



# Differences in pre-imaginal development of the honey bee *Apis mellifera* between in vitro and in-hive contexts

Diego E. VÁZQUEZ<sup>1,2</sup>, Walter M. FARINA<sup>1,2</sup>

<sup>1</sup>Facultad de Ciencias Exactas y Naturales, Departamento de Biodiversidad y Biología Experimental, Laboratorio de Insectos Sociales, Universidad de Buenos Aires, Buenos Aires, Argentina

<sup>2</sup>Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE), CONICET-Universidad de Buenos Aires, Buenos Aires, Argentina

Received 14 June 2019 – Revised 11 February 2020 – Accepted 30 March 2020

**Abstract** – In vitro rearing of honey bees is becoming a widely employed procedure. Nevertheless, few studies have assessed its impact on brood development considering nutritional and social differences between both in vitro and in-hive rearing contexts. We compared developmental parameters between these two contexts using an intra-frame grafting procedure. We confirmed a grafting effect on development. However, pre-imaginal survival did not depend on the rearing procedure. The correlation in mortality between groups of larvae (exposed or not to grafting) was independent of the rearing context suggesting transmission of inheritable and infectious factors. In addition, the comparison between contexts showed that in vitro larvae had delayed molting and achieved emergence 1.57 days later. Furthermore, in vitro imagoes emerged with lower weight and shorter wing length than in-hive bees. Our results strengthen the idea that there is an in vitro phenotype as a consequence of phenotypic plasticity.

*Apis mellifera* / larval rearing / brood grafting / phenotypic plasticity / inter-colony variability

## 1. INTRODUCTION

The increasing disturbance of the agricultural ecosystems negatively affects the health of pollinators as they have to constantly adapt to various environmental challenges. Insect pollinators, including native and managed bees, have declined in abundance and diversity on local and regional scales in North-West Europe and the American continent (IPBES 2016; Kluser et al. 2010; Potts et al. 2010; vanEngelsdorp and Meixner 2010). The decline in honey bee (*Apis mellifera*)

population is well-documented and is thought to be a consequence of multiple concomitant factors, such as exposure to monocultures, agrochemicals, pathogens, parasites, extreme climate conditions, and bad beekeeping practices (IPBES 2016; Kluser et al. 2010). Open-field risk assessments of hives focused on the bias of brood development (Wu et al. 2011) cannot establish causality to a single factor. Additionally, they are not able to assess dose-response relationships due to the complexity of in-hive exposure. As a result, the in vitro rearing procedure (details in Sections 2.2 and 2.3) has been developed as a way to assess potential stressors during the development of bees under laboratory conditions (Becker and Keller 2016; Crailsheim et al. 2013). Nevertheless, only a few studies have compared artificially reared brood with honey bees reared in the hive (Brodtschneider et al. 2009; De Souza et al. 2015; Steijven et al. 2017).

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s13592-020-00767-0>) contains supplementary material, which is available to authorized users.

Corresponding author: W. FARINA,  
WALTER@FBMC.FCEN.UBA.AR  
Manuscript editor: Yves Le Conte

Bees are holometabolous insects (Winston 1987), which means that metamorphosis enables brood to change larval body architecture and habits into an imago phenotype (details in Section 2.5). The larva is the developmental stage of exponential growth in which the future imago determines its morphology and size (de Michelette and Soares 1993; Rembold et al. 1980). As eusocial insects, honey bees live within the nest as a colony with a single reproductive female (queen) and cooperative brood care among sisters (Winston 1987). The social interaction of young adults (nurse bees) with brood influences larval health by supplying defenses (social immunity) and satisfying nutritional requirements (Brodschneider and Crailsheim 2010). At the end of the larval feeding period and prior to metamorphosis, nurse bees seal the brood cells with wax thus reducing the interaction of the developing individuals with the environment. The isolated larvae then begin a process of cell apoptosis and morphogenesis. Pre-pupal (metamorphosis) and pupal stages are critical periods of intense transformation susceptible to environmental disruptions (Groh et al. 2004). The imaginal sensory organs (antenna and eyes), locomotor appendages (legs and wings), and almost all other organs are developed from scratch, while the nervous and digestive systems are simply reorganized (Myser 1954; Oertel 1930; Farris et al. 1999). Adjustments of pre-imaginal development to environmental stimuli thus have short- and long-term consequences for the fate of individuals and colonies.

Every phenotype results from the interaction between an individual's genome and its context. This context covers multiple parameters of both the environment, external to the organism, and the internal state of the organism, e.g., nutritional or hormonal state and the interaction among cells, tissues, and organs. In this sense, the context of early developmental stages is crucial for understanding the phenomenon of phenotypic plasticity (Miner et al. 2005; Moczek 2010; Whitman and Agrawal 2009). Honey bees are a species with high adaptability and resilience (Schwander et al. 2010; Rittschof et al. 2015). They can display changes in phenotypic traits (physiological or behavioral) at the adult stage depending on the pre-

imaginal rearing context. For example, they can show changes in chemosensory and learning abilities (Mustard et al. 2019; Ramírez et al. 2016), aggressive behavior (Rittschof et al. 2015), and resilience to pesticides (Medrzycki et al. 2010) or to starvation (Wang et al. 2016). Female bees can also display two phenotypes or castes with different functions within the colony during the imaginal stage as a consequence of phenotypic plasticity, called polyphenism (Corona et al. 2016; Simpson et al. 2011). The queen is the reproductive phenotype while the workers are facultative sterile females that comply with tasks vital for the colony, from the nursing of brood to foraging for resources. These completely different phenotypes are a result of developmental plasticity (Linksvayer et al. 2011). Although brood show developmental canalization, i.e., a tendency to follow the same developmental pathway to successfully achieve the imago phenotype, sensitive periods (periods of high plasticity) allow the larvae to differentiate into one caste or the other. Diet acts as an environmental stimulus for dimorphism (Winston 1987). Larvae induced to the worker phenotype eat worker jelly (a mixture of glandular secretions of nurse bees) and bee bread (a mixture of pollen and honey). Meanwhile, larvae induced to the queen phenotype eat huge amounts of royal jelly (a different mixture of glandular secretions of nurse bees). Compounds present in larval food (proteins in royal jelly and microRNAs in bee bread) are the environmental stimuli that trigger the shift in phenotype (Kamakura 2011; Zhu et al. 2017). Neurochemical and hormonal pathways mediate this transformation, but genetic and epigenetic mechanisms are involved in initiating and maintaining the polyphenism (Evans and Wheeler 2000; Maleszka 2008; Weiner and Toth 2012). Consequently, the genetic diversity among colonies provides different capabilities to respond to changes in the rearing context.

The aim of the present study was to measure the effects of the rearing context (in-hive or in vitro) on the pre-imaginal development of honey bees regardless of a potential grafting effect. For this purpose, we assessed survival and developmental parameters in brood from the same cohort reared in-hive and in vitro with similar manipulation. In addition, we quantified the grafting

effect by comparing brood reared in-hive with or without transference between cells of the brood frame. We also reared cohorts from different source colonies to take into account the inter-colony variation when the context is the same but the genetic profiles are different.

## 2. MATERIALS AND METHODS

### 2.1. Study site and animals

Experiments were performed from January to March during the summer season of the southern hemisphere. Female larvae (from cells of 5 mm of diameter) were sampled from six colonies (A–C in 2016 and D–F in 2017) of European-derived honey bees (*Apis mellifera* L.) and reared in vitro or in-hive (see below). Colonies were purchased in November of each year and housed in new Langstroth hives at the experimental apiary of the University of Buenos Aires, Argentina (34° 32' S, 58° 26' W). The new six queens were not genetically related (different parents, i.e., inter-colony genetic diversity), and they were naturally inseminated by multiple mates during free flights in the field (i.e., intra-colony genetic diversity).

### 2.2. Rearing context

The in vitro rearing procedure (Crailsheim et al. 2013; Schmehl et al. 2016) was employed by us to study the effects of the rearing context on pre-imaginal development. To compare between different contexts (in vitro or in-hive) with similar manipulation, we performed an intra-frame grafting procedure (i.e., grafting a larva into a different cell within the same brood frame). To avoid variability in grafting effect, the same researcher carried out these procedures. We also monitored an in-hive control group without grafting to account for subtle adverse effects due to the transfer between cells. To sum up, the three groups of larvae were reared in-hive without grafting (control), in-hive with intra-frame grafting (IFG), and in vitro. For that purpose, we introduced an empty frame in a source colony (A–F) and monitored it for 8 h until the queen had laid enough eggs. Three days later, we withdrew the brood frame and carried it to a room with

environmental conditions suitable for grafting (Büchler et al. 2013; Crailsheim et al. 2013; Schmehl et al. 2016). A range of 70–80 cells with newly hatching larvae (0–8-h old post-hatching) was randomly marked and then sorted in each rearing group (Human et al. 2013). This number of larvae represented around 5% of the cohort (eggs laid in 1 day by the queen) and up to 0.5% of the colony in an average hive. For the IFG group, we removed firstly a larva in the first stadium from the target cell, leaving it empty but with a generous amount of worker jelly (around 20 µl). The latter facilitated the next grafting because we could put down gently the larva onto the top of the food without hurting it. Thus, a second larva in the first stadium was grafted from a source cell to the target cell and so on. At this point, the grafting technique and tools are very important (Büchler et al. 2013; Schmehl et al. 2016). Larvae were transferred with careful handling using a stainless steel grafting needle (JRB®, tip with 1 mm of width). They should lie with the same side facing up as they were in the wax cells and should be grafted with a single movement, sliding the grafting tool beneath the larva. We avoided grafting any larvae that were not secured on the first attempt or if her health was believed to be compromised in any way. At the end, we returned the brood frame to the source colony after the grafting procedure for either in-hive or in vitro rearing (Section 2.3). We monitored, in a room with suitable environmental conditions, the marked cells daily until these were sealed (around 120 h post-hatching) to evaluate for survival and development of larvae as well as in larvae reared in vitro (Crailsheim et al. 2013; Human et al. 2013; Schmehl et al. 2016).

### 2.3. In vitro rearing procedure

We grafted 22 first stadium larvae (0–8-h old post-hatching) from the brood frame to plastic cups and placed them in Petri dishes. The same tool and key points for the grafting technique were applied in the in vitro procedure as in Section 2.2 (Büchler et al. 2013; Schmehl et al. 2016). Larvae were reared inside a desiccator (Coltap®, 30 × 15 × 8 cm) with supersaturated salt solution in an incubator with constant temperature (34.5 °C) and

relative humidity (~90% or 75% RH as appropriate) within the desiccator until completing the pre-imaginal development (Crailsheim et al. 2013; Schmehl et al. 2016). To standardize larval food administration, we provided 160  $\mu$ l of food spread in six aliquots of increasing volume to each larva during the 6 days of feeding period: 20  $\mu$ l before grafting, 20  $\mu$ l at 48 h, 30  $\mu$ l at 72 h, 40  $\mu$ l at 96 h, and 50  $\mu$ l at 120 h (Aupinel et al. 2005). We used a previously established diet, i.e., 6% D-glucose, 6% D-fructose, 1% yeast extract (provided by Sigma-Aldrich), and 50% commercial royal jelly (Kaftanoglu et al. 2011; Vandenberg and Shimanuki 1987). The brood cohort completed its larval development into the plastic cups when larvae finished the spinning and defecation periods. Then larvae were transferred carefully with a stainless steel forceps (round tip) to a Petri dish with blotting paper in a second desiccator where they completed metamorphosis (Schmehl et al. 2016). Finally, pupae were transferred carefully with a stainless steel spatula to a new Petri dish with blotting paper in a third desiccator for pupal development (Schmehl et al. 2016). The blotting paper was daily revised and changed when it was dirty. In order to prevent bacterial or fungal contamination and subsequent infection, we maintained sterile conditions and removed daily dead brood (Crailsheim et al. 2013).

## 2.4. Survival

A larva was classified as dead when their color changes to brownish or they develop edema and remain immobile. A pre-pupa or pupa was classified as dead when their pigmentation was unusual or lost cuticle turgor (Aupinel et al. 2005; Crailsheim et al. 2013; Schmehl et al. 2016). We took note of their status daily. For the in-hive rearing, a brood was classified as dead when its cell was empty. However, in the IFG group when cells were empty on the second day, it could be due to unsuccessful grafting or rejection by nurse bees in the source colony (Büchler et al. 2013; Evans and Spivak 2010; Fukuda and Sakagami 1968). Therefore, we analyzed separately the survival after the first day from all groups comparing among colonies.

## 2.5. Pre-imaginal development

Throughout the growth period (from 0 to 96 to 134 h post-hatching), four molts allow a honey bee larva to increase in size, which determines five stadia. A molt normally occurs around every 1 day (17–28 h) up to the 4-day post-hatching (around 85 h of age). Each stadium was identified daily by its morphological traits (Fig. S1) (Human et al. 2013). When a larva had a smaller size or different characteristics from the stadium it was expected to be in, it was classified as delayed (Tavares et al. 2015; Vázquez et al. 2018; Wu et al. 2011). In both rearing contexts (in-hive or in vitro) prior to pupation, delayed or not delayed larvae ate all offered food. The larval stage finishes with a fifth molt after the larva complete the spinning and defecation periods. During the fifth molt occurs the metamorphosis where the brood is called pre-pupa. At the time of the fifth ecdysis (180–248 h post-hatching), the pupal stage starts. During pupal development occurs the eyes and body pigmentation (30–42 h post-pupation). Finally, the sixth and last molt allows a honey bee pupa to complete the pre-imaginal development and appears as an imago after 17 days on average (318–470 h post-hatching). Inside the hive, cells are sealed by nursing bees at the 5-day post-hatching (around 120 h), and brood complete the pre-imaginal development hidden. At the end of the sixth molt, the imago opens the seal and emerges from the cell. These morphological traits and developmental times of reference have been detailed by Bertholf (1925), Myser (1954), Rembold et al. (1980), and Michelette and Soares (1993).

## 2.6. Measurements in the emerged adult

At the 15-day post-hatching, we withdrew the brood frame with the control and the IFG groups from the source colony (A–F). Then, during the next 4 days, we stored the brood frame in an exclusion cage inside an incubator (34 °C and 60% RH). Thus, we monitored it daily in a suitable room for measured emergence and sampled imagoes. We measured morphological traits in the newly emerged adults from both rearing contexts. For that, we used an electronic balance to weigh (Mettler Toledo AG285,  $\pm 0.1$  mg) and a

stereomicroscope (Leica MZ8) to measure appendages (length of antenna and wing) and body size (width and length of thorax and abdomen) (Human et al. 2013).

## 2.7. Statistics

We performed data analysis and graphics in R software (for details see [Supplementary](#)). Survival and developmental data were analyzed with accelerated failure time (ATF) and the Cox proportional hazard (CPH) models, respectively. Weight and morphometric data were analyzed with principal components analysis (PCA). Emergence age was analyzed with generalized linear mixed models (GLMM). Correlation tests were performed with the Pearson or Spearman's rho coefficients depending on normality of data. The alpha level was set at 0.05 and *P* value corrected for multiple post hoc comparisons with Bonferroni procedure.

## 3. RESULTS

### 3.1. Effect of the rearing context on pre-imaginal survival

In order to estimate the impact of the rearing contexts and grafting manipulation on honey bee development, we quantified their survival during the pre-imaginal stage of different colony cohorts (A–F).

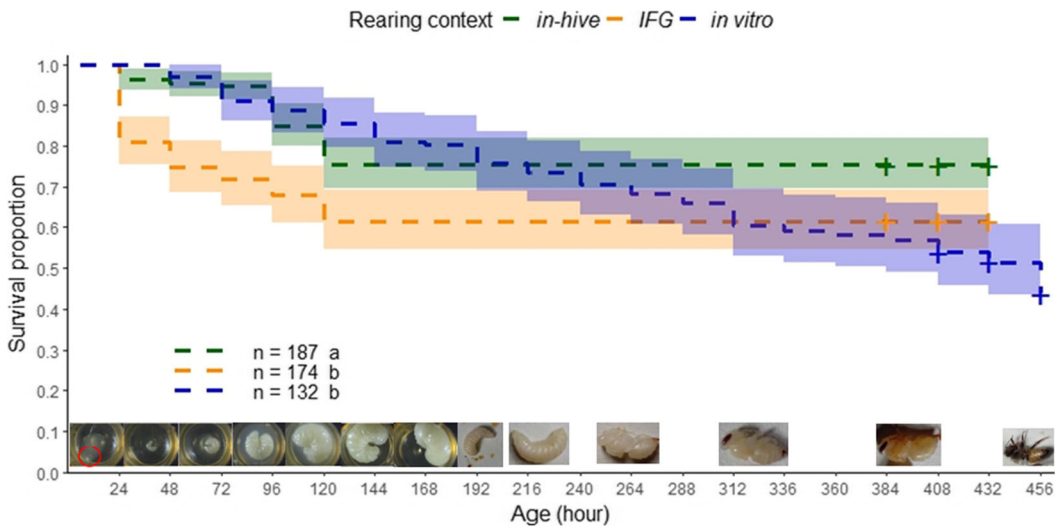
A total of 25% of the tracked brood in the control group without grafting manipulation is dead at the mean age of  $91.92 \pm 36.72$  h that corresponds to the last larval molt before the fifth stadium. Our results showed a significant reduction on pre-imaginal survival due to the grafting (0.6-fold more of deaths) when we compared between broods reared in-hive with or without grafting manipulation (ATF model, survival~rearing context + strata (colony),  $\chi^2(2) = 18.79$ ,  $P < 0.001$ ,  $N = 493$ . Stratification term,  $\chi^2(5) = 17.42$ ,  $P = 0.003$ . Log-rank test for post hoc pairwise comparisons, in-hive vs IFG  $\chi^2(1) = 13.8$ ,  $P < 0.001$ ,  $N = 361$ ; in-hive vs iv  $\chi^2(1) = 12.2$ ,  $P < 0.001$ ,  $N = 319$ ; IFG vs iv  $\chi^2(1) = 0$ ,  $P = 0.933$ ,  $N = 306$ .). Furthermore, no significant differences existed in survival

between rearing contexts with similar manipulation (IFG and in vitro). However, grafting affected differently the temporal pattern of brood mortality through the pre-imaginal development depending on the rearing context (Figure 1). The brood reared in the hive with grafting manipulation (IFG) showed an accelerated reduction in survival during the growth period (0–144 h) than that in the in vitro group (Table I).

For the IFG group, the mean age of death post-grafting was earlier than the group without manipulation, around  $54.72 \pm 34.56$  h, during the third larval stadium before feeding switch and exponential growth. Meanwhile, the brood reared in vitro showed a later mean age of death ( $231.12 \pm 135.36$  h), around the fifth ecdysis during pupation. There was higher brood survival during the growth period in the in vitro rearing (14% of death) than in-hive one even without grafting manipulation (25% of death). Finally, survival, after the first 24 h, was notably lower in the brood reared in the hive with grafting manipulation (19% of death) with respect to in vitro (0% of death) (Table I).

### 3.2. Effect of the rearing context on pre-imaginal development

We identified and recorded the stadium and developmental stage of each individual per cohort daily (A–F) (Fig. S1). We noted some differences in the duration of stadia and developmental stages between bees reared in vitro and in-hive, the latter from previous studies for the pre-pupal and pupal stages (see Section 2.5). For brood reared in-hive in our experiment, the sealing of cells occurred around 120 h post-hatching and did not show delay for most of the brood (90 and 94% of larvae for control and IFG, respectively). In this sense, our results showed no significant differences in the mean age of delayed molting during larval development between broods reared in-hive with or without grafting manipulation (CPH model, successful molting~rearing context + strata(colony),  $\chi^2(2) = 63.4$ ,  $P < 0.001$ ,  $N = 493$ . Log-rank test for post hoc pairwise comparisons, in-hive vs IFG  $\chi^2(1) = 1.7$ ,  $P = 0.197$ ,  $N = 361$ ; in-hive vs iv  $\chi^2(1) = 59$ ,  $P < 0.001$ ,  $N = 319$ ; IFG vs iv  $\chi^2(1) = 32.1$ ,  $P < 0.001$ ,



**Figure 1.** Pre-imaginal survival under different rearing procedures. The proportion of survival during the pre-imaginal period (around 432 h post-hatching) of bees from six colonies (A–F) until emergence is plotted with a photographic sequence of bee development above the x-axis (details in Fig. S1). Survival curves are plotted with their confidence interval (95%) for each rearing group. The number of assessed bees is shown in the graph. Fitting of data to AFT model (survival–rearing context + strata (colony)) followed by a Log-rank test for post hoc comparisons. The curves are plotted with different colors per rearing context, i.e., green for in-hive control, orange for intra-frame grafting (IFG), blue for in vitro. The + indicates time points with censoring data. Different letters indicate significant differences among groups.

$N = 306$ ). Nevertheless, a significant reduction in the proportion of larvae with successful molting was showed due to the artificial rearing context post-grafting (Figure 2). Under in vitro rearing, all the colonies showed an increase in the proportion of larvae with delayed molting (Table I). Furthermore, for 74% of the larvae reared in vitro, the growth period lasted around 144 h post-hatching (10 h more than the maximum value reported previously) or several hours later for the rest. Meanwhile, molting process occurred in 55% of the in vitro larvae until 92 h post-hatching (7 h more than the mean value reported previously) or a few hours later for the rest. During in-hive rearing, 0–7% of larvae showed a delay in the molting process, and there was no variability among colonies. This lack of variability continued after grafting manipulation (IFG) with an increase in the proportion of delayed larvae (4–28%). However, variability in the tolerance to grafting manipulation was found for brood reared in vitro with a conspicuous increase in the range of proportions of delayed larvae (18–68%).

At the end of the growth period, larvae reared in vitro consumed all available food and then started the spinning period into the plastic cup. This period ended around 168 h post-hatching and larvae start defecation. The larval stage in vitro finished with a fifth molt when metamorphosis occurs and the stage is called pre-pupa (after 192 h reared in vitro). The pupal stage started after 240 h reared in vitro when the fifth ecdysis finished (26 h more than the mean value reported previously). The eyes pigmentation of pupae reared in vitro occurred around 312 h and the body pigmentation around 360 h (62 h more than the mean value reported previously). Moreover, 37% of pupae started pigmentation with delay respect to the remainder brood reared in vitro. Finally, pupae finished their maturation and started the last molt eating away its pupal cuticle. A walker bee with extended wings was recorded as an emerged adult.

The proportion of emerged adults was similar between rearing contexts with equal grafting manipulation (in vitro and IFG) (Table I and

**Table 1.** Differences in pre-imaginal development among colonies.

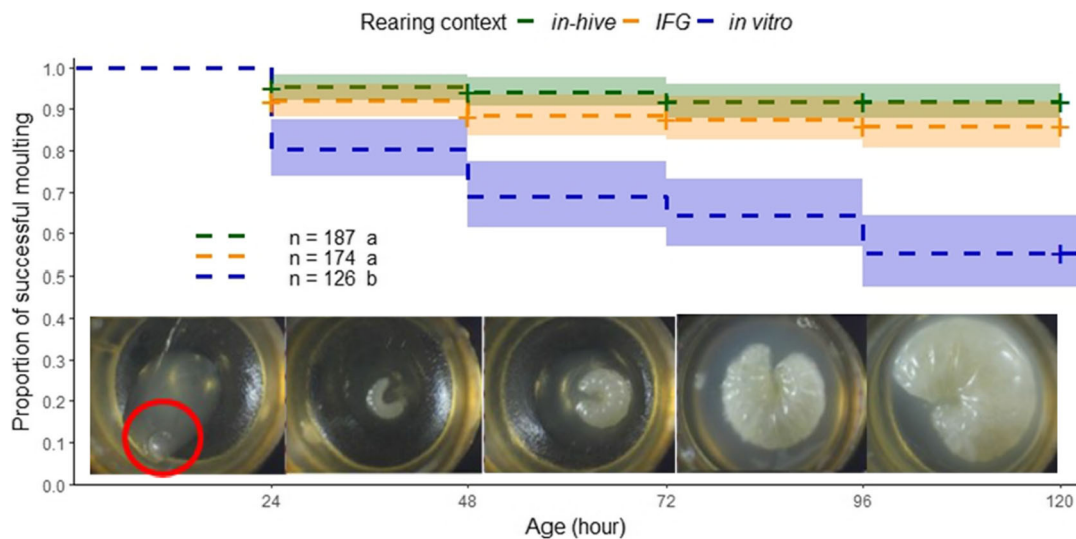
Year	In-hive control						Intra-frame grafting (IFG)						In vitro					
	2016		2017		2017		2016		2017		2017		2016		2017		2017	
Colony	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
Survival	0.90	1.00	1.00	1.00	1.00	0.87	0.93	0.93	0.46	0.88	0.72	0.93	1.00	1.00	1.00	1.00	1.00	1.00
			0.96						0.81						1.00			
0–144 h	0.87	0.93	0.68	0.92	0.37	0.73	0.80	0.83	0.39	0.69	0.37	0.61	0.95	1.00	0.91	0.86	0.64	0.77
			0.75						0.61						0.86			
Successful molting rate	0.93	0.93	1.00	0.94	0.93	0.77	0.87	0.87	0.86	0.92	0.72	0.93	0.64	0.82	0.59	0.45	0.41	0.41
			0.92						0.87						0.55			
Emergence Rate	0.87	0.93	0.68	0.92	0.37	0.73	0.80	0.73	0.39	0.69	0.38	0.61	0.55	0.45	0.36	0.59	0.45	0.50
			0.75						0.60						0.48			
Mean age (day)	16.2	16.0	17.1	17.0	17.0	17.0	16.2	16.4	17.2	17.0	17.0	17.0	18.7	18.7	18.6	18.0	17.7	17.9
			16.7 ± 0.5						16.7 ± 0.5						18.3 ± 0.6			
No. of brood	30	30	31	36	30	30	30	30	28	26	32	28	22	22	22	22	22	22

Proportion of bees with successful development post-hatching per colony (A–F) measured within different rearing contexts: in-hive (without manipulation), intra-frame grafting (within the same colony), and in vitro

The endpoints are delay in the larval molting process (0–96 h) or death measured in each bee during the growth period (0–144 h) and its emergence

Survival on the first 24 h was segregated due to the possibility of an unsuccessful grafting or rejection by nurse bees in the source colony

The number of brood assessed for each colony is shown in the bottom of the table



**Figure 2.** Pre-imaginal development under different rearing procedures. The proportion of larvae without delay in molting for each day during the growth period (around 144 h post-hatching) of bees from six colonies (A–F) until emergence is plotted with a photographic sequence of larval development above the  $x$ -axis (details in Fig. S1). Curves of successful molting are plotted with their confidence interval (95%) for each rearing group. The number of assessed bees is shown in the graph. Fitting of data to CPH model (successful molting~rearing context + strata (colony)) followed by a Log-rank test for post hoc comparisons. The curves are plotted with different colors per rearing context, i.e., green for in-hive control, orange for intra-frame grafting (IFG), blue for in vitro. The + indicates time points with censoring data. Different letters indicate significant differences among groups.

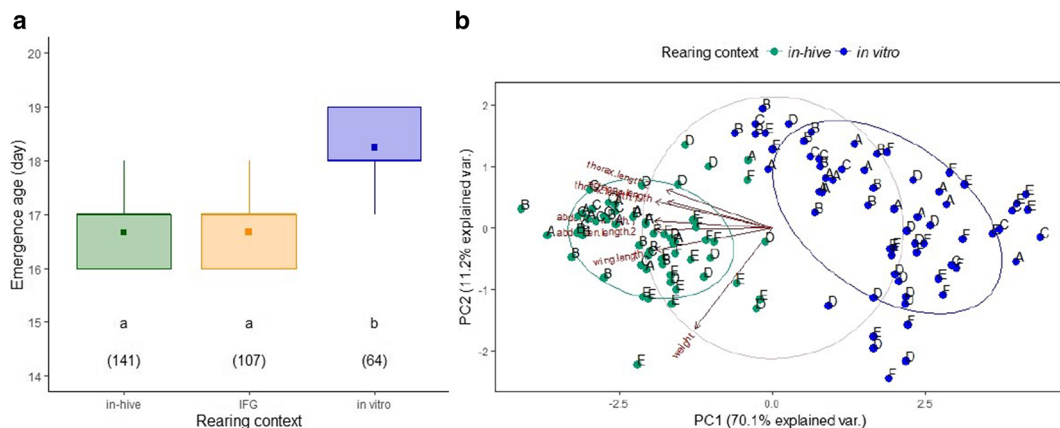
Section 3.1). However, bees reared in vitro emerged in mean 1.57 days later than bees reared in the hive (Figure 3a) regardless of grafting manipulation (GLMM model, emergence age~rearing context + (1|colony). Rearing context term,  $F(2, 304) = 289.53$ ,  $P < 0.001$ . Variance structure, 26% among colonies, Tukey's test for post hoc pairwise comparisons, in-hive vs IFG  $Z = 1.16$ ,  $P = 0.474$ ,  $N = 248$ ; in-hive vs iv  $Z = 22.88$ ,  $P < 0.001$ ,  $N = 205$ ; IFG vs iv  $Z = 20.9$ ,  $P < 0.001$ ,  $N = 171$ ). Furthermore, we measured weight and morphometric indicators of size (Table S1) in the newly emerged adults from both rearing contexts (in-hive and in vitro). We performed a PCA with these variables and a single principal component (PC1) achieved 70% of the cumulative proportion of deviation (Table S2). Although, no variable had a high correlation with the PC1 so all measurements were equal explanatory. Two groups that are easily distinguishable were displayed along the PC1 associated with the rearing procedure (Figure 3b). Our results showed a notorious reduction on the size of bees reared

in vitro reflected mainly in the loss of weight (25.8%) and the shortest lengths of the wings (33.7%) and abdomen (24.5%). In addition, bees exhibited an increase in variation of weight and abdomen length reared in vitro (Table S1).

### 3.3. The dependency of the pre-imaginal development on the source colony

Under the same field conditions, control cohorts reared in-hive showed a different brood mortality (7–67%) per colony (A–F) (Table I). Moreover, larval mortality during the growth period (144 h post-hatching) was strongly positively correlated among the six colonies (A–F) among groups with or without grafting manipulation regardless of the rearing context post-grafting (IFG,  $r = 0.87$ ,  $t(4) = 3.48$ ,  $P = 0.01$ ; in vitro,  $r = 0.84$ ,  $t(4) = 3.15$ ,  $P = 0.02$ ; Figure 4a). Apart from that, in the in vitro context, the larval survival, and successful molting showed inter-colony variability (Table I) with a strong positive non-linear correlation between both endpoints ( $\rho = 0.99$ ,  $S =$





**Figure 3.** Effect of rearing procedure in newly emerged adults. **a** Dispersion of the emergence age of adults reared in the different rearing procedures. For in-hive bees, emergence age was measured when the target cell was empty. Boxplot of data is plotted with its mean (point) and median (thick line). The number of assessed bees is shown in the graphs. **b** PCA plot based on morphological measurements (length of antenna and wing, and width and length of thorax and abdomen) and weight of newly emerged adults reared in-hive or in vitro during the pre-imaginal period. The points and boxplots are plotted with different colors per rearing procedure, i.e., green for in-hive control, orange for intra-frame grafting (IFG), blue for in vitro. Different letters indicate significant differences among groups.

0.5,  $P < 0.001$ ). There was no correlation between endpoints in cohorts reared in-hive (with manipulation,  $\rho = 0.06$ ,  $S = 32.97$ ,  $P = 0.46$ ; without manipulation,  $\rho = -0.15$ ,  $S = 40.31$ ,  $P = 0.61$ ). Finally, there was a strong positive correlation between the emergence proportions of bees with or without grafting manipulation among the six colonies (A–F) when brood were reared in-hive post-grafting ( $r = 0.85$ ,  $t(4) = 3.27$ ,  $P = 0.02$ ; Figure 4b). However, there was no correlation when brood were reared in vitro post-grafting ( $r = 0.47$ ,  $t(4) = 1.07$ ,  $P = 0.17$ ).

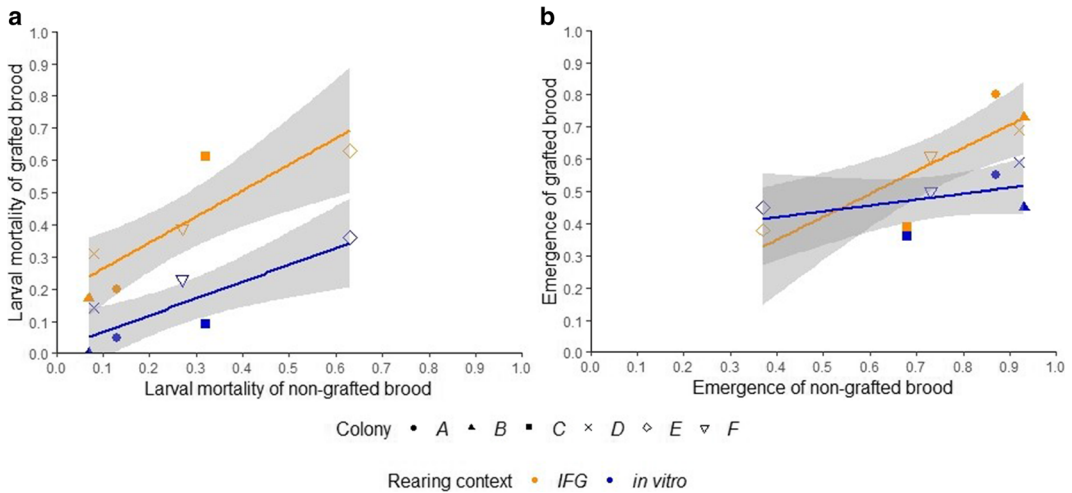
## 4. DISCUSSION

### 4.1. Effect of grafting manipulation

Previous studies have highlighted the importance of the handling when researchers transfer brood between containers with the priority not to injure larvae or pupae (Büchler et al. 2013; Crailsheim et al. 2013; Schmehl et al. 2016). Brood must be transferred with careful handling and with a single movement. It is important that the larvae are not immersed in the food but are floated off the grafting tool on the top of the target drop. Besides, they should lie with the same side

facing up as they were in the wax cells to prevent their drowning. Clumsy handling of the brood (larva or pupa) can hurt them and cause an injury in the cuticle with bleeding. A great loss of hemolymph leads inevitably to death within a short time. Conversely, a microinjury is not lethal to the brood because of coagulation and melanization mechanisms. Both of them heal without great bleeding but induce a humoral immune response (Bidla et al. 2005; Negri et al. 2014; Randolt et al. 2008; Theopold et al. 2004). As a consequence, the transfer of brood may induce a subtle response to mechanical stress. Thereupon, the exclusion of the grafting effect by ignoring dead or abnormal larvae from the experiment in the first days (Crailsheim et al. 2013) introduces bias into the interpretation of measurements, mainly because the stress susceptibility is variable among larvae and it was reported as age-dependent (Staron et al. 2019; Vázquez et al. 2018). In this vein, the effect of grafting on development has been generally ignored.

In our experiment, when bees were reared in the hive, the grafting manipulation promoted a reduction in survival after the first 24 h and an increase in the proportion of bees with delayed molting during larval development. However, all



**Figure 4.** The selection of a source colony determines survival and emergence ratios for in-hive and in vitro rearing context post-grafting. **a** Correlation between survival proportions with (IFG or in vitro) or without manipulation (in-hive) during the growth period (144 h post-hatching) among the six colonies (A–F). Regression equations, IFG  $y = 0.81x + 0.18$ ,  $R^2 = 0.75$ ; in vitro  $y = 0.52x + 0.01$ ,  $R^2 = 0.71$ . **b** Correlation between emergence proportions with (IFG or in vitro) or without manipulation (in-hive) among the six colonies (A–F). Regression equations, IFG  $y = 0.71x + 0.07$ ,  $R^2 = 0.73$ ; in vitro, n.s. The graphs are plotted with different shapes per colony (orange for in-hive context and blue for in vitro context). Only best-fit lines that have significant correlations are reported (regression parameters from GLM).

the brood reared in vitro survived during this period with exactly the same manipulation as the reared in-hive. Hence, there could be no physical damage in larvae grafted in the hive. The apparent mortality recorded during the first 24 h in the intra-frame grafting procedure is probably a consequence of social modulation by nurse bees (Büchler et al. 2013; Evans and Spivak 2010; Fukuda and Sakagami 1968). In this sense, the acceptance of grafted larvae for queen rearing inside a hive depends on different environmental factors. Nevertheless, 20% of the worker larvae in the first stadium are rejected even under well-managed conditions (Büchler et al. 2013).

The comparison between contexts with similar manipulation showed a higher percentage of delays in larval molting during in vitro rearing. Thus, the absence of social compensatory mechanisms may change the way that the stress symptoms are observed. Accordingly, the survival of larvae and successful molting showed a strong positive correlation for the in vitro context but not for the in-hive context. Inside the hive, nurses might be able to detect the internal state of brood

and thus reject stressed larvae or customize the larval food to compensate the stress response (Free and Winder 1983; Le Conte et al. 1995; Schmickl and Crailsheim 2002). In other words, the grafting effect on survival and development depends on the care taken in the manipulation of brood but it cannot be removed.

#### 4.2. Effect of rearing context

Artificially reared bees have environmental, nutritional, and social differences compared with in-hive-reared bees. In order to compare both rearing contexts independently of the grafting effect, we proposed the intra-frame grafting procedure.

Pre-imaginal survival does not depend on the rearing context. Larval survival during the growth period in the in vitro rearing context did not differ from larval survival in the in-hive context without grafting manipulation. However, the comparison between contexts with similar manipulation shows that in vitro rearing delays larval molting. The larger percentage of delayed molting

occurred during the immature stadia, first 48 h, which corresponds to the period closer to the grafting event. A second large increase in the accumulated number of bees with delayed molting occurred around the last larval molt, when larvae start an exponential growth, i.e., the gorging period. Social and nutritional factors are crucial at this point. Nurse bees provide immunity and modulate feeding and the type of diet administered to larvae (Evans and Spivak 2010; Huang and Otis 1991). Consequently, individual compensatory mechanisms are enhanced during *in vitro* rearing (Vázquez et al. 2018). Brood reared under these conditions displays a slower growth rate than brood reared in the hive (Wang 1965). This could be due to the restrictive and uniform diet, which could not meet the energetic demands of larvae. As a result, the emerged adults show reduced size and delayed emergence (1.57 days later than larvae reared in the hive).

It is well known that diet composition causes caste determination during larval development in the hive (Kamakura 2011; Zhu et al. 2017). The queen phenotype is characterized by a shorter post-hatching developmental time (12 days) and a larger abdomen (housing reproductive organs) than the worker phenotype (Bertholf 1925). The artificial diet of the current *in vitro* procedure differs in composition and amount from the natural diet of both castes (Dietz and Lambremont 1970). In our experiment, bees reared *in vitro* showed a longer developmental time and smaller abdomens than the worker phenotype. This prolonged duration is caused by the slower growth and delayed pigmentation during pre-imaginal development. Both are hormone-regulated processes that depend on the internal state of brood (Bitondi et al. 1998; Schmickl and Crailsheim 2002).

*In vitro*-reared bees showing a mixture of traits from both castes have been previously called intercaste (Kaftanoglu et al. 2011; Linksvayer et al. 2011). Recent studies have assessed morphological and behavioral traits to estimate similarity among the *in vitro*-reared bees and the two in-hive castes (Brodshneider et al. 2009; De Souza et al. 2015; Steijven et al. 2017). A multivariate analysis with anatomical and geometrical morphometric measures (De Souza et al. 2015) showed that the *in vitro*-emerged adults become

part of a morphotype gradient between worker and queen phenotypes because of phenotypic plasticity (Leimar et al. 2012). In addition, artificially reared bees showed smaller heads and mushroom body lateral calyces than in-hive bees (Steijven et al. 2017). This may be due to the stimulus-depleted environment inside the incubator. Furthermore, the *in vitro* procedure could disrupt the metamorphosis causing death or deformations. Previous reports showed that thorax size (Steijven et al. 2017) and fore- and hindwing surface areas (Brodshneider et al. 2009) of bees reared *in vitro* were slightly smaller than those of in-hive bees. The adult bee body is morphologically adapted for flight. Dry weight and wing size of an imago are accurate indicators of nutritional investment during the larval development (Daly et al. 1995; Eishchen et al. 1982). The flight muscles housed in the thorax and the wings are formed during pupal development (Oertel 1930). The *in vitro*-reared brood in our experiment achieved a mean dry weight of 76.08 mg and a wing length of 9.55 mm, while in-hive-reared bees achieved a mean dry weight of 102.54 mg and a wing length of 14.4 mm (Table S1). Thus, deficiencies in pupal development appear to be caused by malnutrition of larvae, the *in vitro* procedure itself, or both. Researchers cannot accurately mimic the colony environment, but more research is needed to improve the procedure and get closer to natural conditions. For this reason, future research using the *in vitro* rearing should bear in mind the differences between both contexts to avoid misleading results.

#### 4.3. Influence of source colony in the *in vitro* procedure

In adverse environmental conditions, honey bees display variability among colonies in susceptibility to different stressors such as diseases (Jensen et al. 2009; Palmer and Oldroyd 2003) and pesticides (Poquet et al. 2016; Tahori et al. 1969). In our experiment, each colony (A–F), subjected to the same concomitant stressors under open-field conditions shows varying survival and emergence rates. Although standard apicultural practices were used, the developmental parameters of brood depend on colony fitness, queen

reproductive success, and allostatic load due to the asymptomatic prevalence of previous pathologies (McEwen 1998). Moreover, the allocation of brood in the nest also affects these developmental parameters due to the hygienic behavior of nurse bees when brood is in peripheral frames (Fukuda and Sakagami 1968). Despite the homogeneous environment in the incubator, variability in larval survival and emergence rate during *in vitro* rearing has been reported (Aupinel et al. 2010; Vázquez et al. 2018). Low values in the survival of the control group may reflect intrinsic properties of the source colony rather than a flaw in the *in vitro* procedure. Differences in genetic diversity (Oldroyd and Fewell 2007; Simone-Finstrom et al. 2016; Tarry and Pettis 2013) could explain inter-colony variation in larval survival during *in vitro* rearing.

In our experiment, larval mortality in the in-hive rearing (i.e., mainly when brood is in unsealed cells) strongly positively correlated among the six colonies (A–F) between larvae exposed or not to grafting manipulation, results which are in agreement with Mortensen and Ellis (2018). Our experiment also shows that this result is independent of the rearing context. This suggests a transmission among rearing contexts of the susceptibility of each colony to stress caused by inheritable and infectious factors (Al-Lawati and Bienefeld 2009; De Miranda et al. 2013). Consequently, the health and number of source colonies should be carefully considered in experimental designs using the *in vitro* procedure.

Nevertheless, when we analyzed the emergence rate, there was no correlation among brood cohorts reared *in vitro*, which contrasted with our findings in the in-hive context. This context-dependent indicates a higher susceptibility of the pre-pupal and pupal stages to the *in vitro* procedure (Jay 1965a, b). The *in vitro* emergence rates of our assay are within the 80% of assays carried out in previous related studies, which have reported emergence rates between 40 and 90% (Aupinel et al. 2010). However, the OECD has determined a conservative threshold of emergence (70%) as a standard for good quality for the *in vitro* procedure (OECD 2016). This point is controversial because emergence is affected by both source colony and rearing procedure. Considering these

outcomes, risk assessments that do not take into account the underlying inter-colony variation may result in sampling bias, only assessing strong and tolerant colonies.

#### 4.4. Conclusions

The results in the intra-frame grafting procedure allow us to conclude that the current *in vitro* procedure for rearing honey bee brood affects their pre-imaginal development. The main effects are prolonged duration of growth in larvae and delayed pigmentation in pupae. These results are in line with the reduced size of the *in vitro* imago and its delayed emergence. Therefore, these facts support the hypothesis that there is an *in vitro* phenotype as a consequence of the phenotypic plasticity in the artificially reared bees. Nevertheless, our measurements related to morphology and developmental time showed that the *in vitro* phenotype is similar to the worker bee caste.

On the other side, our results strengthen the importance of the grafting effect on the development of brood that it cannot be removed. Finally, the larval mortality during the growth period was positively correlated between broods exposed or not to grafting manipulation from the same colonies. This result is independent of the rearing context, which suggests transmission of inheritable and infectious factors that conditioned larval survival. However, the correlation in emergence rate showed context dependency that indicates a higher susceptibility of the pre-pupal and pupal stages to the *in vitro* procedure. As a consequence, the health and number of source colonies should be carefully considered in experimental designs. Meanwhile, more research is carried out to improve the *in vitro* procedure and get closer to the hive environment.

#### ACKNOWLEDGMENTS

We thank H. Verna and R. Velarde for the technical assistance. We thank M.C. Estravis Barcala and M.S. Balbuena for the language review of the manuscript. D.E.V. and W.M.F. thank the University of Buenos Aires, CONICET, and ANPCYT for the support.

## AUTHOR'S CONTRIBUTIONS

Conceptualization: DEV and WMF Design, experiments and data analysis: DEV Supervision of the project and funding acquisition: WMF The manuscript was drafted and revised by DEV and WMF. Data availability

The datasets generated and analyzed during the current study are available in the supporting information.

## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of interest** The authors declare that they have no conflict of interest.

**Différences dans le développement post-embryonnaire de l'abeille *Apis mellifera* in vitro et en ruche**

***Apis mellifera* / élevage larvaire / greffage de couvain / plasticité phénotypique / inter-colonie**

**Unterschiede in der Präimaginalentwicklung zwischen im Volk und in vitro aufgezogenen Honigienen, *Apis mellifera***

***Apis mellifera* / Larvenaufzucht / Umlarven / phänotypische Plastizität / Variabilität zwischen Völkern**

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