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Abstract. Ecdysteroids are a family of insect hormones that may play a role in modulating aggressive behavior in reproductive contexts. In Hymenoptera, the few studies investigating the link between ecdysteroid titers, reproduction and aggressiveness during contests concern solely eusocial species. Here, we explored whether ecdysteroid titers influenced female reproductive status as well as aggressiveness and resolution of conflict in a solitary ectoparasitoid, *Eupelmus vuilleti* (Hymenoptera: Eupelmidae). *Eupelmus vuilleti* females parasitize and feed upon juvenile stages of *Callosobruchus maculatus* (Coleoptera: Bruchidae). When two *E. vuilleti* females are simultaneously present on a patch, they tend to protect the host they exploit by displaying aggressive behaviors

towards conspecific competitors. To our knowledge, nothing is known about the association between ecdysteroids and aggressiveness or the outcome of contests for host access in solitary insects. First, we quantified ecdysteroid titers that naturally circulate in females without fighting experience and after a contest over host access. Ecdysteroid titers measured after the contest did not correlate with female aggressiveness during the contest, but winner wasps had higher titers than both losers and females that did not fight. Then, we manipulated hormone titers via injection: ecdysone favored egg maturation (i.e., gonadotropic effect) within 24h and increased almost immediately the females' probability of winning host access without affecting their aggressiveness. Our results represent an important step in understanding how hormones, such as ecdysteroids, mediate insect behavior during intraspecific competition.

Key words: ecdysteroids, egg maturation, aggressive behaviors, conflict resolution, hymenopteran parasitoid

1. Introduction

Intraspecific competition for indivisible resources may lead to direct interactions between individuals during contests (Kokko, 2013; Hardy and Briffa, 2013). Winning a contest generally provides profound fitness benefits to animals as they can get access to food (Barton, 1993; Vogel, 2005), a sexual partner (West-Eberhard, 1979; Schwagmeyer and Woontner, 1985; Simmons, 1986), dominance status (Chase, 1974) or a territory (Festa-Bianchet *et al.*, 1990). Because aggressiveness may help individuals to obtain such resources, many vertebrate and invertebrate taxa commonly exhibit aggressive behaviors during contests (Collias, 1944; Breed and Bell, 1983; Hardy and Briffa, 2013). For example, in parasitoid insects, females compete to lay their eggs on hosts from which their offspring develop, and aggressive interactions between females for host access have been observed in different parasitoid species (Hughes *et al.*, 1994; Petersen and Hardy, 1996; Field and

Calbert, 1999; Goubault *et al.*, 2007; Mohamad *et al.*, 2013, Mathiron *et al.*, 2018). However, being aggressive can also be costly, as associated physical injuries can lead to death (Palombit, 1993; Innocent *et al.*, 2011) and the inherent decrease in vigilance may increase predation risk (Jakobsson *et al.*, 1995; Díaz-Uriarte, 1999).

Hormones function, in part, to allow animals to match their physiology to the situations they face and adjust their behavior accordingly. Males of many vertebrates respond to social challenges from conspecific with a rapid increase in androgen titers such as testosterone, which supports and stimulates the expression of aggressive behaviors (reviewed in Wingfield *et al.*, 1990; Moore *et al.*, 2019). In insects, there is growing evidence that hormones may respond to social challenge in ways that parallel androgens in vertebrates (reviewed in Tibbetts and Crocker, 2014; Tibbetts *et al.*, 2019), such as in male and female burying beetles *Nicrophorus orbicollis* (Scott, 2006), male lobster cockroaches *Nauphoeta cinerea* (Kou *et al.*, 2008, 2019) and female wasps *Polistes dominulus* (Tibbetts and Huang, 2010). The majority of studies investigating how hormones mediate insect behaviors in competitive contexts have focused on juvenile hormone (JH), but another important class of hormones are the ecdysteroid hormones (ESH) – mainly ecdysone and 20-hydroxy-ecdysone (20E). In adults, ESH are produced by the fat body, gonads or epidermis (Festucci-Buselli *et al.*, 2008; Bloch *et al.*, 2009) and act in almost all body tissues, such as central nervous system, fat body, midgut and reproductive tissues in both males and females (reviewed in Schwedes and Carney, 2012). Similar to androgens, ESH pass into the cell and bind to nuclear receptors: in this case, a heterodimer composed of the ecdysone receptor (EcR) and the Ultraspiracle (USP; Thomas *et al.*, 1993, Yao *et al.*, 1992). Finally, the receptor–hormone complex binds to specific sequences of the DNA to regulate gene transcription (Bloch *et al.*, 2009; Roy *et al.*, 2018).

In adult females, ESH are mainly involved in the control of reproduction and reproductive behaviors (reviewed in Nijhout, 1994; Elekonich and Robinson, 2000; Ringo, 2002). For example, in eusocial Hymenoptera, *Bombus terrestris,* egg-laying mated queens have higher ESH titers than

76 virgin queens (Bloch *et al.*, 2000; Geva *et al.*, 2005), and ESH titers are higher in egg-laying worker
77 honeybees than in both nurses and foragers, and even higher in egg-laying queens (Robinson *et al.*,
78 1991). By contrast, Hartfelder *et al.* (2002) found no difference between virgin queens and mated
79 egg-laying queens of the stingless bee, *Melipona quadrifasciata*. In addition, ESH have rapid actions
80 on cell-membrane properties that might be particularly relevant for understanding their role as
81 behavioral mediators during intraspecific competition (Bloch *et al.*, 2009), but the few studies
82 measuring links between ESH titers and the expression of behaviors such as aggressiveness have
83 found inconsistent results. In eusocial hymenopterans, ESH titers are elevated in highly aggressive,
84 high-ranking workers relative to low-ranking workers in the queenless ant *Streblognathus peetersi*
85 (Brent *et al.*, 2006). In *Polistes dominula*, overwintered foundresses females injected with 20E in the
86 pre- and early-nesting phases were more likely to become dominant (i.e., displaying the highest
87 number of aggressive behaviors). In contrast, 20E titers in the haemolymph are very low and are not
88 associated with dominance rank of females in the late pre-emergent stage in the same species, as well
89 as in the South African species *Polistes smithii* (Kelstrup *et al.*, 2015).
90 Here, we explored how ESH mediate female reproductive status, aggressive behaviors during
91 contests over host access and the outcome of such contests in the parasitoid wasp *Eupelmus vuilleti*
92 Crawford (Hymenoptera: Eupelmidae). *E. vuilleti* is a solitary ectoparasitoid (i.e., only one offspring
93 can develop per host, with supernumerary juveniles being killed during larval competition; Fisher,
94 1961) that parasitizes and feeds upon larvae and pupae of the cowpea seed beetle *Callosobruchus*
95 *maculatus* (Coleoptera: Bruchidae), which infest seeds of *Vigna unguiculata* (Fabaceae). Female *E.*
96 *vuilleti* are known to protect the host they exploit by displaying aggressive behaviors towards
97 conspecific competitors (Mohamad *et al.*, 2010; Mathiron *et al.*, 2018). Previous studies have shown
98 that one major factor affecting aggressiveness and contest outcomes in *E. vuilleti* females is their
99 number of mature eggs (i.e., egg load): females are usually more aggressive and are more likely to
100 win contests when they have a greater egg load (Mohamad *et al.*, 2010, 2012; Mathiron *et al.*, 2018,

2019). Moreover, Mathiron *et al.* (2019) showed that wasps fighting over host access were more aggressive when they had more ready-to-lay eggs, and this effect was stronger when females were injected with JH. Finally, it is known that ecdysone is the main ESH secreted by reproductively active females (Bodin *et al.*, 2007, 2009), and that simple contact with a host can trigger an increase in its levels, correlated with the maturation of a single egg within 24 hours (Casas *et al.*, 2009). Altogether, these results indicate the potential of ESH as a behavioral mediator in *E. vuilleti*. Surprisingly, nothing is known about the role of this hormone family in aggressiveness and contest outcome during competition for host access.

We designed a first experiment to quantify natural ESH titers circulating in *E. vuilleti* females both without a host and after fighting over a host, and the extent to which contest aggressiveness and outcome relate to these titers. We expected post-contest ESH titers to vary positively with the number of aggressive behaviors displayed by females, and that winner females would have higher titers than losers. Another common method that enables understanding of how hormonal change affects individual responses is hormone manipulation via administration of a given amount of hormones (Tibbetts and Izzo, 2009). We therefore set up a second experiment to determine the effect of ESH, at different titers and time delays, on the number of mature eggs. This permitted us to define the ESH injection conditions to study aggressive behavior independent of a gonadotropin effect. We then studied whether aggressiveness and outcome of contest between wasps were influenced by ecdysone treatment. We expected that individuals treated with ecdysone would display more aggressive behaviors, and would ultimately be more likely to win contests for hosts than control females (i.e., treated with vehicle).

2. Material and Methods

2.1. Laboratory breeding

We collected *E. vuilleti* on cowpea seeds infested by the bruchid beetle *Callosobruchus maculatus* in Togo (West Africa) in 2007. According to the methodology described by Jaloux *et al.* (2004), we then bred parasitoids in the laboratory (IRBI, University of Tours, France) on larvae and pupae of *C. maculatus*. We dissected cowpea seeds to collect hosts for *E. vuilleti*, which were then placed individually in a standard transparent gelatin capsule (length: 2 cm, diameter: 0.6 cm). By mimicking a cowpea seed, this system facilitates observation of the number of eggs laid by females, without significantly altering oviposition behavior (Gauthier and Monge, 1999). Previous studies have shown that after a period of acclimation, parasitoid females behave in the same manner to the presence of hosts inside seeds and capsules (Gauthier and Monge, 1999; Jaloux *et al.*, 2004), although the number of eggs laid on hosts is slightly lower inside capsules (Damiens *et al.*, 2001).

2.2. Females' preparation for behavioral observations

During the following three experiments, we isolated parasitoid females directly at their emergence to prevent them from having any previous experience of oviposition and competition. We then placed them individually in petri dishes (diameter: 8.5 cm; height: 2.7 cm), provided with a piece of cotton soaked in water and one pupa of *C. maculatus* located in a gelatin capsule that was renewed each day preceding the experiments. *E. vuilleti* is a synovigenic parasitoid species (Jervis *et al.*, 2008): emerging females only have a few ready-to-lay eggs and they mature additional eggs throughout their adult life (oviposition activity increases over time and peaks three days after emergence; Jaloux *et al.*, 2004). We also provided females with one male during the first day for mating and oogenesis stimulation (Terrasse and Rojas-Rousse, 1986). We carried out all experimental procedures in a climate-controlled room at 30°C, 12:12 h light: dark and constant 70% relative humidity.

2.3. Experiment 1: ESH titers, aggressiveness and outcome of contests

2.3.1. Experimental procedure

To facilitate wasps' identification during contests, we marked them on the dorsal part of their thorax with a dot of bright yellow or bright red acrylic paint. On the day of behavioral observations, we deprived females of hosts for at least 2h before the experiment so that they were more ready to oviposit. All females were of the same age (3 days old), and marked with different colors. Paint color did not affect contest outcomes (red-painted wasps won 15 of 25 contests; binomial test: $P = 0.42$). At the beginning of the observation, we simultaneously introduced wasps into an arena consisting of a plastic block made of three chambers linked by a narrow channel (adapted from Petersen and Hardy, 1996; see Mathiron *et al.*, 2019). Females could freely move throughout the entire arena.

We placed either one (N = 31) or two females (N = 30) in the central chamber already containing a 4th instar larva of *C. maculatus* previously placed in a capsule. A previous study showed that females that experienced oviposition on *C. maculatus* pupae are more motivated to get access to a 4th instar larva (Mathiron *et al.*, 2018). In a patch with hosts, females often stop their behavioral oviposition sequence after detecting a conspecific (females raised their antennae in the direction of their opponent; Mohamad *et al.*, 2010). Wasps then either simply kick their opponent with their legs without taking their ovipositor out of the capsule (defensive behavior) or can escalate to a full attack, in which case one female generally chases her opponent away from the capsule, hits her with her head or mounts her (Mathiron *et al.*, 2018). Chasing, hitting and mounting can lead to the loser leaving the central chamber of the arena. We therefore recorded oviposition during all tests, which contestant female displayed first oviposition behavior, and aggressive behaviors displayed by each contestant female. All observations lasted 1h or were stopped when i) one contestant female pushed her opponent out of the central chamber, ii) one exited the central chamber for at least 2 min or iii) neither contestant female touched the host nor displayed agonistic interactions for at least 2 min. When both contestant females stayed in the central chamber during the whole time of observation,

174 the winning female was determined as the one displaying oviposition behaviors (N = 22; mean ± S.E.
175 eggs laid: 1.32 ± 0.19).

176 We immediately froze wasps at -20°C after the experiments, weighed them using an electronic
177 balance (Ohaus Discovery® model, accuracy: 0.01mg), and stored them at -20°C until sample
178 preparation for hormonal quantification. Due to the small body of *E. vuilleti* females (mean body
179 mass ± S.E. = 1.52 ± 0.03 mg; N = 91), we were not able to collect the hemolymph from individuals
180 both before and after behavioral observations. In order to keep as much biological material as
181 possible for subsequent ESH quantification, we also did not dissect females to determine their egg
182 load.

183

184 *2.3.2. ESH quantification using Enzyme Immunoassays*

185 We placed each parasitoid in a 1.5 mL conical microcentrifuge tube with 0.5 mL of methanol
186 (99% HPLC grade), and we crushed them with a polypropylene pestle. After one hour at ambient
187 temperature, we centrifuged samples (10,000 *x g* for 5 min), and we transferred the supernatant
188 methanol into a new tube. We dried down these extracts in a SpeedVac (AES 1010 Thermo Savant),
189 and we then stored them at -20°C, until resuspending the samples in 0.180 mL of phosphate buffer
190 (0.01 M) before quantification of ESH titers.

191 Immunoassays (EIA) used for quantification of ESH were conducted according to the method of
192 Porcheron *et al.* (1989) modified for the use of a peroxidase conjugate of 20E as a tracer (De Reggi
193 *et al.*, 1992) and adapted for *E. vuilleti* (Bodin *et al.*, 2007). The L2 polyclonal antibody (gift from
194 Dr. Delbecque, University of Bordeaux, France) is very sensitive to ecdysone, 2-deoxyecdysone and
195 3-dehydro-ecdysone, but ca. six times less sensitive to 20E. Because ecdysone is the main compound
196 found in reproductive *E. vuilleti* female, ESH titers are expressed in ecdysone equivalents (E-eq.,
197 expressed in units of pg/ mg of female parasitoid), as this compound was used for reference curves.
198 To verify the reproducibility of our results, each sample measured by EIA was analyzed in duplicate.

199

200 ## 2.4. Experiment 2: Effect of ecdysone on females' reproductive status

201 To investigate the influence of ecdysone manipulation on female reproductive status, we
202 randomly assigned two-day old wasps to one of four treatments: they received either 0.04 µL of
203 HPLC grade methanol (no female died due to methanol injection during this experiment) or 0.04 µL
204 of three different quantities of ecdysone (i.e. α-ecdysone, purity ≥ 90.0 %, purchased from Sigma-
205 Aldrich, Inc.) dissolved in methanol (14, 64 or 120 pg). Immediately after being immobilized on a
206 plate that was cooled by ice, we injected wasps into the abdomen through the intersegmental
207 membrane using the nanoliter injector Nanoject III (Drummond Scientific Company). We then
208 placed females individually in a petri dish and we froze them at -20°C, either 4h (N = 59, 60, 59 and
209 57 for injection of 0, 14, 64 and 120 pg, respectively) or 24h (N = 68, 67, 67 and 62 injection of 0,
210 14, 64 and 120 pg, respectively) after injection. We deprived wasps of hosts so that no mature eggs
211 could be laid during this period. Finally, after weighing all females, we dissected them (N = 119 and
212 118 for 4h and 24h post-injection treatment, respectively) to count mature (i.e., ready-to-lay)
213 oocytes.

214

215 ## 2.5. Experiment 3: Effect of ecdysone on female aggressiveness and contest outcome

216 To investigate the influence of ecdysone manipulation on female aggressive behavior and their
217 probability of winning a contest, we randomly assigned three-day old wasps to two different
218 injection treatments: either with 0.04 µL of ecdysone diluted in methanol (quantity = 64 pg) or 0.04
219 µL of methanol alone (no female died due to methanol injection during this experiment). We marked
220 wasps the day before behavioral observations to allow individual identification during contests. We
221 deprived females of hosts for at least 1h before the start of the contest so that they were more ready
222 to oviposit. Based on our results from Experiment 2 (see section 3.1.), we observed contests between
223 females from 1h to 3h post-injection to avoid an effect of ecdysone treatment on females' mature egg

224 load. As described in experiment 1, we then simultaneously introduced two wasps into the central
225 chamber of the contest arena at the beginning of the observation.

226 We ran contests between either i) one control female (i.e., injected with methanol only) and one
227 test female (i.e., injected with ecdysone; N = 28), or ii) between two control females (N = 25),
228 challenging for a 4th instar larva of *C. maculatus* located in gelatin capsule and previously placed in
229 the central chamber of the contest arena. Contests occurred between females of the same age (3 days
230 old), marked with different colors and visually matched for size; post-experiment data analysis
231 confirmed that contestants of the same dyad did not differ significantly in body mass (paired t-test:
232 $t_{52} = 0.42$, $P = 0.68$). Both females could freely move throughout the entire contest arena.
233 Observations lasted 1h maximum. During all tests, we recorded oviposition behaviors, which
234 contestant female displayed first such behaviors, and attack behaviors displayed by each female.
235 When both females of a dyad stayed in the central chamber during the whole time of observation, we
236 determined the winning female as the one exhibiting oviposition behaviors (N = 44; mean ± S.E.
237 eggs laid: 0.65 ± 0.1).

238 After each contest, we counted the number of eggs laid on the host by the winning female, and we
239 froze all wasps at -20°C. We finally weighed them and dissected them to count the number of mature
240 eggs in their ovaries. The initial mature egg load of winning females was calculated as the number of
241 mature eggs in their ovaries + the number of eggs laid on the host.

242

243 2.6. Statistical analysis

244 We performed data analyses with the software Rstudio (RStudio Team, 2016), using $\alpha = 0.05$.
245 Our general approach was to use, when possible, parametric analyses in which the assumed
246 distribution of residuals was matched to the data rather than transforming data to fit standard
247 assumptions (Wilson and Hardy, 2002; Hardy and Briffa, 2013). However, we used non-parametric
248 tests when parametric conditions were not satisfied.

Experiment 1: For the 30 contests that we observed during experiment 1, 25 contests were resolved (i.e., there was a clear winner and loser female). Therefore, we only considered these contests in the analysis. We first randomly assigned one of the 2 contestant females of each replicate as the focal female, using the ALEA function of Microsoft Excel. We then performed a generalized linear model (GLM) assuming a quasi-Poisson distribution of errors (link function = 'log') to test the association between post-trial ESH titers of focal females and their contest experience (i.e., no contest, winner or loser). Pairwise *post hoc* comparisons (function 'emmeans') were used to determine significant difference between treatments. We ran the same procedure to test the effect of an interaction between after ESH titers of focal contestant females and ESH of their opponent on the number of attacks displayed by focal contestant females. Then we calculated the relative number of attacks of focal contestant females as the ratio: 'number of attacks of the focal female / (number of attacks of the focal female + number of attacks of the opponent)' and the relative difference of post-contest ESH titers between contestant females as the ratio: '(post-contest ESH titer of the focal female – post-contest ESH titer of the opponent) / (post-contest ESH titer of the focal female + post-contest ESH titer of the opponent)'. We also defined contest outcome as a binary response: 0 = the focal contestant female lost, 1 = the focal contestant female won. We ran a GLM assuming a binomial distribution of errors (link function = 'logit') to investigate whether the interaction between the relative number of attacks of focal females and the relative difference in post-contest ESH titers between females affected the probability of the focal female winning. We used the same procedure to test whether the probability of being first to display oviposition behavior (binary response: 0 = the focal female was not first to display oviposition behavior, 1 = the focal female was the first to display oviposition behavior) was significantly associated with the relative difference in post-contest ESH titers between contestants.

Experiment 2: We first investigated whether the number of mature eggs differed significantly between females receiving different ecdysone quantities, 4h after injection, by performing a GLM

assuming a quasi-Poisson distribution of errors (link function = 'log'). We then ran a generalized polynomial model (GPM) with a Poisson distribution of errors (link function = 'log') to explore the effect of ecdysone treatments on the number of mature eggs 24h after injection.

Experiment 3: Of the 74 contests that we observed during experiment 3, 53 contests were clearly resolved. We only considered these contests in the following analysis. We first verified that initial egg load did not differ between ecdysone-treated females (N = 28; mean egg load ± S.E.: 2.6 ± 0.3) and control females (N = 78; mean egg load ± S.E.: 2.4 ± 0.2; Mann-Whitney test: U = 1197.5, P = 0.44). We then ran a GLM with a quasi-Poisson distribution of errors (link function = 'log') to test the effect of injection treatment, initial egg load of focal females, initial egg load of opponent females and two-way interactions between these variables on the number of attacks displayed by focal females. We also calculated the relative number of attacks of focal females as described for experiment 1, and we determined the relative difference in initial mature egg load between females as the ratio: '(initial egg load of the focal female – initial egg load of the opponent) / (initial egg load of the focal female + initial egg load of the opponent)'. We then performed a GLM with a binomial error distribution (link function = 'logit') to explore the influence of injection treatment, the relative number of attacks of focal females and the relative difference in egg load between females on the probability of the focal female winning. The model included the three main effects and the two-way interactions between variables. We used the same procedure to investigate whether the probability of being first to display oviposition behavior varied with the injection treatment received by focal females.

For all analyses, likelihood ratio and F statistics were calculated using the 'Anova' function (package car), which performs type 2 analysis of variance for GLM models, that is, invariant to the order in which effects are entered into the model (Fox and Weisberg, 2019).

3. Results

3.1. Experiment 1: ESH titers, aggressiveness and outcome of contests

We found a significant difference in post-trial ESH titers between focal females with different contest experience (GLM with quasi-Poisson distribution of residuals: $F_{2,53} = 4.48$, $P = 0.02$; **Fig. 1**): winner females had significantly higher post-trial ESH titers (mean ± S.E. = 127.2 ± 25.2 pg E-eq / mg of female) than losers (mean ± S.E. = 53.6 ± 13.6 pg E-eq / mg of female; pairwise *post hoc* comparisons: $P = 0.029$) and females with no contest experience (mean ± S.E. = 77 ± 9.9 pg E-eq / mg of female; pairwise post-hoc comparisons: $P = 0.047$). However, post-trial ESH titers were not significantly different between loser females and females with no contest experience (pairwise post-hoc comparisons: $P = 0.519$).

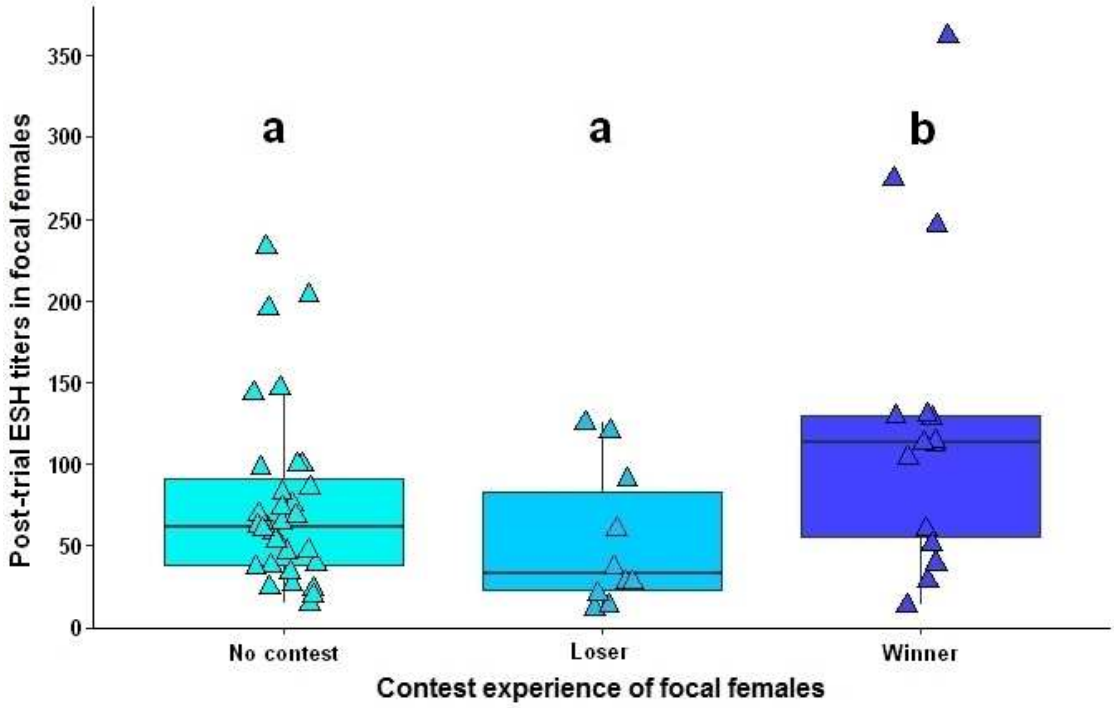


Figure 1. Post-trial ESH titers (pg E-eq / mg of female) in focal females with different contest experience (no contest: N = 31, loser: N = 10 or winner: N = 15). *: $P < 0.05$. Different letters indicate significant statistical differences.

We found that the number of attacks displayed by focal contestant females was independent of their post-contest ESH titers (GLM with quasi-Poisson distribution of residuals: $F_{1,21} = 0.01$, $P = 0.91$), their opponent's post-contest ESH titers ($F_{1,21} = 0.32$, $P = 0.58$) or the interaction between these variables ($F_{1,21} = 0.03$, $P = 0.86$).

The probability of a female winning a contest was not significantly affected by the interaction between the relative number of attacks and the relative difference in post-contest ESH titers between females (**Table 1**). However, it was significantly influenced by both factors independently (**Table 1**): winner females displayed significantly more attacks (mean ± S.E. = 18 ± 3 attacks) than their opponent (mean ± S.E. = 9 ± 2 attacks) compared to loser females (mean ± S.E. = 9 ± 2 attacks) with their opponent (mean ± S.E. = 21 ± 7 attacks; **Fig. 2A**). In addition, focal females were more likely to win contest when they had higher post-contest ESH titers (mean ± S.E. = 127.3 ± 25.2 pg E-eq / mg of female) than their opponent (mean ± S.E. = 74.9 ± 8.8 pg E-eq / mg of female) compared to loser females (mean ± S.E. = 53.6 ± 13.8 pg E-eq / mg of female) with their opponent (mean ± S.E. = 95 ± 24.3 pg E-eq / mg of female; **Fig. 2B**).

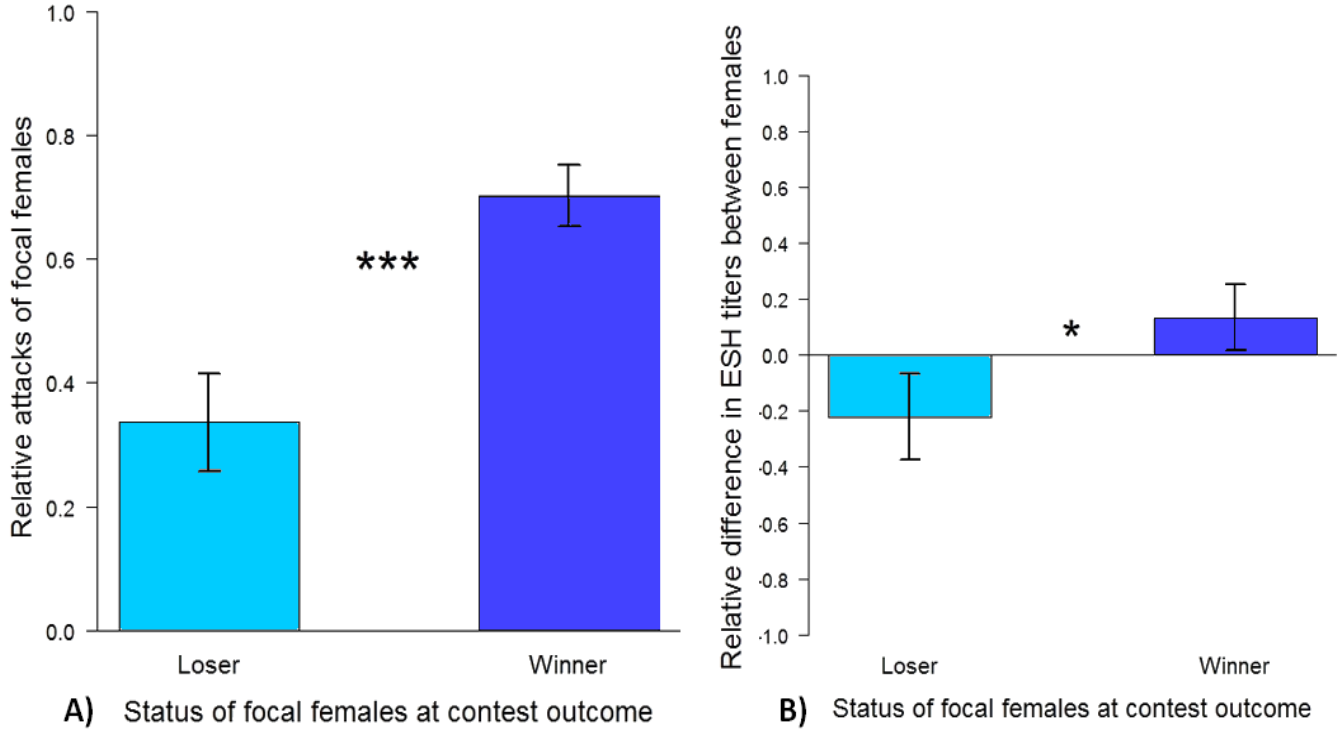
Table 1. Effect of relative number of attacks and relative difference in post-contest ESH titers on winning probability over host of focal females.

Factors affecting contest outcomes	**Estimates**	**d.f.**	**G**	***P***
Intercept	-5.782			
Relative attacks	13.81	1	13.9	**< 0.001**
Relative difference in after contest ESH titers	-2.638	1	**4.57**	**0.03**
Relative attacks x Relative difference in after contest ESH titers	13.31	1	2.35	0.12

	Residuals	21
	Total	24

330 *P*-values of significant explanatory variables are highlighted in bold font.

331



332

333 **Figure 2.** Status of focal females (loser: N = 10 or winner: N = 15) after conflict resolution (N = 25). A) Mean
334 (± S.E.M.) relative attacks of focal females. ***: $P < 0.001$. B) Mean (± S.E.M.) relative difference in post-
335 contest ESH titers (pg E-eq / mg of female) between females. *: $P < 0.05$.

336

337 The probability of focal females being first to display oviposition behavior was significantly
338 associated with the relative difference in post-contest ESH titers between contestants (GLM with
339 binomial distribution of error: $G_1 = 4.58$, $P = 0.03$). Focal females were more likely to be first to
340 display such behavior when they had higher ESH titers than their opponent.

341

342 ## 3.2. Experiment 2: Effect of ecdysone treatment on females' reproductive status

343 The number of mature eggs in females did not vary across treatments 4h after injection ($F_{1,117}$ =
344 0.09, P = 0.77; **Fig. 3A**). However, egg load was significantly influenced by treatment 24h after
345 injection, with a dose-dependent effect of ecdysone injection on egg maturation reaching the highest
346 positive effect at 64 pg (G_1 = 14.33, P < 0.001; **Fig. 3B**).

347

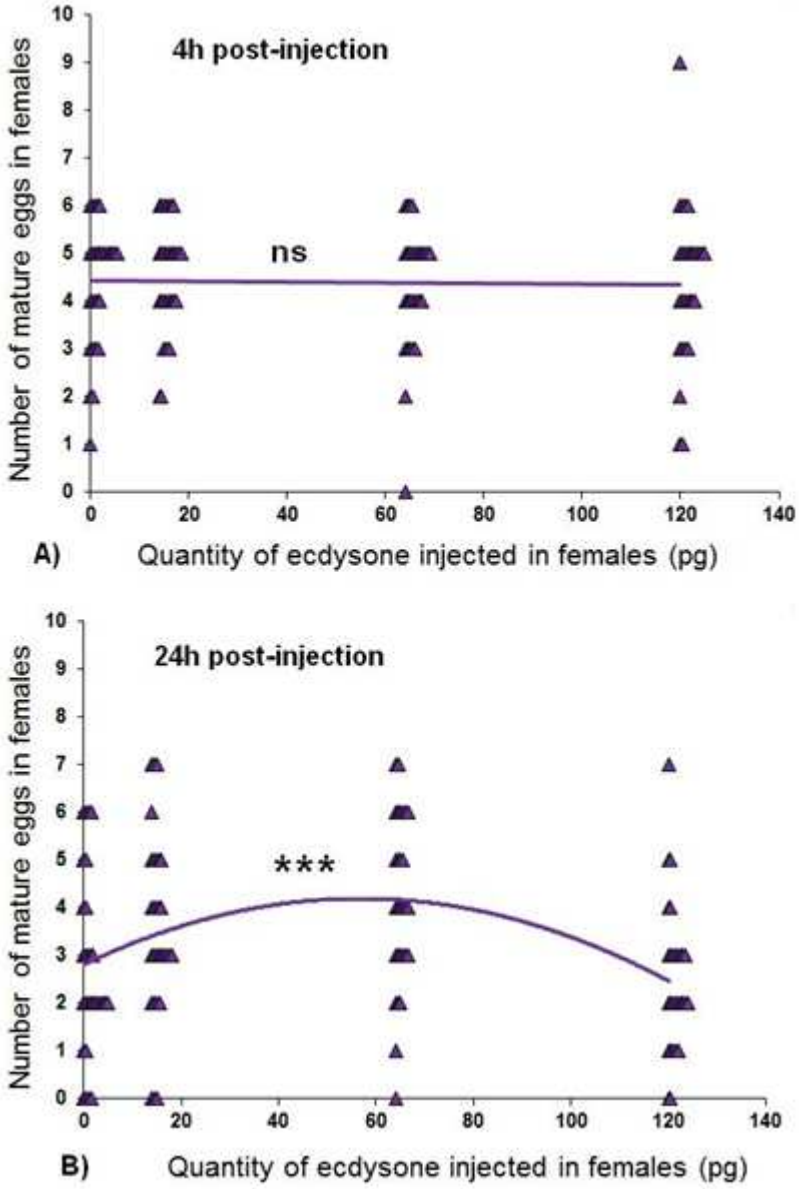


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Figure 3. Number of mature eggs in females injected with different quantities of ecdysone. A) 4h after injection. ns: non-significant. B) 24h after injection. ***: $P < 0.001$. Data points have been horizontally displaced from their position to show the numbers of observations.

3.3. Experiment 3: Effect of ecdysone treatment on female aggressiveness and contest outcome

The number of attacks displayed by females was not influenced by injection treatment, initial egg load of females, initial egg load of their opponent, or any interactions between these variables (**Table 2**).

Table 2. Effect of injection treatment, initial egg load of focal females and initial egg load of the opponent females on the number of attacks of focal females.

Factors affecting aggressiveness	**Estimates**	**df**	**F**	***P***
Intercept	1.5133			
Injection treatment (control)	-0.6853	1	3.0776	0.09
Egg load of focal females	0.3183	1	3.0297	0.09
Egg load of opponent females	0.0833	1	2.8708	0.10
Injection treatment (control) x Egg load of focal females	0.0402	1	0.0303	0.86
Injection treatment (control) x Egg load of opponent females	-0.0241	1	0.0090	0.92
Egg load of focal females x Egg load of opponent females	-0.0983	1	1.2683	0.27
Residuals		46		

Total	52

Focal females: ecdysone-treated: N = 28; methanol-treated: N = 25)

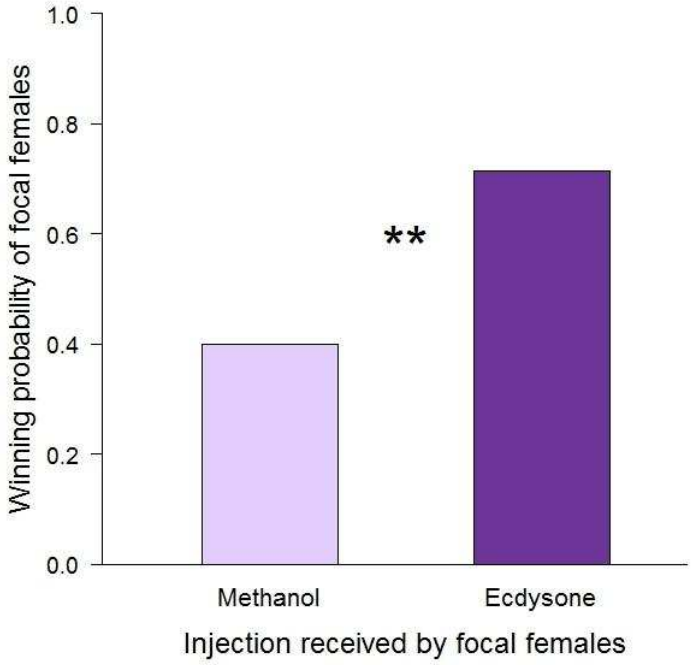
The probability of females winning contests over hosts did not vary significantly with the difference in egg load between contestants but was significantly influenced by ESH injection (**Table 3**): wasps injected with ecdysone were more likely to win contests than females injected with vehicle alone (**Fig. 4**). Moreover, there was an interaction between the relative attacks of females and the relative difference in egg load between contestants. Aggressive females had a greater chance of winning the contest, and this probability increased further when they had more ready-to-lay eggs than their opponent (see **Table 3**). There were no significant interactions between injection and either relative attacks or difference in egg load on winning probabilities (**Table 3**).

Table 3. Effect of injection treatment, relative number of attacks and relative difference in egg load on the winning probability over host of focal females.

Factors affecting contest outcomes	**Estimates**	**df**	**G**	***P***
Intercept	-1.8814			
Injection treatment (control)	-0.4766	1	9.88	**0.002**
Relative attacks	8.8585	1	19.1	**< 0.001**
Relative diff. in egg load	-2.9157	1	0.04	0.84
Injection treatment (control) x Relative attacks	-5.9728	1	3.78	0.05
Injection treatment (control) x Relative diff. in egg load	1.8775	1	1.08	0.30

Relative attacks x Relative diff. in egg load	7.7581	1	4.70	**0.03**
Residuals		46		
Total		52		

374 *P*-values of significant explanatory variables are highlighted in bold font. Focal females: ecdysone-treated: N
375 = 28; methanol-treated: N = 25)
376



377

378 **Figure 4.** Winning probability of focal females according to the injection treatment they received before a
379 contest (methanol (control): N = 25 or ecdysone in methanol: N = 28). **: $P < 0.01$

380

381 The probability of a contestant displaying first oviposition behavior varied significantly with
382 injection treatment (GLM with binomial distribution of error: $G_1 = 4.66$, $P = 0.03$): females injected
383 with ecdysone were more frequently the first to display such behavior (16/28 contests) than control
384 females (7/25 contests).

385

4. Discussion

Our work aimed to explore the role of ESH in female aggressiveness and conflict resolution over oviposition sites (hosts) in the solitary ectoparasitoid *E. vuilleti*. First, we showed no link between naturally circulating post-contest ESH titers in females and their aggressiveness when fighting for host access, but winner females had higher ESH titers than losers after the contest and females that did not experience contest. Second, when measuring how ecdysone manipulation affects egg maturation, we found that ecdysone injection had a dose-dependent gonadotropin effects at 24h post-injection. Finally, ecdysone injections had no effect on female aggressiveness during contests over a host, but ecdysone-injected females were more likely to win contests over a host.

4.1. ESH, aggressiveness and outcome of contest over host

One main finding of our study – supported by both hormone quantification and hormone manipulation – is that ESH are involved in the outcome of contests over hosts in *E. vuilleti*. First, we found that females that won fights over hosts had higher post-contest ESH titers than both loser females and females that did not experience a contest. This agrees with previous works in bumblebees (Bloch *et al.*, 2000; Geva *et al.*, 2005) and queenless ants, *Streblognathus peetersi* (Brent *et al.*, 2006), showing that ESH titers are higher in high-ranking than in low-ranking workers. Here, we are not able to determine whether ESH titers were already high in eventual winner prior to the contests or ESH titers increased as a result of winning. Supporting one hypothesis than another would need hormones quantification both before and after contest, which is not yet technically feasible in small animals such as *E. vuilleti*. Nevertheless, our results demonstrate that ESH titers are associated with the outcome of contests over hosts. Moreover, in our study, contestant females injected with ecdysone were more likely to win compared to control females. In the only one experiment that directly tested the effect of ESH injection on contests between conspecific individuals in insects, Röseler *et al.* (1984) also found that injecting 20E in females of the eusocial

wasp *Polistes dominula*, alone or coupled with JH-I, increased the probability of attaining dominant status compared to control individuals. To our knowledge, our study shows, for the first time, the importance of ESH in outcomes of intraspecific competition for the access to an indivisible resource in a solitary insect species.

In both vertebrate and invertebrate taxa, it is well known that individuals displaying higher levels of aggressiveness during intraspecific competition are more likely to win access to indivisible resources (Breed and Bell, 1983; Hsu *et al.*, 2006; Hardy and Briffa, 2013; but see Scott, 2006). Here, we also found such a relationship between aggressiveness and contest outcome, with *E. vuilleti* females being more likely to win when showing relatively more attacks. However, this link appeared to be independent of ESH titers. Indeed, post-contest ESH titers in fighting wasps did not correlate to their level of aggressiveness and the relative number of attacks displayed by contestants did not vary significantly with the injection treatment they received. Similarly, Kelstrup *et al.* (2014a, b) showed no relationship between ESH titers and aggressive behaviors, neither in females of the caste-flexible epiponine wasp *Synoeca surinama*, nor in queens and workers of the swarm-founding eusocial wasp *Polybia mycans*. Altogether, these data support the idea that ESH are not involved in modulating aggressive behaviors in these insects. In *E. vuilleti,* such agonistic behaviors are mediated by JH (Mathiron *et al.*, 2019). Although we still lack information about the interplay between both families of hormones, our present work might suggest that ESH did not stimulate JH sufficiently to mediate female aggressiveness within the time frame of our experiments (under 3 hours).

4.2. ESH, egg load and contest outcome

As observed in many other insect species (reviewed in Nijhout, 1994; Swevers and Iatrou, 2009), ecdysone has a gonadotropin effect in *E. vuilleti* females. By manipulating ESH levels, we showed that wasps' egg load increased with the quantity of ESH injected after 24h, with the greatest effect at 64 pg. The absence of a measurable response of egg load to ecdysone injection at the highest dose

(i.e., 120 pg) may result from negative feedback driven by ecdysone binding its receptors (Beydon and Lafont, 1983; Parvy *et al.*, 2014). Interestingly, this gonadotropin effect was observed 24h after injection but not 4h post-injection, suggesting that egg maturation takes longer than 4h in *E. vuilleti* (see also Mathiron *et al.*, 2019). By observing wasps fighting over hosts between 1h and 3h post-injection in our last experiment, we were thus able to disentangle the effects of ecdysone and egg load on aggressiveness and contest outcome.

Female fertility is known to strongly affect behavioral strategies during contests over hosts in *E. vuilleti*, with wasps with a higher egg load than their opponent being more likely to win host access (Mohamad *et al.*, 2010, 2012; Mathiron *et al.*, 2018, 2019). Game theory models predict that the outcome of a conflict is influenced by the existing asymmetry between competitors in terms of subjective resource value (Maynard Smith and Parker, 1976; Enquist and Leimar, 1987). Parasitoid females with more ready-to-lay eggs should place a higher value in the hosts, which should lead them to win more often, as observed in different parasitoid species (e.g. Stokkebo and Hardy, 2000; Mohamad *et al.*, 2010; but see Goubault *et al.*, 2007). The underlying mechanisms remain, however, unknown. Because ecdysone is produced by active ovaries and partially stored in the eggs of *E. vuilleti* females (Mondy *et al.*, 2006; Bodin *et al.*, 2007), this effect of egg load asymmetry on contest outcome may actually result from the action of ESH produced by mature eggs. In experiment 1, we thus quantified post-contest ESH titers in females without determining their initial egg load (we could not dissect them without impairing ESH quantification). It is therefore possible that winner females were the ones with more ready-to-lay eggs prior to the contest; these females may have had higher ESH titers and attacked more to get access to the host. The fact that we did not observe a relationship between the number of mature eggs and winning probability in our last experiment also supports this idea: the ecdysone we injected in females bypassed the actions of ecdysone naturally produced by eggs, masking the usual link between egg load and winning success.

As a result, ESH would not solely have an autocrine and/or paracrine action in the ovaries, but also an endocrine role in the mediation of female *motivation* to get access to the host.

4.3. How can ecdysone increase the probability of winning contests?

The mechanism underlying the association between wasps with higher ESH titers and their elevated winning probability remains to be determined. From our results, it appears to be independent of the modulation of aggressive behaviors, because ESH did not drive variation in aggressiveness in our experiments. An alternative explanation would be that ecdysone increases female motivation to lay their eggs, leading them to exploit the host faster than control conspecifics. This would lead them to win host access more often. Previous studies investigating the role of ESH on *E. vuilleti* oogenesis found an increase in ecdysone titers after simply contacting a host, which correlated with maturation of an egg within 24 hours (Bodin *et al.*, 2007, 2009; Casas *et al.*, 2009). As such, females with higher ESH titers than their opponent would be more ready to lay eggs, and would thus be more likely to be first to display oviposition behavior (i.e., before their opponent). In the same way, ecdysone-injected wasps should be more likely to be the first female of the dyad to display oviposition behavior than methanol-injected females. Our analyses seem to support these expectations: focal females were more likely to be first to display oviposition behavior when they have higher ESH titers than their opponent (Experiment 1). Moreover, females injected with ecdysone were more frequently the first female of the dyad to display such behavior than control females (Experiment 3). Even if in Experiment 1 we cannot determine whether the relative difference in ESH titers between females after contest resulted from displaying oviposition behavior or the oviposition behavior associated with winning host access resulted from difference in ESH titers before contests, we hypothesize that ESH mediate female winning success by increasing their propensity to display oviposition behavior quickly. While it is well known that *subjective resource value* drives individuals' behavior during contests (Briffa & Hardy 2013), the underlying

mechanisms remain poorly investigated, particularly in insects. The endocrine action of ESH appears to be one important pathway mediating contest behavior.

5. Conclusion

By testing the role of ESH on both fertility and behavioral decisions during contests over hosts in female *E. vuilleti*, we have shown for the first time that ecdysone is involved in the outcome of conflicts in a solitary insect. However, we found no evidence that ecdysone fuels aggressive behaviors like JH does in this species (Mathiron *et al.*, 2019a). This suggests that the two family hormones, ESH and JH, interact to mediate wasps' behavior during intraspecific competition for reproductive resources. Interactions between JH and ESH are already known to play a central role in different aspects of insect biology, such as metamorphosis in juveniles (reviewed in Riddiford, 2008) and the regulation of vitellogenesis and ovarian development in adults (reviewed in Bloch *et al.*, 2009; Roy *et al.*, 2018; see also Santos *et al.*, 2019). In *E. vuilleti*, JH-III and ecdysone both favor egg maturation (Mathiron *et al.*, 2019 and this study, respectively). Direct competition over host access between *E. vuilleti* females is closely related to reproduction. Thus, ESH and JH effects on behavioral strategies during contests may have been co-opted because of the joint role of these hormones on reproductive status, but particular selective advantages and constraints may have selected each one for the mediation of different behaviors. Future experiments exploring the relative and/or joint action of ESH and JH in this species would broaden our understanding of the hormonal mediation of aggressiveness and conflict resolution over indivisible resources in solitary insects.

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