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Experimental Ecotoxicology Procedures Interfere with Honey Bee Life History

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Abstract: *Apis mellifera* was used as a model species for ecotoxicological testing. In the present study, we tested the effects of acetone (0.1% in feed), a solvent commonly used to dissolve pesticides, on bees exposed at different developmental stages (larval and/or adult). Moreover, we explored the potential effect of in vitro larval rearing, a commonly used technique for accurately monitoring worker exposure at the larval stage, by combining acetone exposure and treatment conditions (in vitro larval rearing vs. in vivo larval rearing). We then analyzed the life-history traits of the experimental bees using radio frequency identification technology over three sessions (May, June, and August) to assess the potential seasonal dependence of the solvent effects. Our results highlight the substantial influence of in vitro larval rearing on the life cycle of bees, with a 47.7% decrease in life span, a decrease of 0.9 days in the age at first exit, an increase of 57.3% in the loss rate at first exit, and a decrease of 40.6% in foraging tenure. We did not observe any effect of exposure to acetone at the larval stage on the capacities of bees reared in vitro. Conversely, acetone exposure at the adult stage reduced the bee life span by 21.8% to 60%, decreased the age at first exit by 1.12 to 4.34 days, and reduced the foraging tenure by 30% to 37.7%. Interestingly, we found a significant effect of season on acetone exposure, suggesting that interference with the life-history traits of honey bees is dependent on season. These findings suggest improved integration of long-term monitoring for assessing sublethal responses in bees following exposure to chemicals during both the larval and adult stages. *Environ Toxicol Chem* 2024;00:1–12.

Keywords: Acetone; *Apis mellifera*; Flight activities; In vitro larval rearing; Life history traits; Radio frequency identification (RFID)

INTRODUCTION

Bees play a critical role in global food production by promoting the pollination of many plants, on which many crops depend directly (Garibaldi et al., 2014; Ollerton et al., 2011). Indeed, 90% of all flowering plants and 75% of our main crops require animal pollination (Klein et al., 2007; Tepedino, 1979). However, pollinators are facing increasing threats, particularly exposure to pesticides used to protect crops; and major concerns about their impact on bees have been raised (Goulson et al., 2015; Sanchez-Bayo & Goka, 2014).

Pesticides are generally composed of active substances that are toxic to pests as well as inert substances that are designed to improve their effectiveness (US Environmental Protection Agency [USEPA], 2013). Although classified as inert ingredients, these additives are used by the main manufacturers of agricultural pesticides, and some have been shown to have harmful effects on the health of humans and the environment (Cox & Surgan, 2006; Pezzoli & Cereda, 2013). The global use of inert ingredients is very unclear, and in Europe, no data are available on the quantities used, only on their number (e.g., 70 in France, 294 in the United Kingdom; Agence nationale de sécurité sanitaire, de l'alimentation, de l'environnement et du travail, 2023; Straw et al., 2022). Almost all active ingredients are applied with inert ingredients, and it is assumed that their quantities are proportional to or probably greater than those of

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the active ingredients (Straw et al., 2022). The identities of inert ingredients contained in commercial solutions are confidential, which makes it challenging to understand how they affect biodiversity and the environment (European Commission, 2009). Nevertheless, volatile organic compounds such as xylene, toluene, and acetone have been found in the environment (Wang et al., 1995).

These inert ingredients are also widely used in regulatory ecotoxicological tests of the active ingredients (e.g., dissolving, emulsifying, and decanting). The Organisation for Economic Co-operation and Development (OECD) empirically recommends that bee tests should incorporate <1% to 5% of inert ingredients in the overall volume of the exposure solution (OECD, 1998a, 1998b, 2017). Overall, 19 studies between 1973 and 2021 directly tested the effects of inert ingredients on bees (Straw et al., 2022). Thus, some inert ingredients (e.g., Citowett®, Pulse®, Boost®) have been shown to be toxic when administered topically or orally (Goodwin & McBrydie, 2000). In addition, nonlethal effects of specific inert ingredients were observed. These compounds can modify the behavior of *Apis mellifera*; for example, the proboscis extension reflex is impaired by the ingestion of inert ingredients, such as organosilicons and tensioactives (Ciarlo et al., 2012). Many active ingredients are not miscible in water, so protocols must use an inert ingredient, such as a solvent, to incorporate them into test solutions. Acetone is an inert ingredient that is commonly used as a control and is considered to have low direct toxicity (Dai et al., 2019; Kablau et al., 2023; Rembold et al., 1974). Many scientific studies do not evaluate the effects of acetone because they do not include unexposed controls in their experiments (li & Rangel, 2018; Rinkevich et al., 2015). This can lead to potential biases because the ingestion of acetone can have consequences on organisms and modify the intestinal microbiota (Quinn et al., 2018) of adult bees, and acetone inhibits glandular growth during larval exposure (Salles & Cruz-Landim, 2004). It is therefore relevant to better assess the consequences of exposure to acetone and its effects on the behavior and life-history traits of bees.

In addition to the common use of acetone in ecotoxicological tests involving bees, the in vitro larval rearing method is commonly used for the assessment of environmental risks associated with bees, particularly pesticides (Aupinel et al., 2007; Fourrier et al., 2015). Ingestion of acetone could be one of the causes of developmental disturbance in the larval stage, which results in an increase in mass (Rembold et al., 1974). However, only a few studies have examined how the rearing method affects the behavior of adult bees, and this is important for better assessing the long-term consequences of the rearing method on the life of bees.

Few studies follow bees individually throughout their lives (Brodschneider et al., 2009; Fisher et al., 2021; Mortensen & Ellis 2023; Schilcher et al., 2022). Most of these studies have focused on a single stage of bee life, such as the larval, adult, or forager stage (Fine et al., 2017; Kralj & Fuchs, 2006; Scofield & Mattila, 2015). In vitro larval rearing allows monitoring of the development of larvae in the laboratory until they emerge as young adult bees and analyses of the physiology of larvae and adults in the context of biotic or abiotic stresses (Aupinel et al., 2007; Schmehl et al., 2016). The use of radio frequency identification (RFID) devices provides automatic recording of the in-and-out activity of adult bees to estimate flight activity (Nunes-Silva et al., 2019; Requier et al., 2020; Streit et al., 2003). For instance, this system is applied to assess the impact of sublethal doses of pesticides on the flight activity of bees (Bagnis et al., 2010; Henry et al., 2012; Prado et al., 2020).

Unfortunately, the environment used for larval rearing differs between in vivo and in vitro conditions and can affect the morphology, physiology, and behavior of bees at the adult stage. For instance, bees reared larval in vitro (RL_{vitro}) can have smaller body sizes and smaller lateral calyces than bees reared larval in vivo (RL_{vivo}; Steijven et al., 2017). The flight behavior and performance of suckling and foraging tasks are reduced in RL_{vitro} bees compared with RL_{vivo} bees (Brodschneider et al., 2009; Schilcher et al., 2022). This is because larvae are deprived of social contact in in vitro larval rearing methods, either through chemical interactions with pheromones or through the maintenance of nurse bees (Schmehl et al., 2016). Moreover, the use of artificial food in in vitro larval rearing methods could lead to nutritional stress, affecting the physiology and morphology of adult bees (Mortensen et al., 2019). Thus, combining the in vitro larval rearing method with RFID monitoring could help researchers explore whether the larval development environment can affect the flight activity of adult bees.

The aim of the present study was to test whether acetone, an inert ingredient commonly used in bee ecotoxicological tests to solubilize pesticides, could affect larval and adult bee life history. Therefore, we combined the use of the in vitro larval rearing method to monitor the survival of larvae and the use of the RFID technique to monitor the life history of adult bees. We exposed bees to acetone during their larval and/or adult stage (RL_{vitro}) or adult stage (RL_{vitro} and RL_{vivo}), and we compared their life histories to those of bees (RL_{vitro} and RL_{vivo}, respectively) that were not exposed to acetone (Supporting Information, Figure S1). We measured the core life-history traits of all the bees, including the life span (LSP), age at first exit (AFE), age at onset of foraging (AOF), foraging tenure (FT), and foraging intensity (FI) of the adult bees (Prado et al., 2020; Requier et al., 2020). Moreover, we tested whether the potential effect of acetone could be dependent on the season by replicating the experiment over three sessions (in May, June, and August).

MATERIALS AND METHODS

All experiments were conducted in the entomological experimental unit of INRAE, Le Magneraud (46°8′59,104″N, 0°41′ 28,609″W), France, between April and August 2021. The apiary is located in an intensive cereal farming system with a high proportion of wheat, sunflower, oilseed rape, and maize (Odoux et al., 2014; Requier et al., 2015). We used *A. mellifera mellifera* × *caucasica* colonies for the experiment. Bees were treated with *Varroa destructor* before winter but not during the spring or summer of the present study.

In vitro larval rearing and exposition to acetone

One-day-old larval workers were collected from three honey bee colonies and reared in vitro until emergence following the method described by Aupinel et al. (Aupinel et al., 2007; Medrzycki et al., 2013; Papach et al., 2017). The first-cycle larvae (L1) were obtained by enclosing the queen with a frame of built-up brood in an isolated Italian cage for 30 h. Then, the queen was released, and the freshly laid frame was left in the hive for 1 more day to obtain L1 for grafting. All graft procedures were performed in the laboratory at room temperature (~20–24 °C). The same experimenters performed all transplants. The larvae (L1) were transferred to disinfected plastic grafting dishes. The L1 cups were maintained in an incubator at 34.5 ± 5 °C from day 1 to day 8 with a relative humidity (RH) of 95 ± 5% (K_2SO_4 solution). Cups were filled with a mixture of royal jelly, aqueous sugar, and yeast on days 1, 3, 4, 5, and 6. Each larva was unexposed (control) or chronically exposed to 0.1% acetone for 4 days in their diet (day 3 to day 6). At the first grafting session, on day 8, the plates containing the pupae were capped with a layer of wax. Pupal plates were placed vertically in crystal polypropylene emergence boxes (11 × 15 × 12 cm) with a 5-mm piece of Be-Boost® (PseudoQueen) attached to a piece of wax (~5 × 5 cm). The emergence boxes were transferred to a pupae incubator at 34.5 ± 5 °C with 80 ± 5% RH (saturated NaCl solution). Prior to emergence (day 15), the emergence boxes were placed at 34.5 ± 5 °C and 50 ± 5% RH with 50% agarose–sugar solution ad libitum. The second and third grafting sessions were performed with the same protocol to obtain adult bees. The first part of the protocol was similar to the first grafting session until days 7 and 8. At days 7 and 8, the larvae were transferred to a new clean plate when they had finished feeding, and the plates containing the pupae were transferred to a pupae incubator. Prior to emergence (day 15), the pupae plates were placed in a crystal polypropylene emergence box equipped in the same way as those used for the first grafting (equipped in the same format as in Session 1). Individuals that died before the start of feeding on day 2 were removed from the analysis. The percentages of larvae that died before ingesting the first feed were 4.3%, 2.8%, and 3.2% in May, June, and August, respectively. For these individuals reared in vitro, we monitored daily mortality and the days of emergence. Only in May was the mortality of the capped plates from day 7 to day 20 subsequently assessed according to the stage of development. Overall, 984 individuals were reared in May, 998 individuals in June, and 994 individuals in August.

Adult bee exposition to acetone and release

At emergence (days 17–20), the RL_{vitro} bees were marked with an RFID transponder (see below, *RFID monitoring*). The RL_{vitro} bees were directly introduced to a single host hive after RFID was marked. The RL_{vitro} bees were placed in small wire frames, which were sealed with candy (8 h after the first tagging). The frames were then placed directly in a bait comb host hive equipped with RFID readers. On day 18, some of the marked bees were kept in polypropylene boxes equipped in the same way as the emergence boxes under laboratory conditions for 48 h at 34.5 ± 5 °C and 50 ± 5% RH. The plants were then exposed to 0.1% acetone for 48 h in a sugar solution (50% w/v sucrose). After exposure, the RL_{vitro} bees were introduced into the hives with a small wire frame. Thus, we used four modalities: (i) RL_{vitro} bees that were never exposed to acetone, called control (C); (ii) RL_{vitro} bees that were exposed to acetone only during the larval phase, called larvae exposed (L); (iii) RL_{vitro} bees that were exposed to acetone only during the adult phase, called adult exposed (A); and (iv) RL_{vitro} bees that were exposed to acetone during the larval and adult phases, called larvae and adult exposed (L + A; Supporting Information, Figure S1). Overall, 43 C RL_{vitro}, 52 L RL_{vitro}, 83 A RL_{vitro}, and 77 L + A RL_{vitro} bees were released in May. In June, 103 C RL_{vitro}, 98 L RL_{vitro}, 99 A RL_{vitro}, and 92 L + A RL_{vitro} bees were released. In August, 100 C RL_{vitro}, 104 L RL_{vitro}, 98 A RL_{vitro}, and 101 L + A RL_{vitro} bees were released.

On the other hand, RL_{vivo} bees that were less than 12 h old were collected from the hives that provided the larvae for in vitro rearing. The RL_{vivo} bees were marked with an RFID transponder and subjected to the same treatment as the RL_{vitro} bees at emergence: direct release or exposure for 48 h and release (see above). There were two modalities: RL_{vivo} bees that were never exposed to acetone (C), and RL_{vivo} bees that were exposed to acetone during the adult phase (A) (Supporting Information, Figure S1). Finally, 104 C RL_{vivo} and 104 A RL_{vivo} bees were released in May, 104 C RL_{vivo} and 90 A RL_{vivo} bees were released in June, and 102 C RL_{vivo} and 90 A RL_{vivo} bees were released in August.

RFID monitoring

The adult bees were immobilized on their thoraxes using a piston cage to glue a mic3®-TAG 16-kbit RFID transponder (HF-RFID 13.56 MHz; Microsensys mic3 Technology). The transponders were glued to the thorax using biocompatible dental cement (TempoSIL2). The tags were 1.6 × 1.9 × 0.5 mm^3 in size and weighed approximately 3 mg (Streit et al., 2003). The impact of the weight of the tag may be marginal or null, given that it represents 3% of a bee's weight without considering its capacity to transport nectar or pollen (Marden, 1987; Winston, 1991). The operation and ID of the tags were checked and saved before they were attached to the bees with a portable USB holder (ID®PENmini 7.0; Microsystems).

All tagged bees from the three donor colonies were released into a single host colony equipped with an RFID system. The 10-frame Dadan hive equipped with the RFID system contained five brood frames, three honey+pollen reserve frames, and two empty wax frames. The queen of the host colony was born in 2020. Two sets of four RFID readers (iID2000, 2k6 HEAD; Microsensys) were placed at the entrance to the hive (with a total of eight readers). The two lines of readers allowed us to distinguish the direction of the activity (i.e., an entrance or an exit; Requier et al., 2020). Each well passed through a tunnel 80 × 8 mm high. The reader recorded the identity, date, and time at each passage of a tagged bee. The data were collected every 7 days.

Life-history traits

We measured the core life-history traits of 604 RL_{vitro} bees and 1050 RL_{vivo} bees based on the RFID data after cleaning the raw data set following Requier et al. (2020)'s method. The LSP was measured in days up to the last recorded RFID hit for each bee. The other traits were computed after removing bee trips shorter than 2 s and trips longer than 180 min (Prado et al., 2020). The AFE of the bees was defined as the number of days from the first recorded RFID hit (the first flight). We then calculated the FT as the time span from the AFE to the LSP. We also analyzed the proportion of bees lost after their first exit from the hive (L-AFE; Coulon et al., 2020). We measured FI as the total number of flights performed by bees (see also Prado et al., 2020). Finally, we computed the AOF of the bees in days using the *aof* R package (Requier & Rebaudo, 2020) for 338 RL_{vitro} bees and 410 RL_{vivo} bees.

Statistical analyses

All statistical analyses were performed using R software (Ver. 4.2.2; 2022). The significance level for the statistical tests was set at 5% for the probability of rejecting the true null hypothesis. Graphs were generated with the *ggplot2* R package (Wickham et al., 2023). The compact letters of all pairwise comparisons were generated with the *multcomp* R package (Piepho, 2004).

Effects on bee larvae. We generated a Cox proportional hazards model using the *coxme* package (Therneau, 2022) to assess whether larval survival in hazard ratios (HRs) was affected by acetone exposure (C, L), session (May, June, and August), and the hive of origin of the larvae (R3, R4, and R6). We considered the rearing plate as a random factor in the model (standard deviation = 0.250, variance = 0.062). The impact of technicians transferring larvae to day 7 to day 8 (June and August sessions only) had a significant effect on larval survival and was considered in a specific model ($\chi^2 = 74.7523$, $df = 3$, $p < 0.001$; Supporting Information, Table S1). We used a linear mixed model (LMM) to assess the emergence day of RL_{vitro} bees using the *lme4* R package (Bates et al., 2023). The explanatory variables considered for this model are similar to those of the survival model (see above). We considered the rearing plate as a random factor in the model (standard deviation = 0.010, variance = 0.10; Supporting Information, Table S2). For both models, we performed post hoc (Tukey) pairwise comparisons with the *emmeans* R package (Lenth et al., 2023) to identify differences between sessions, acetone exposure, and the impact of the hive of origin.

Effects of the experimental procedure on adult bees. We generated different models to test the effects of the larval rearing protocol (i.e., in vitro and in vivo), the origin of the larvae (i.e., R3, R4, and R6), acetone exposure (i.e., C, L, A. and L + A) and their interactions with the sessions (i.e., May, June, and August) on bee life-history traits (see above). Survival analysis was performed using a Weibull hazard model with the *survival* package (Therneau et al., 2023) to test the effects of these explanatory variables on LSP. The AFEs of bees were compared with generalized LMMs (GLMMs) using the *glmmTMB* R package (Brooks et al., 2023). The L-AFEs (binary response variables) were compared with binomial GLMMs from the *ade4* R package (Dray & Dufour, 2007). For bee flight capacity, FT and FI were analyzed with a negative binomial 1 GLMM (we included in the formula "ziformula~0"). For foraging activities, the proportion of forager bees (binary response variables) was analyzed with binomial GLMM. Finally, AOF was analyzed with gamma GLMM. We carried out pairwise comparisons to analyze the effects of the different explanatory variables. We calculated the value of the Akaike information criterion (AIC) to compare the log-likelihood of GLMM error distributions (AFE and AOF: gamma and Gaussian; FT and FI: Poisson, zero-inflated, negative binomial 1 and negative binomial 2) and selected the lowest AIC (Sakamoto et al., 1986). The residuals were checked, and analysis of variance was performed using the *DHARMa* R package (Hartig, 2022) and *car* (Fox et al., 2023).

RESULTS

Effect of acetone on bee larvae

The survival of in vitro–reared larvae was affected by the session of the experiment ($\chi^2 = 40.306$, $df = 2$, $p < 0.001$; Figure 1; Supporting Information, Table S1). In May, larval mortality was significantly greater than that in June and August (May 1.863 ± 0.194 HR, June 0.925 ± 0.100 HR, and August 0.587 ± 0.065 HR; $p < 0.01$; Supporting Information, Table S1).

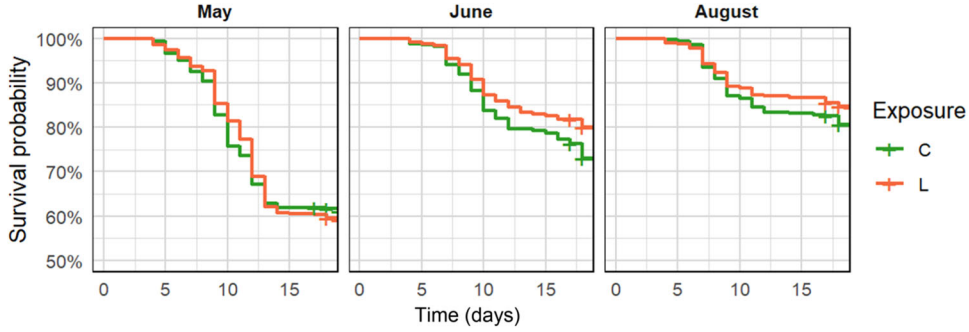

FIGURE 1: Effect of acetone on bee larvae survival. Larvae were not exposed (control) or were chronically exposed to 0.1% acetone in their diet on days 3, 4, and 5 for each session (May, June, and August). C = control; L = larvae exposed.

TABLE 1: Type II analysis of variance for the different generalized mixed effect models

Models	Session 2 *df*		Exposure 3 *df*		Session:exposure 6 *df*		Rearing 1 *df*		Hive 2 *df*	
	χ^2	*p*	χ^2	*p*	χ^2	*p*	χ^2	*p*	χ^2	*p*
LSP	126.6	***	55.6	***	89.3	***	165.1	***	2.6	
AFE	57.4	***	83.4	***	122.0	***	20.4	***	0.7	
L-AFE	80.0	***	2.0		23.4	***	153.0	***	12.0	**
FT	37.8	***	23.5	***	18.2	**	26.0	***	0.4	
FI	51.8	***	6.8		12.3		47.9	***	0.1	
Foragers	60.9	***	13.1	**	8.4		27.5	***	4.4	
AOF	57.7	***	16.3	**	3.7		5.4	*	12.4	**

For all generalized linear models, the χ^2 tests of Wald's likelihood were calculated with their associated *p* values (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).
LSP = life span; AFE = age at first exit; LAFE = lost after first exit; FT = foraging tenure; FI = foraging intensity; AOF = age at onset of foraging.

Acetone exposure had no impact on larval survival ($\chi^2 = 1.23$, $df = 1$, $p < 0.267$; Figure 1; Supporting Information, Table S1). The hives used to graft the larvae had no effect on larval survival ($\chi^2 = 5.422$, $df = 2$, $p = 0.066$; Supporting Information, Table S1).

Experiment session was impacted on the emergence day of the RL_{vitro} bees (LMM, $\chi^2 = 27.878$, $df = 2$, $p < 0.001$; Supporting Information, Table S2). The average bee emergence days for the May, June, and August sessions were 19.13 ± 0.030 days, 19.18 ± 0.027 days, and 18.98 ± 0.027 days, respectively. In the August session, the bees emerged earlier than in the May and June sessions ($p < 0.001$; Supporting Information, Table S2). Chronic exposure of larvae to acetone had no effect on bee emergence day (LMM, C 19.07 ± 0.023 and L 19.12 ± 0.023; $\chi^2 = 2.833$, $df = 1$, $p < 0.092$; Supporting Information, Table S2). The hives of origin of the larvae impacted the days of emergence of the bees (LMM, $\chi^2 = 35.564$, $df = 2$, $p < 0.001$; Supporting Information, Table S2). The days of emergence of the R6 bees were later than those of the R3 and R4 hives (R3 18.98 ± 0.023 days, R4 19.09 ± 0.028 days, and R6 19.22 ± 0.028 days; $p < 0.001$; Supporting Information, Table S2).

Effects of experimental procedures on adult bees

Impact of larval rearing conditions on adult bees. Larval rearing type had a significant effect on all GLMMs: LSP, AFE, L-AFE, FT, FI, foragers, and AOF ($\chi^2 > 5.4$, $df = 1$, $p < 0.020$; Table 1). The survival model indicated that the adult RL_{vitro} bees had significantly shorter life spans than did the adult RL_{vivo} bees (11.0 ± 0.294 days for the RL_{vitro} bees, 21.2 ± 0.901 days for the RL_{vivo} bees, $p < 0.001$; Figure 2; Supporting Information, Table S3). In terms of the first flight activity, the RL_{vitro} bees exhibited a reduced AFE and a significant increase in L-AFE compared with the RL_{vivo} bees (AFE 5.66 ± 0.102 days for the RL_{vitro} bees, 6.58 ± 0.201 days for the RL_{vivo} bees, $p < 0.001$; L-AFE $68.6 \pm 1.52\%$ for the RL_{vitro} bees, $30.4 \pm 2.44\%$ for the RL_{vivo} bees, $p < 0.001$; Figure 3; Supporting Information, Tables S4 and S5).

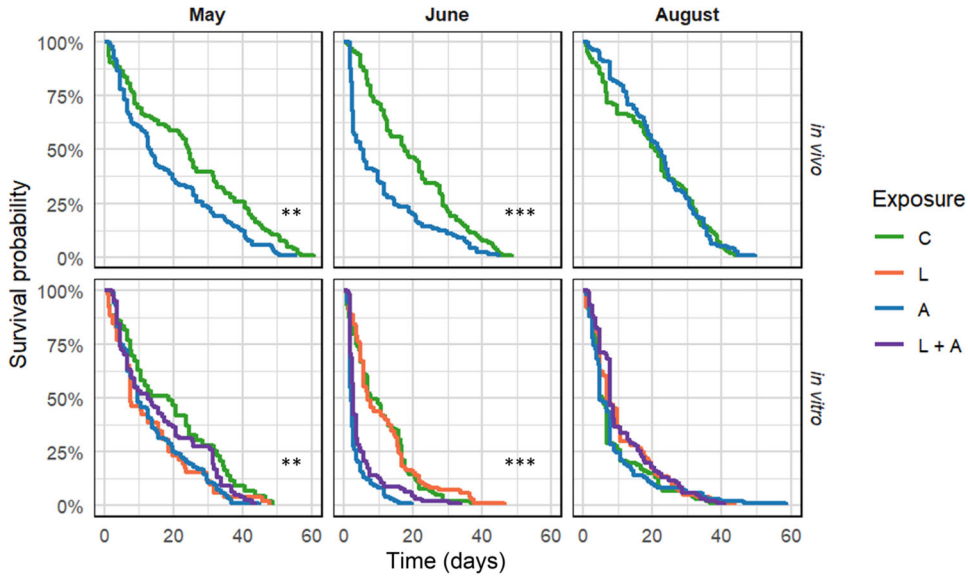

FIGURE 2: Effect of acetone on the survival of adult bees. Adult bees were reared following the larval rearing protocol (i.e., in vitro and in vivo) for each experimental session (May, June, and August). Bees were in the unexposed control group (C) or were exposed to acetone at the larval stage, at the adult stage, or at both the larval and adult stages. **$p < 0.01$; ***$p < 0.001$—if acetone exposures affect life span for each session. C = control; L = larval stage; A = adult stage.

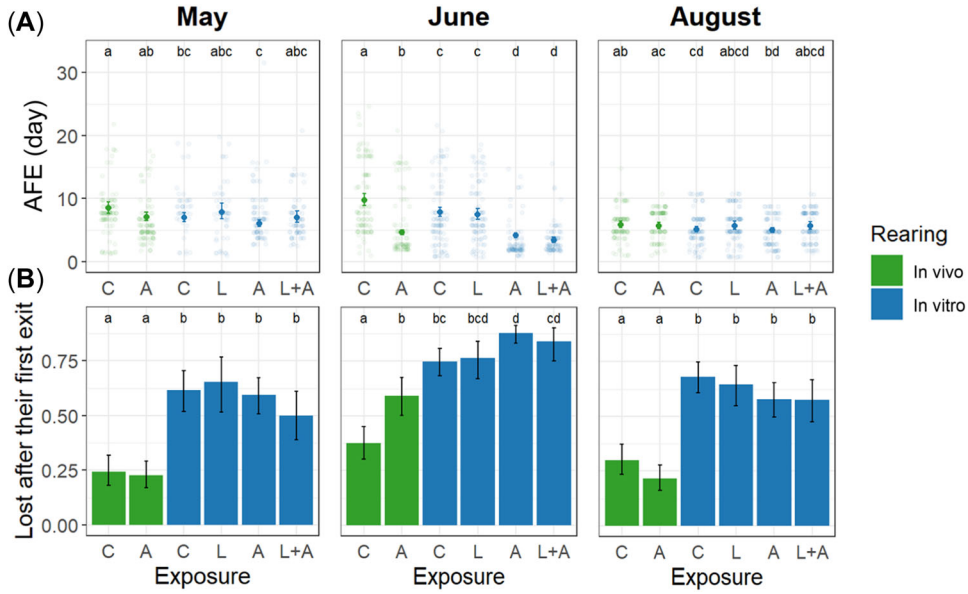

FIGURE 3: Effect of acetone on the first flight activity of adult bees. (**A**) Age at first exit in days. (**B**) Lost after first exit in proportion. Bees were in the unexposed control group or were exposed to acetone at the larval stage, at the adult stage, or at both the larval and adult stages for each experimental session (May, June, and August). The colors show that the adult bees followed the larval rearing protocol (in vitro and in vivo). Transparent dots represent the raw data. Larger dots or bars represent model estimates ± 95% confidence intervals of the models. Significant differences according to Tukey's pairwise comparisons are indicated with letters for each session. AFE = age at first exit; C = control; A = adult stage; L = larval stage.

Regarding total flight activity, the RL_{vitro} bees had a significantly lower FT and FI than did the RL_{vivo} bees (FT 13.5 ± 0.571 days for the RL_{vitro} bees, 18.5 ± 0.976 days for the RL_{vivo} bees, $p < 0.001$; FI 18.0 ± 0.962 trips for the RL_{vitro} bees, 30.3 ± 1.852 trips for the RL_{vivo} bees; $p < 0.001$; Figure 4; Supporting Information, Tables S6 and S7). For foraging activities, the RL_{vitro} bees exhibited significantly fewer foragers and earlier AOFs compared with the RL_{vivo} bees (foragers 54.4 ± 3.03% for the RL_{vitro} bees, 77.9 ± 2.93% for the RL_{vivo} bees, $p < 0.001$; AOF 18.7 ± 0.565 days for the RL_{vitro} bees, 20.9 ± 0.825 days for the RL_{vivo} bees, $p < 0.05$; Supporting Information, Figures S2 and S3 and Table S9).

Effects of seasonal variation on adult bees. The session of the experiment had an impact on all GLMMs: LSP, AFE, L-AFE, FT, FI, foraging, and AOF ($\chi^2 > 37.8$, $df = 2$, $p < 0.001$; Table 1). The average LSP differed significantly among adult bees in May, June, and August (May 20.6 ± 0.928 days, June 11.0 ± 0.421 days, and August 15.8 ± 0.529 days, $p < 0.001$; Figure 2; Supporting Information, Table S3). The AFE of the bees was significantly greater in the May session than in the June and August sessions (7.57 ± 0.239, 5.36 ± 0.161, and 5.78 ± 0.150, respectively, $p < 0.001$; Figure 3A; Supporting Information, Table S4). The L-AFE of the bees was significantly greater in the June session than in the May and August sessions (May 39.3 ± 2.74%, June 68.6 ± 0.015%, and August 42.3 ± 2.39%, $p < 0.001$; Figure 3B; Supporting Information, Table S5). The FT of the bees was significantly lower in the June session than in the May and August sessions (May 18.7 ± 0.983 days, June 12.6 ± 0.909 days, and August 16.4 ± 0.753 days, $p < 0.004$; Figure 4A; Supporting Information, Table S6). The FI of the bees was significantly greater in the August session than in the May and August sessions (May 22.0 ± 0.151 trips, June 19.4 ± 1.57 trips, and August 29.7 ± 1.55 trips, $p < 0.01$; Figure 4B; Supporting Information, Table S7). The proportion of foragers was significantly lower in the June session than in the May and August sessions (May 70.5 ± 3.59%, June 47.3 ± 4.86%, and August 80.1 ± 2.55%, $p < 0.001$; Supporting Information, Figure S2 and Table S8). The AOF of the bees was significantly earlier in the August session than in the other sessions (May 22.5 ± 0.858 days, June 20.5 ± 1.245 days, and August 17.1 ± 0.494 days, $p < 0.05$; Supporting Information, Figure S3 and Table S9).

Effects of exposure on adult bees. Acetone exposure had significant effects on the GLMM LSP, AFE, FT, foraging, and AOF ($\chi^2 > 13.1$, $df = 3$, $p < 0.004$; Table 1). In addition, an interaction effect between experimental session and exposure was reported for GLMM LSP, AFE, L-AFE, and FT ($\chi^2 > 18.2$, $df = 6$, $p < 0.006$; Table 1). Larval acetone exposure had no discernible effect on the parameters examined in the present study; the RL_{vitro} bees and those exposed as larvae exhibited similar LSP, AFE, L-AFE, FT, foraging, and AOF to the control RL_{vitro} bees across all the experimental sessions ($p > 0.05$; Supporting Information, Tables S3–S9). The life cycle of the bees exposed to acetone in the adult stage was affected; the bees exposed as adults had a shorter LSP than did the control bees but only during the May and June sessions (in May, C 21.72 ± 1.477 HR, A 17.05 ± 1.022 HR; in June, C 15.53 ± 0.882 days, A 6.90 ± 0.418 days; $p < 0.007$; Figure 2; Supporting Information, Table S3). Bees exposed as adults exhibited a significant decrease in AFE compared with control bees during the May and June sessions (in May, C 7.68 ± 0.359 days, A 6.56 ± 0.273 days; in June, C 8.69 ± 0.344 days, A 4.35 ± 0.227

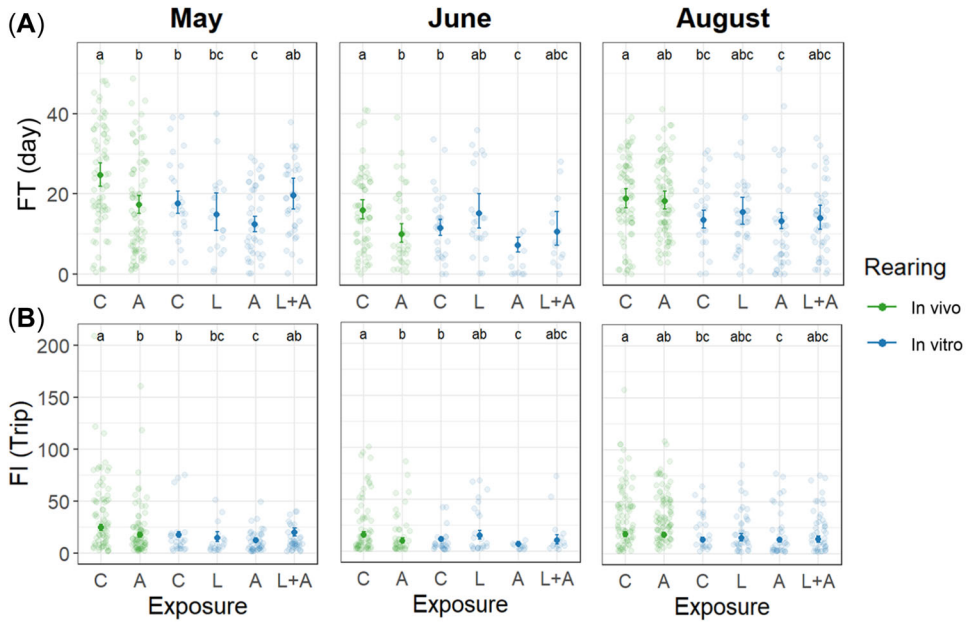

FIGURE 4: Effect of acetone on the total flight capacity of adult bees. (**A**) Foraging tenure in days. (**B**) Foraging intensity in total number of trips. Bees were in the unexposed control group or were exposed to acetone at the larval stage, at the adult stage, or at both the larval and adult stages for each experimental session (May, June, and August). The colors show that the adult bees followed the larval rearing protocol (in vitro and in vivo). Transparent dots represent the raw data. Larger dots represent model estimates ± 95% confidence intervals of the models. Significant differences according to Tukey's pairwise comparisons are indicated with letters for each session. FT = foraging tenure; C = control; A = adult stage; L = larval stage; FI = foraging intensity.

days; $p < 0.001$; Figure 3A; Supporting Information, Table S4). Bees exposed as adults showed a significant increase in L-AFE compared with the control bees in the June and August sessions (in June, C 57.2 ± 3.71%, A 76.4 ± 3.21%; in August, C 49.0 ± 3.82%, A 38.0 ± 3.70%; $p < 0.05$; Figure 3B; Supporting Information, Table S5). Bees exposed as adults significantly reduced FT compared with control bees during the May and June sessions (in May, C 20.83 ± 1.293 days, A 14.56 ± 0.951 days; in August, C 13.49 ± 1.012 days, A 8.41 ± 1.009 days; $p < 0.001$; Figure 4A; Supporting Information, Table S6). Bees exposed as adults had significantly fewer foragers compared with control bees (C 67.0 ± 3.22%, A 53.8 ± 3.74%; $p < 0.05$; Supporting Information, Figure S2 and Table S8). Bees exposed to acetone as larvae and adults experienced an impact on their life cycle compared with control bees. During the June session, the adult RL_{vitro} bees that were exposed as larvae + adults had a shorter LSP than did the RL_{vitro} bees, but their LSP was equal to that of the RL_{vitro} bees that were exposed as adults (C RL_{vitro} bees 11.19 ± 0.689 HR, A RL_{vitro} bees 4.97 ± 0.336 HR, and L + A RL_{vitro} bees 5.69 ± 0.448 HR; $p < 0.001$ and $p < 0.2132$, respectively; Figure 2; Supporting Information, Table S3). The RL_{vitro} bees that were exposed as larvae + adults showed a significant decrease in AFE compared with the control RL_{vitro} bees and the RL_{vitro} bees that were exposed as adults during the May session (C RL_{vitro} bees 7.85 ± 0.345 days, A RL_{vitro} bees 4.13 ± 0.171 days, and L + A RL_{vitro} bees 3.42 ± 0.206 days; $p < 0.01$; Figure 3A; Supporting Information, Table S4).

Effect of hive origin on adult bees. The hives of origin had a significant effect on the GLMM L-AFE and AOF of adult bees ($\chi^2 = 12.0$, $df = 2$, $p < 0.002$; $\chi^2 = 12.4$, $df = 2$, $p < 0.002$; Table 1). Bees from hive R4 exhibited a significantly greater L-AFE than did bees from hives R3 and R6 (R3 44.7 ± 2.47%, R4 55.9 ± 2.56%, and R6 47.7 ± 2.63%; $p = 0.042$; Supporting Information, Table S4). Bees from hive R4 had a significantly earlier AOF than did bees from hives R3 and R6 (R3 18.5 ± 0.574 days, R4 21.2 ± 0.774 days, and R6 19.8 ± 0.774 days; $p < 0.021$; Supporting Information, Table S9).

DISCUSSION

Effect of acetone on bee larvae

During in vitro larval rearing, survival rates varied between experimental sessions, with higher mortality occurring in May. Regarding development time and bee emergence day, bees from the August session emerged earlier than those from the May and June sessions. We fed bee larvae a regular diet containing 0.1% acetone, and we did not observe any effect of this chronic exposure on larval survival or development time with respect to bee emergence day. The hive of origin influenced survival, with larvae from R6 having a higher mortality rate and bees having longer development times and emerging later than bees from the two other hives.

The higher mortality in the May session is associated with a protocol that was under development. Vertical capping plates are thought to significantly reduce the deformation rate of newly emerged adults but, in our experience, increase mortality and do not allow us to monitor mortality on a daily basis (Kim et al., 2021). During the June and August sessions, we applied the modification proposed by Schmel et al. to transfer the larvae at day 8, as has been done in other publications on

larval rearing (Mortensen & Ellis, 2023; Mortensen et al., 2019; Schmehl et al., 2016). This greatly reduced larval mortality during our experimental sessions, but we were slightly above the OECD recommendation of <15% in negative controls (OECD, 2013). In terms of larval life-history traits, acetone had no negative effect on survival or developmental delay with respect to the date of emergence. This observation is consistent with numerous in vitro larval rearing studies that used dual controls: unexposed and exposed to acetone (Dai et al., 2019; Kablau et al., 2023; Rembold et al., 1974). It cannot be ignored that a low dose of acetone could inhibit the development of certain pathogens and bacteria present in the larval gut (Ola-Fadunsin & Ademola, 2013). In the long term, this disruption could have an impact on immunity because symbionts and intestinal bacteria play essential roles in the digestion and assimilation of nutrients, including pollen (Daisley et al., 2020). It has also been shown that some larvae gain weight as a result of this exposure (Rembold et al., 1974). However, the effects of this acetone on other larval parameters remain unknown.

Effects of experimental procedure on adult bees

Impact of larval rearing conditions on adult bees. The method of rearing larvae has a profound influence on various vital aspects of the life cycle of bees. The RL_{vitro} bees may experience nutritional deficits during their development, potentially resulting in underdeveloped adult bees having notably shorter life spans than RL_{vivo} bees. In addition, the microbiota of RL_{vitro} and RL_{vivo} bees differ, but such differences are subsequently reversed by individual transfer after emergence (Kowallik & Mikheyev, 2021). In terms of their first flight activity, RL_{vitro} bees may experience nutritional deficits during their development, potentially resulting in underdeveloped RL_{vitro} bees that initiate their first flight earlier and have a higher proportion of bees that are lost after the first flight. In addition, they show a reduction in total flight activity and foraging behaviors, including fewer foragers and an earlier onset of foraging. These results suggest that the larval rearing method plays a crucial role in shaping bee life-history traits, particularly in terms of their flight patterns and foraging behaviors.

Morphologically, RL_{vitro} bees can suffer nutritional deficits during development, potentially resulting in underdeveloped bees with marked differences (Scofield & Mattila, 2015). This leads to variations in forewing length and width, dry body weight, and hypopharyngeal gland size (Mortensen et al., 2019). However, in line with the literature, the average flight times of the RL_{vitro} and RL_{vivo} bees were generally similar (Brodschneider et al., 2009). Nevertheless, it is essential to note that in vitro rearing may deprive bees of essential behavioral and social learning experiences that occur in colonies and could lead to differences in their suckling and foraging behavior (Scofield & Mattila, 2015). In particular, a decrease in the foraging capacity and activity of RL_{vitro} bees has already been noted (Schilcher et al., 2022).

An interesting juvenile rearing environment did not significantly alter the behavioral repertoire of adult bees (Mortensen et al., 2019). The RL_{vitro} bees continue to perform tasks such as assisting the queen, collecting pollen, performing dances, and engaging in defense against parasites (Mortensen et al., 2019). Thus, rearing in vitro is a well-established method that allows for precise control and rigorous monitoring of developmental factors, including the targeted application of pesticides or pathogens. However, it is imperative to acknowledge the substantial differences between RL_{vitro} bees and those naturally developing in hives. These distinctions underscore the critical importance of considering these variations when conducting ecotoxicological tests under properly controlled environments.

Effects of seasonal variation on adult bees. These results highlight the impact of season on the life-history characteristics of bees. The life span of adult bees varied significantly between the experimental sessions. In particular, the age at first flight significantly differed, with the age at first flight being older during May than during June and August. Similarly, June was characterized by a greater proportion of bees lost after their first flight and reduced overall foraging tenure. Furthermore, the proportion of foragers was significantly greater in the May and August sessions than in the June session. In addition, the intensity of foraging is greater and the age at which foraging begins is earlier in August than in other months.

The observed variations were consistent with natural fluctuations in bee behavior throughout the season, a phenomenon previously described (Fukuda & Sekiguchi, 1966). Life-history traits are known to be influenced by environmental factors such as climate effects, seasonality, landscape structure, and weather conditions (Goulson et al., 2015; Henry et al., 2014; Steffan-Dewenter & Kuhn, 2003; Vanbergen, 2014). During our study period, rainfall levels were highly variable at 75, 141, and 32 mm in the May, June, and August sessions, respectively (Infoclimat, 2021). These climatic differences could have a significant impact on bee activity because their first flight activity is essential for bees. Conversely, temperatures that are too high or too low lead to a reduction in foraging activity (Abou-Shaara et al., 2017).

For example, approximately 20 °C is the optimum temperature for foraging activity by bees (Tan et al., 2012). The landscape in relation to the season and availability of flowers has a major influence on the behavior of bee colonies (Danner et al., 2016; Free & Winder, 1983; Klein et al., 2007; Steffan-Dewenter & Kuhn, 2003). The diversity of the local landscape structure can influence bee pollen–based diets, as well as periods of resource depletion. In agricultural landscapes, it has been reported that colony dynamics are largely influenced by the phenology of the main massive-flowering crops foraged by bees, which at our experimental site were oilseed rape and sunflower (Odoux et al., 2014). Thus, agricultural landscapes in late spring colonies suffer a period of food shortage between the two massive flowerings, which would modify foraging behavior (i.e., between late May and early July; Requier et al., 2015). These results highlight the importance of considering colony dynamics, seasonal variations, climatic conditions and the availability of floral resources when analyzing bee life-history traits and colony management.

Effects of exposure on adult bees. Exposure to acetone had a significant impact on various life-history parameters of bees. However, exposure of larvae to acetone did not influence the studied parameters. These results highlight the sensitivity of bees to acetone exposure and its consequences for their life cycle and foraging activities, with variations between the different experimental sessions. Adult exposure to acetone resulted in a shorter life span and a significant reduction in age at first flight (May and June sessions). In addition, the proportion of bees lost after the first flight increased when they were exposed to acetone as adults (June and August). Total flight capacity and foraging activities (fewer foragers) were reduced in adult bees exposed to acetone (during the May and June sessions). Exposure to acetone during the larval and adult stages led to a shorter life span for adult bees (June session) and earlier first flight (May session). The age at first exit could be attributed to the inhibition of glandular growth (Salles & Cruz-Landim, 2004) and the development of the hypopharyngeal gland (Sigg et al., 1997). In addition, similar concentrations of acetone altered morphological and histochemical digestive cells but also had no impact on the brain cells of Africanized bees (Oliveira et al., 2014). However, in *A. mellifera*, the neuropil of the antennal lobe was impacted by acetone (Sigg et al., 1997).

Our results show that acetone, an inert ingredient commonly used in pesticide validation tests and in commercial pesticide formulations, can have sublethal effects. Thus, scientists' preoccupation regarding the potential toxicity risks of inert ingredients in pesticide formulations is justified (Mullin, 2015; Straw & Brown, 2021; Straw et al., 2022). Regulatory agencies (i.e., the European Food Safety Authority in Europe and the USEPA) evaluate the risk of the active ingredient before approval. Thus, potential effects of commercial formulations may be underestimated (Fine et al., 2017; Straw & Brown, 2021).

Effect of hive origin on adult bees. The bees used in this experiment came from three different colonies whose queens were genetically related. However, significant variations were observed in several aspects of their life cycle, including the duration of larval development, the proportion of bees lost after the first flight, and the age at which foraging begins.

Indeed, genetically related colonies can show notable variations in key aspects of the bee life cycle (George et al., 2020; Junca et al., 2019; Page et al., 1995), including behavioral differences such as aggressive colony behavior in response to stress (Provost et al., 2003). Genetic diversity has also been identified as a major explanatory factor for variations in foraging behavior (Dreller et al., 1995). To clarify the extent to which our results have a genetic basis, future work could compare the behavior of queens inseminated by a monodrone or use genomic sequencing to determine the paternal lineage of each bee (Junca et al., 2019). In addition, certain genetic characteristics could increase the susceptibility of some individuals to health pressures (Kralj & Fuchs, 2006). Our rearing hives showed no visible symptoms, but we do not know about nonsymptomatic infections such as viral infections or parasites such as *V. destructor*, which affect the capabilities of the bee (Penn et al., 2022). It is important to consider the diversity of genetic patterns when studying different stresses because it is essential for colony-level functioning (Mattila & Seeley, 2007).

Experimental recommendation

Notably, larval rearing conditions have a significant impact on the life characteristics of adult bees. Consequently, it is important to recognize the differences between RL_{vitro} and RL_{vivo} bees, particularly in the context of ecotoxicological or behavioral studies. This would make it more efficient to transfer the effects observed in the laboratory to field conditions. In the future, it would be wise to explore the mechanisms and consequences underlying the morphological variations observed in RL_{vitro} bees and their influence on flight and foraging behavior in adult bees. When studying the effects of biotic or abiotic stresses on bee life characteristics, it is essential to consider seasonal variations and genetic diversity as potential synergistic effects. Chronic exposure to acetone during the larval phase had no significant effect on the survival, developmental duration, or day of emergence of bee larvae. Acetone did not affect life-history traits, despite variations between the different sessions. Future studies could examine other physiological or molecular parameters that might be influenced by larval exposure to acetone to better understand its effects on bee development. Finally, exposure to acetone in the adult stage had a significant impact on the different life-history traits of the bee. These results underline the fact that experiments using only acetone controls without unexposed controls need to be conducted. Exposure to acetone can influence the life history of bees. For future research, it would be relevant to examine other physiological parameters, such as the development of mandibular glands in adult bees. We highlight the necessity of having a control solvent, if any, to better evaluate the risk of active ingredients.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5872.

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Author Contribution Statement—**Victor Desclos le Peley**: Conceptualization; Data curation; Methodology; Formal analysis; Investigation; Visualization; Writing–original draft; Writing–review & editing. **Stéphane Grateau, Carole Moreau-Vauzelle, Daniel Raboteau, Colombe Chevallereau**: Resources, Writing–review & editing. **Pierrick Aupinel**: Conceptualization; Funding acquisition; Supervision; Writing–review & editing. **Fabrice Requier**: Conceptualization; Supervision; Formal analysis; Methodology; Writing–review & editing. **Freddie-Jeanne Richard**: Conceptualization; Funding acquisition; Investigation; Supervision; Project administration; Methodology; Writing–review & editing.

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