam | M2<br>M2 |
| M22 | fam (4 VCs)<br>f.fam | 0 | 1 | | |
| M31 | fam<br>f.fam | 1.3 | 0.3822 | fam (4 VCs) | M22 |
| M32 | fam (4 VCs) | 18.4 | 0.00389 | f.fam | M22 |
| M41 | f.fam | 1.2 | 0.65 | fam | M31 |
| M42 | fam | 18.4 | 0.00023 | f.fam | M31 |

So the best model is the model with 'food.family' as random effect.

*Fixed effects*

The score of the best model in terms of $AIC_c$ is displayed in bold.

| | Variable length: fixed effects | |
|---|---|---|
| Model | Description | $AIC_c$ |
| c + f + s + c.f + f.s + c.s + c.f.s | Three main effects plus three interactions plus one triple interaction | −85.2 |
| c + f + s + c.f + f.s + c.s | Three main effects plus three interactions | −87.2 |
| c + f + s + c.f + f.s | Three main effects plus two interactions | −91.5 |
| c + f + s + c.f + c.s | Three main effects plus two interactions | −78.8 |
| c + f + s + f.s + c.s | Three main effects plus two interactions | −89.3 |
| c + f + s + c.f | Three main effects plus one interaction | −83.3 |
| c + f + s + f.s | Three main effects plus one interaction | **−93.5** |
| c + f + s + c.s | Three main effects plus one interaction | −80.9 |
| c + f + s | Three main effects | −85.3 |
| c*f | Two main effects plus one interaction | 349.3 |
| c*s | Two main effects plus one interaction | −33.8 |
| f*s | Two main effects plus one interaction | −82.7 |
| c + f | Two main effects | 346.1 |
| c + s | Two main effects | −38.1 |
| f + s | Two main effects | −74.4 |
| c | One main effect | 390.2 |
| f | One main effect | 356.8 |
| s | One main effect | −36.1 |
| . | Intercept | 391.6 |


 

### (2) SurvStarv

*Random effects*

| | Variable SurvStarv: random effect | | | | |
|---|---|---|---|---|---|
| Model | Description | LRT | *P*-value | Effect removed | Ref. |
| M1 | fam (4 VCs)<br>s.fam<br>f.fam<br>s.f.fam | . | . | None | . |
| M2 | fam (4 VCs)<br>s.fam<br>f.fam | 0 | 1 | s.f.fam | M1 |
| M21 | fam (4 VCs)<br>s.fam | 8.5 | 0.247 | f.fam | M2 |
| M22 | fam (4 VCs)<br>food.fam | 4.6 | 0.653 | s.fam | M2 |
| M23 | fam<br>s.fam<br>f.fam | 7.8 | 0.016 | fam (4 VCs) | M2 |
| M24 | fam (4 VCs) | 11.8 | 0.001 | s.fam<br>f.fam | M2 |

Regarding the model selection concerning random effects for the variable SurvStarv, one can note that the removal of one of the random effects either 'sex.family' or 'food.family' did not worsen significantly the fit of the model while the removal of both effects led to a model significantly worst (LRT = 11.8 $P = 0.01$). Thus, we were left as best covariance structure model with either the model including 'sex.family' and 'family (4 VCs)' or the model including 'food.family' and 'family (4 VCs)', both models including the four variance components for the crosses. However, the estimates of variance components between the two models were very similar with, in particular, the same ranking among crosses (results not shown). Therefore, in the following steps of model selection we kept the model including 'food.family' and 'family (4 VCs)' (its deviance value was indeed slightly better; 2774.0 vs. 2777.9). At the end of the model selection process, the best covariance structure had the random effects 'food.family' and 'family (4 VCs)' including a different variance component for each cross.

*Fixed effects*

The score of the best model in terms of $AIC_c$ is displayed in bold.

| | Variable SurvStarv: fixed effects | |
|---|---|---|
| Model | Description | $AIC_c$ |
| c + f + s + c.f + f.s + c.s + c.f.s | Three main effects plus three interactions plus one triple interaction | 2855.6 |
| c + f + s + c.f + f.s + c.s | Three main effects plus three interactions | 2851.6 |
| c + f + s + c.f + f.s | Three main effects plus two interactions | 2847.4 |
| c + f + s + c.f + c.s | Three main effects plus two interactions | 2853.1 |
| c + f + s + f.s + c.s | Three main effects plus two interactions | 2848.8 |
| c + f + s + c.f | Three main effects plus one interaction | 2848.9 |
| c + f + s + f.s | Three main effects plus one interaction | 2844.8 |
| c + f + s + c.s | Three main effects plus one interaction | 2850.1 |
| c + f + s | Three main effects | 2846.1 |
| c*f | Two main effects plus one interaction | 2861.2 |
| c*s | Two main effects plus one interaction | 2848.1 |
| f*s | Two main effects plus one interaction | 2845.1 |
| c + f | Two main effects | 2858.7 |
| c + s | Two main effects | **2844.2** |
| f + s | Two main effects | 2846.4 |
| c | One main effect | 2856.7 |
| f | One main effect | 2859.1 |

| | Variable SurvStarv: fixed effects | |
|---|---|---|
| Model | Description | $AIC_c$ |
| s | One main effect | 2844.5 |
| . | Intercept | 2857.1 |

The best model in terms of $AIC_c$ is displayed in bold in the table and has cross and sex as fixed effects. However, the evidence for the inclusion of factor cross was weak (model 'c + s' vs. model 's') and thus for the sake of parsimony we used the model 's' for inferences.

### (3) ReproInvest

*Random effects*

| | Variable ReproInvest: random effects | | | | |
|---|---|---|---|---|---|
| Model | Description | LRT | *P*-value | Effect removed | Ref. |
| M1 | fam (4 VCs) f.fam | . | . | None | . |
| M2 | fam (4 VCs) | 0 | 1 | f.fam | M1 |
| M3 | fam f.fam | 2.5 | 0.2095 | fam (4 VCs) | M1 |
| M4 | fam | 2.5 | 0.295 | f.fam and fam (4 VCs) | M1 |

So the best model is the model with 'family' as random effect.

*Fixed effects*

The score of the best model in terms of $AIC_c$ is displayed in bold.

| | Variable ReproInvest: fixed effects |
|---|---|
| Model | $AIC_c$ |
| f*c | 116.7 |
| f + c | 116.2 |
| f | 115.9 |
| c | 115.5 |
| Intercept | **115.3** |

### (4) LarvSurv

*Random effects*

| | Variable LarvSurv: random effects | | | | |
|---|---|---|---|---|---|
| Model | Description | LRT | *P*-value | Effect removed | Ref. |
| M1 | fam (4 VCs) f.fam | . | . | None | . |
| M2 | fam (4 VCs) | 0 | 1 | f.fam | M1 |
| M3 | fam f.fam | 1.4 | 0.3632 | fam (4 VCs) | M1 |


 

| | Variable LarvSurv: random effects | | | | |
|---|---|---|---|---|---|
| Model | Description | LRT | *P*-value | Effect removed | Ref. |
| M4 | fam | 1.4 | 0.4745 | f.fam and fam (4 VCs) | M1 |

So the best model is the model with 'family' as random effect.

*Fixed effects*

The score of the best model in terms of $AIC_c$ is displayed in bold.

| | Variable LarvSurv: fixed effect |
|---|---|
| Model | $AIC_c$ |
| f*c | −23.8 |
| f + c | −30.2 |
| f | **−34.2** |
| c | −11.9 |
| Intercept | −15.8 |

**(5) DvptTime**

*Random effects*

| | Variable DvptTime: random effects | | | | |
|---|---|---|---|---|---|
| Model | Description | LRT | *P*-value | Effect removed | Ref. |
| M1 | fam (4 VCs) f.fam | . | . | None | . |
| M2 | fam (4 VCs) | 146.8 | 0 | f.fam | M1 |
| M3 | fam f.fam | 1 | 0.4466 | fam (4 VCs) | M1 |
| M4 | f.fam | 7.6 | 0.03871 | fam | M3 |
| M5 | fam | 147.3 | 0 | f.fam | M3 |

The random effects were kept as 'food.family' and 'family'.

*Fixed effects*

The score of the best model in terms of $AIC_c$ is displayed in bold.

| | Variable DvptTime: fixed effects |
|---|---|
| Model | $AIC_c$ |
| f*c | 5553.1 |
| f + c | **5552.3** |
| f | 5562.5 |
| c | 5618.9 |
| Intercept | 5624.4 |

### (6) SurvCold

*Random effects*

| | Variable SurvCold: random effects | | | | |
|---|---|---|---|---|---|
| Model | Description | LRT | *P*-value | Effect removed | Ref. |
| M1 | fam (4 VCs) f.fam | . | . | None | . |
| M2 | fam (4 VCs) | 0 | 1 | f.fam | M1 |
| M3 | fam f.fam | 2.5 | 0.2095 | fam (4 VCs) | M1 |
| M4 | fam | 2.5 | 0.2950 | f.fam and fam (4 VCs) | M1 |

So the best model is the model with 'family' as random effect.

*Fixed effects*

The score of the best model in terms of $AIC_c$ is displayed in bold.

| | Variable SurvCold: fixed effects |
|---|---|
| Model | $AIC_c$ |
| f*c | 85.4 |
| f + c | 80.2 |
| f | **77.4** |
| c | 92.4 |
| Intercept | 90.0 |

## Literature cited

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## Appendix B: Results of ANOVAs with full models for the six traits studied

| Source | Degrees of freedom | Test statistic | *P* |
|---|---|---|---|
| *(A) Larval survival (LarvSurv)* | | | |
| Fixed effects | | Type III *F* | |
| Cross | 3 | 1.10 | 0.3722 |
| Food | 1 | 28.26 | <0.0001 |
| Food × Cross | 3 | 0.45 | 0.7160 |
| Random effect | | Wald test | |
| Fam (BIOBIO) | | 1.27 | 0.1016 |
| Fam (BIOINV) | | 0.92 | 0.1786 |
| Fam (INVBIO) | | 1.06 | 0.1452 |
| Fam (INVINV) | | 0.30 | 0.3823 |
| Food × Fam (cross) | | * | * |
| *(B) Development time (DvptTime)* | | | |
| Fixed effects | | Type III *F* | |

| Source | Degrees of freedom | Test statistic | *P* |
|---|---|---|---|
| Cross | 3 | 5.86 | 0.0047 |
| Food | 1 | 185.03 | <0.0001 |
| Food × Cross | 3 | 1.86 | 0.1529 |
| Random effect | | Wald test | |
| Fam (BIOBIO) | | 1.38 | 0.0844 |
| Fam (BIOINV) | | 1.39 | 0.0822 |
| Fam (INVBIO) | | 0.70 | 0.2407 |
| Fam (INVINV) | | 1.33 | 0.0920 |
| Food × Fam (cross) | | 3.63 | 0.0001 |
| *(C) Reproductive investment (ReproInvest)* | | | |
| Fixed effects | | | |
| Cross | 3 | 2.15 | 0.1248 |
| Food | 1 | 1.66 | 0.2009 |
| Food × Cross | 3 | 2.06 | 0.1089 |
| Random effect | | Wald test | |
| Fam (BIOBIO) | | 0.37 | 0.3555 |
| Fam (BIOINV) | | 0.44 | 0.3283 |
| Fam (INVBIO) | | 1.27 | 0.1021 |
| Fam (INVINV) | | 1.35 | 0.0890 |
| Food × Fam (cross) | | * | * |
| *(D) Survival in starvation (SurvStarv)* | | | |
| Fixed effects | | Type III *F* | |
| Cross | 3 | 1.61 | 0.2176 |
| Food | 1 | 0.09 | 0.7616 |
| Sex | 1 | 10.14 | 0.0027 |
| Food × Cross | 3 | 2.19 | 0.1032 |
| Cross × Sex | 3 | 0.58 | 0.6341 |
| Food × Sex | 1 | 3.71 | 0.0548 |
| Food × Cross × Sex | 3 | 0.93 | 0.4261 |
| Random effect | | Wald test | |
| Fam (BIOBIO) | | 0.60 | 0.2747 |
| Fam (BIOINV) | | 1.61 | 0.0542 |
| Fam (INVBIO) | | 1.56 | 0.0593 |
| Fam (INVINV) | | * | * |
| Food × Fam (cross) | | 2.09 | 0.0185 |
| Fam × Sex (cross) | | 1.66 | 0.0487 |
| Food × Fam × Sex (cross) | | * | * |
| *(E) Survival in cold conditions (SurvCold)* | | | |
| Fixed effects | | Type III *F* | |
| Cross | 3 | 1.34 | 0.2909 |
| Food | 1 | 19.29 | <0.0001 |
| Food × Cross | 3 | 0.85 | 0.4736 |
| Random effect | | Wald test | |
| Fam (BIOBIO) | | 1.04 | 0.1501 |
| Fam (BIOINV) | | 1.57 | 0.0579 |
| Fam (INVBIO) | | 1.30 | 0.0960 |
| Fam (INVINV) | | 0.64 | 0.2621 |
| Food × Fam (cross) | | * | * |
| *(F) Body length (Lgth)* | | | |
| Fixed effects | | Type III *F* | |
| Cross | 3 | 5.01 | 0.0006 |
| Food | 1 | 88.37 | <0.0001 |
| Sex | 1 | 943.25 | <0.0001 |
| Food × Cross | 3 | 1.80 | 0.1638 |
| Cross × Sex | 3 | 0.65 | 0.5831 |
| Food × Sex | 1 | 10.22 | 0.0015 |
| Food × Cross × Sex | 3 | 1.56 | 0.2000 |

| Source | Degrees of freedom | Test statistic | *P* |
|---|---|---|---|
| Random effect | | Wald test | |
| Fam (BIOBIO) | | 0.04 | 0.4829 |
| Fam (BIOINV) | | 0.61 | 0.2693 |
| Fam (INVBIO) | | 1.08 | 0.1406 |
| Fam (INVINV) | | 0.31 | 0.3787 |
| Food × Fam (cross) | | 2.40 | 0.0082 |
| Fam × Sex (cross) | | * | * |
| Food × Fam × Sex (cross) | | 0.33 | 0.3705 |


 