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Oviposition behavior of the pollen beetle (*Meligethes aeneus*): a functional study

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Abstract

The recognition by female phytophagous insects of a plant as a ‘good’ or ‘bad’ host for egg laying is based on a variety of cues (either visual, physical or chemical). Specific cues are often looked for during stereotypic oviposition behaviors, composed of several phases having their own function(s). In this study the oviposition behavior of the pollen beetle *Meligethes aeneus*, a pest which lays eggs in flower buds of only some brassicaceous plants, was described in detail on five oilseed rape (*Brassica napus*) genotypes. In parallel, setae borne by the ovipositor were characterized by scanning electron microscopy. Observations showed that the stereotypic oviposition sequence is functionally divided into three independent phases: external inspection, internal inspection and egg laying. The ovipositor plays a role in all phases by gaining information about external and internal bud parts. This role appears to be only physical since all the setae it bears are mechanoreceptors. Despite the fact that the pollen beetle is a specialist for oviposition, important variations in secondary metabolites that are typical of its host plant family (i.e. glucosinolates) on the bud did not influence clutch size. The crucial phase in the oviposition sequence seems to be the external inspection, during which poor and high-quality host plants are probably discriminated. Chemical information on bud surface is likely to be determinant in this process.

Keywords: Oilseed rape (*Brassica napus*); Insect pest; Plant acceptability; Behavioral sequence; Scanning Electron Microscopy

Introduction

The classical 'preference - performance' hypothesis (e.g. Thompson 1988) predicts that female insects should be selected to lay eggs preferentially on oviposition sites that are favorable for offspring development. In phytophagous species, this hypothesis has been supported by a recent meta-analysis (Gripenberg et al. 2010). The recognition of a plant as a host or non-host, and more precisely of the quality of a host plant, is based on cues (i.e. plant traits) used by females. These can be either chemical and/or visual, acting at distance (e.g. volatile compounds, size or architecture) and/or at contact (e.g. primary and secondary metabolites inside and on surface of plant tissues, trichome density, tissue thickness or toughness).

In many insect species, females show a stereotypic oviposition behavior (e.g. the cabbage root fly *Delia radicum* (Städler and Schöni 1990), the mustard leaf beetle *Phaedon cochleariae* (Müller and Rosenberger 2006) or the cabbage seedpod weevil *Ceutorhynchus obstrictus* (Ulmer and Dossall 2006)). Such behavioral sequence is often divided into several phases, each of them having one or several precise function(s). Specific host cues are detected during these phases, by means of sensory organs such as mechano- or chemoreceptors borne by antennae, tarsi or the ovipositor. Describing stereotypic oviposition behaviors, deciphering their function(s) and characterizing sensory organs that are used by females is the first step before identifying specific cues used by females to evaluate plant quality.

The pollen beetle (*Meligethes aeneus* F.; Coleoptera: Nitidulidae) is one of the major insect pests of oilseed rape (*Brassica napus* L.; Brassicaceae) (OSR) crops. Females lay eggs only on brassicaceous plants (Ekbom and Borg 1996; Free and Williams 1978). Adults colonize OSR fields after their winter diapause, when plants are at the bud stage. They destroy flower buds to reach the pollen inside, from which they feed. This destruction sometimes leads to

important yield losses (Nilsson 1987). Mating occurs on the plant and females oviposit inside buds, after having made a small hole at its base. Reproduction goes on until death of the individuals, in summer. Buds are not destroyed during oviposition and usually continue their normal development. Larvae hatch inside the bud and feed from the pollen contained in anthers during their first instar. Transition to the second (and last) instar occurs approximately as the flower opens. Second-instar larvae move from one flower to another, still feeding on pollen. They finally drop from the plant at the end of their development and pupate in the soil (Williams 2010).

Not all brassicaceous plants are accepted for oviposition by females even when they are attractive at distance (Cook et al. 2004; Kaasik et al. 2014a, 2014b; Veromann et al. 2012). Cues present on the buds and acting upon contact play a key role in determining oviposition. White mustard *Sinapis alba* is, for example, especially known to be a low-quality host for the pollen beetle (Ekbom 1998; Ekbom and Borg 1996; Hopkins and Ekbom 1996, 1999; Hopkins et al. 1998). Borg and Ekbom (1996) characterized for the first time the oviposition behavior of pollen beetle females and proved that *S. alba* is of inferior acceptability (in the strict sense of Singer (2000)) compared to *Brassica* spp. They showed that the flower bud is inspected in a stereotypic sequence before oviposition, and that this inspection can lead to females stopping the sequence and leaving the plant. Although contact cues appear essential in determining plant acceptability, these authors were not able to identify the cues females use to make their decision.

The aims of this study were (i) to describe in more detail the oviposition behavior of the pollen beetle, (ii) to give a functional interpretation of the different steps of the behavioral sequence, and (iii) to identify the critical step(s) of this sequence determining the acceptance of the plant. For this purpose, five OSR genotypes for which the biochemical composition of buds is known (Hervé et al. 2014a) were compared in a no-choice experiment, as precisely as

possible. As the ovipositor is known to bear setae in its distal part in the Meligethinae subfamily (Audisio et al. 2009), morphology of these setae was also characterized by scanning electron microscopy (SEM) to understand their function.

Material and methods

Plants

All genotypes used in this study were lines from the INRA OSR collection (BraCySol Center for Genetic Resources, INRA, Le Rheu, France). Both winter (genotypes ‘Darmor’, ‘Express’ and ‘Mar’) and spring (genotypes ‘Liho’ and ‘Yudal’) OSR genotypes were used. Plants were produced in controlled conditions as described in Hervé et al. (2014a) and used at BBCH stage 55-57 (Lancashire et al. 1991), i.e. the ‘green bud stage’. To keep the interaction natural, entire intact plants were used.

Insects

Overwintered pollen beetle females were collected from an unsprayed winter OSR crop near Le Rheu (Brittany, France), in April-May. Females were identified by observing mating behavior. Experiments took place within 3 h after field collection.

Oviposition behavior characterization

One female was placed on the main inflorescence of an intact OSR plant, in a plastic pot (diameter 6.5 cm, height 9 cm) isolating this inflorescence from the rest of the plant. As described by Borg and Ekbohm (1996), the beginning of the oviposition sequence is discernible when, after walking on several buds, a female walks circuitously on the same bud. Observations were carried out by constantly following the female with a hand magnifier (x 5),

recording the sequence and the duration of each behavior with a handheld recorder, and transcribing it later with the interface SequenceR (Hervé 2013). Based on the previous characterization of Borg and Ekbom (1996) and our preliminary observations, six behaviors were considered (Table 1). After the end of the sequence, the bud in which the female oviposited was measured and dissected to count the number of eggs laid. A different plant was used for each female. Thirty different individuals were recorded per OSR genotype. Replicates were conducted randomly through time (total study period: about one month) and experiments took place at 20 °C.

Scanning electron microscopy of the ovipositor

About 40 ovipositors were dissected, dehydrated by successive alcohol-bath (70 %, 80 %, 90 %, 96 % and 100 %), critical-point dried and coated with gold-paladium. Observations were then performed with a JSM-7100F (Jeol) microscope.

Statistical analysis

All statistical analyses were performed using R software (R Core Team 2013). The proportion of females completing their oviposition sequence was compared among genotypes using a likelihood ratio test on a Generalized Linear Model (GLM) (distribution: binomial, link function: logit). Only females that completed their sequence were included in subsequent analyses. Number of eggs laid was analyzed using a likelihood ratio test on a GLM (distribution: Poisson, link function: log) taking into account OSR genotype, size of the bud in which oviposition took place and duration of each behavior. ANOVAs were used to compare genotypes for the size of the bud in which the female oviposited, the duration of each behavior (durations of ‘Walking with ovipositor’ and ‘Resting’ had to be log-transformed for a better model fit) and the total duration of the sequence. When needed, pairwise comparisons

of Least Squares Means were performed using the function 'lsmeans' (package 'lsmeans' (Lenth 2013)) and the False Discovery Rate (FDR) correction for P -values (Benjamini and Hochberg 1995). Pearson's correlation tests were used to assess the relationship between duration of all pairs of behaviors. The P -value of each test was adjusted with the FDR correction.

Results

Oviposition behavior characterization

The mean length (\pm SE) of the bud in which pollen beetle females laid eggs was 4.0 (\pm 0.07) mm. Although the size of all buds forming an OSR raceme are quite variable, ranging between less than 1 mm and 7-8 mm long, only a narrow size range was used by females (90 % of chosen buds had a length between 3 and 5.5 mm). No difference was observed among the five OSR genotypes for the length of the chosen bud ($F_{4,128} = 0.80$, $P = 0.525$).

The observed sequences were consistent with the description of Borg and Ekbom (1996), but we recorded finer details during two particular steps. Firstly, Borg and Ekbom (1996) described a behavior of "walking with the abdomen touching the bud surface". Our observations showed that during this step, the abdomen is very close to the bud surface but does not touch it, and the ovipositor is partly extruded, tapping for a few seconds with its distal end on the bud surface. Secondly, Borg and Ekbom (1996) described a behavior consisting of "placing the abdomen over the bite hole". Our observations showed that the abdomen is not only placed over the hole, but that the ovipositor is fully extruded and inserted inside the hole, tapping on bud organs we were not able to identify. Finally, we add that antennae were constantly used to tap the bud surface throughout the 'Walking' and 'Walking with ovipositor' behaviors.

No difference was found among OSR genotypes for the duration of ‘Walking’, ‘Walking with ovipositor’, ‘Resting’ and ‘Oviposition’ (Table 2). On the contrary, the mean duration of ‘Biting’ and ‘Ovipositor inside hole’ was significantly longer in genotypes ‘Darmor’ and ‘Mar’ compared to ‘Express’, ‘Liho’ and ‘Yudal’. Consequently, the mean total time of the sequence was greater in ‘Darmor’ and ‘Mar’ than in the three other genotypes. Pairwise correlations between durations of each behavior (Table 3) revealed that all durations were independent, except for two pairs. First, the time spent walking was highly positively correlated to the time spent walking with the ovipositor tapping on the bud surface. Secondly, the time spent biting the perianth was highly positively correlated to the time spent with the ovipositor tapping inside the bud.

A high proportion of females completed their oviposition sequence (overall proportion [95 % CI]: 0.89 [0.82 - 0.93]). This proportion was not statistically different among the OSR genotypes (Table 2). Altogether, 17 females left the bud before laying eggs. Six left it before biting any hole, after a mean time (\pm SE) of 561.2 (\pm 94.0) s (26.6 % of the mean total time of completed sequences). The other eleven females left the bud after at least starting biting, after a mean time of 1,250.4 (\pm 269.2) s (59.4 % of the mean total time of completed sequences). Between one-six eggs were laid, with a mean (\pm SE) of 2.88 (\pm 0.11). The number of eggs laid was not influenced by any variable except the duration of the ‘Oviposition’ behavior (Genotype: $\chi^2 = 3.07$, $df = 4$, $P = 0.547$; Size of the bud in which eggs were laid: $\chi^2 = 0.02$, $df = 1$, $P = 0.890$; ‘Walking’: $\chi^2 = 0.001$, $df = 1$, $P = 0.971$; ‘Walking with abdomen’: $\chi^2 = 0.88$, $df = 1$, $P = 0.349$; ‘Resting’: $\chi^2 = 0.03$, $df = 1$, $P = 0.866$; ‘Biting’: $\chi^2 = 0.24$, $df = 1$, $P = 0.625$; ‘Abdomen over hole’: $\chi^2 = 0.50$, $df = 1$, $P = 0.481$; ‘Oviposition’: $\chi^2 = 4.37$, $df = 1$, $P = 0.037$). The more time a female spent laying eggs, the more eggs were laid.

Scanning electron microscopy of the ovipositor

Sixteen setae were found on each half of the ovipositor. They were located at the end of the gonostyloid (i.e. the distal end of the gonocoxites), for one part on the gonostyloid themselves and for the other part on two cylindrical styli (Fig. 1b, c). Two types of sensilla were observed: long trichoid sensilla (between 10-15 μm long; Fig. 1d) and short basiconic sensilla (2-3 μm long; Fig. 1e, f). Both types were strictly aporous.

Discussion

Functional organization of the oviposition sequence

Borg and Ekbom (1996) made the first, broad description of the oviposition behavior of the pollen beetle. Based on our results on the correlation and the transitional frequencies between behaviors, we were able to go further and draw a general functional view of the oviposition sequence of this species (Fig. 2). This sequence is divided into three independent steps: the first comprising alternate walking and walking with the ovipositor tapping on the bud surface. This probably represents 'external inspection' of the bud. If the female did not leave the bud during this first step, the second step started. This comprised alternate biting of the oviposition hole and, after a U-turn, placing the ovipositor inside this hole and tapping on internal organs of the bud. We called this phase 'internal inspection'. Finally, if the female did not leave the bud during the second step, the third and final step (consisting of laying eggs) started.

External inspection

It is very likely that external inspection of the bud surface has several functions. Pollen beetle females oviposited only in a narrow bud size range, which supports previous results (Ekbom

and Borg 1996; Ferguson et al. 2014; Nilsson 1989). This bud selection is considered as an adaptive compromise between the protection of larvae against natural enemies until bud opening, and the amount of food (i.e. pollen) available for these larvae during the first part of their development (Ekbom and Borg 1996). The external inspection, as it is the first step of the oviposition sequence, probably plays a crucial role in evaluating the size of the bud. The process by which the female assesses this size is unknown, but it might be based on a comparison with its own size.

The majority of females oviposited on the five studied OSR genotypes, confirming that OSR is a host species of high acceptability. Borg and Ekbom (1996) showed that during the external inspection, *S. alba*, a known low-quality host plant species for the pollen beetle (Ekbom 1998; Ekbom and Borg 1996; Hopkins and Ekbom 1996, 1999) was systematically rejected after a very short walk on the bud surface. A second function of this phase of the oviposition sequence could hence be to discriminate between poor and high-quality host plants. Cues that are used by the female to perform such discrimination are likely to be multiple. Indeed, phytophagous insects and plant surfaces interact in a complex manner, as both physical and chemical parameters can influence insect behavior (reviewed *in* Müller and Riederer (2005)). However, as the pollen beetle oviposits only on certain brassicaceous plant species, it is likely that chemical cues (e.g. surface metabolites), which are more specific than physical ones (e.g. trichome density), are of primary importance. Interestingly, we observed that female's antennae are constantly used to tap the bud surface throughout the external inspection. This suggests that surface compounds are sampled during this phase of the oviposition sequence.

Our results revealed that the female's ovipositor plays an active role in the external inspection, by tapping on the bud surface. This organ is known to bear sensilla at its distal end (Audisio et al. 2009), although their nature remains unknown. Our observations showed that

all of them are totally aporous, clearly indicating that they have a sole, mechanosensory role (Chapman 2013). The function of the behavior ‘Walking with ovipositor’ is probably therefore to get physical information from the bud. It may either be the toughness or the thickness of the perianth which has to be pierced to bite the oviposition hole.

Internal inspection

The internal inspection consists of alternating biting of the oviposition hole and tapping inside the bud with the ovipositor. During biting, it is possible that the female would be influenced by cues from the perianth, which could either be physical or chemical. Although often neglected, plant toughness can negatively influence chewing insects by reducing consumption rate (Clissold et al. 2009). On the other hand, mouth parts of insects are a ‘hot spot’ of chemosensory receptors (Chapman 2013) and the pollen beetle is not an exception (Błażejczak-Zawadziska and Błażejczak 2002). Interestingly, clear differences appeared among OSR genotypes, dividing them into two groups. The biochemical composition of the perianth of all of these genotypes has previously been characterized by Hervé et al. (2014a) and showed two clear results. Firstly, ‘Yudal’ is much more concentrated in total glucosinolates – secondary metabolites typical of a few plant families including Brassicaceae (Fahey et al. 2001) – than the other genotypes (in descending order and relatively to ‘Yudal’: ‘Express’ 0.22, ‘Darmor’ 0.11, ‘Liho’ 0.04 and ‘Mar’ 0.04). Glucosinolate profiles are similar; differences are essentially quantitative. Biting duration being equivalent on ‘Yudal’, ‘Express’ and ‘Liho’, this suggests that there is no link between glucosinolate content of the perianth and biting duration (Fig. 3a). Moreover, the number of eggs laid was not different among genotypes, which also indicates the absence of link between glucosinolate content of the perianth and clutch size (Fig. 3b). These results confirm those of Hervé et al. (2014b) that an important increase in glucosinolate content (maximum - minimum ratio: 23.4) does not

further stimulate oviposition of the pollen beetle. Such a pattern was previously reported for another insect specialized on brassicaceous plants, the cabbage seedpod weevil *Ceutorhynchus obstrictus* (Ulmer and Dosdall 2006). This conclusion is rather unexpected, as increased amounts of glucosinolates generally stimulate feeding and oviposition of phytophagous insects specialized on brassicaceous plants (reviewed in Hopkins et al. 2009). It has to be noted that since we did not use glucosinolate-free plants, it cannot be concluded that these compounds do not stimulate oviposition at all. Secondly, Hervé et al. (2014a) showed a gradient of feeding stimulation among the same five genotypes: ‘Express’ is the most stimulant, ‘Liho’ the least and the three others are intermediate. Again, biting duration does not seem to be linked with perianth biochemistry. All of these results suggest that biting duration could be independent of the biochemical composition of the perianth and is possibly influenced only by its structural characteristics. Finally, since no difference in the acceptability or in the number of eggs laid has been observed among the five OSR genotypes, it seems that oviposition is not determined by cues present in the perianth of this host plant. Further studies on a greater number of genotypes are needed to confirm this hypothesis. During the internal inspection, pollen beetle females tap inside the flower bud with their ovipositor. In a study comparing two OSR genotypes producing male-fertile or male-sterile flowers (Cook et al. 2004), pollen beetle females made the same number of oviposition holes on buds from the two types of plants. However, oviposition took place more often on male-fertile plants (i.e. the likelihood of laying eggs after having making a hole was greater in pollen-containing buds). These results, combined with our observations on the mechanosensory function of ovipositor’s sensilla, suggest that the behavior ‘Ovipositor inside hole’ is likely to be no more than a simple assessment of the presence (and possibly the size) of anthers.

Finally, it seems that during the second phase of the oviposition sequence of the pollen beetle, chemical (i.e. specific) information provided by the bud does not play an important role. It suggests that this phase is not decisive in the assessment of host quality. This is concurrent with the results of Borg and Ekbom (1996) who found that even on the low-quality host *S. alba*, once females started this phase they did not stop the sequence until its end. In our study, it is likely that the few interruptions that occurred after females started biting their oviposition hole were accidental and not purposeful. Some disturbance in our experimental conditions may have occurred.

Oviposition

The third and last phase of the oviposition sequence consists of laying eggs. We found no difference among OSR genotypes in terms of number of eggs laid. Borg and Ekbom (1996) showed that for females completing their sequence, there was no difference among host species differing in their quality. In the same manner, Cook et al. (2004) showed that for females having oviposited, the same number of eggs was laid in male-fertile and male-sterile buds. These results suggest that clutch size of the pollen beetle is not influenced by immediate cues obtained during the oviposition sequence. The daily egg load of individual pollen beetle females has previously been shown to be between one and five eggs (Ekbom and Ferdinand 2003; Ferguson et al. 2014; Hopkins and Ekbom 1996). Our results suggest that, if a host plant is accepted, all available mature eggs are laid.

Conclusion

Our observations have resulted in a more detailed characterization of the behavioral sequence of oviposition in pollen beetle, and highlighted the importance of females' ovipositor in gaining information from the oviposition site. Combined with results of previous studies, we

were able to draw a functional scheme of the oviposition sequence, divided into three phases. Oviposition starts with an external inspection of the flower bud, in which the acceptability of the plant is assessed. This assessment is likely to be influenced mainly by chemical information, i.e. surface metabolites. This is likely to be the critical step, discriminating low and high-quality host plants. The second phase is an internal inspection of the bud, probably essentially influenced by structural parameters of the perianth and during which the presence (and possibly the size) of anthers (i.e. food for larval development) is assessed. Finally, the third phase consists of laying probably all the mature eggs the female is carrying.

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Table 1. Behaviors used to characterize the oviposition sequence of *M. aeneus*

Behavior	Description
Walking	All locomotion except for “Walking with ovipositor”
Walking with ovipositor	Walking with ovipositor tapping on the bud surface
Resting	No locomotion
Biting	Female stays at the same place and chews on the bud; once a hole is initiated all biting behavior occurs at the same location
Ovipositor inside hole	Female stays at the same place, inserts her ovipositor inside the hole bitten and taps on internal bud organs. The female is wiggling and antennae are constantly agitated
Oviposition	Ovipositor is inserted into the hole and egg(s) laid. The female is completely immobile, including antennae

Table 2. Results of (i) ANOVAs comparing durations of each behavior and total oviposition sequence of *M. aeneus* on oilseed rape; (ii) likelihood ratio tests comparing number of eggs and number of females having completed their oviposition sequence. Mean (SE) values are given. For significant ANOVAs, different letters show statistically different means. *P*-values less than $\alpha = 0.05$ are represented in bold

	Genotype						
	$F_{4,128}$	<i>P</i>	Darmor	Express	Liho	Mar	Yudal
Behavior duration (s)							
Walking	0.844	0.500	456.2 (50.4)	363.5 (46.8)	369.9 (39.5)	382.1 (54.3)	447.1 (50.7)
Walking with ovipositor	1.440	0.225	39.1 (9.3)	22.5 (5.0)	37.1 (3.9)	42.3 (11.9)	33.7 (5.4)
Resting	1.503	0.205	22.3 (7.1)	8.4 (3.9)	17.9 (5.3)	21.8 (8.7)	26.2 (12.4)
Biting	8.780	< 0.001	1256.1 (128.2) a	705.8 (83.9) b	629.0 (84.3) b	1181.9 (126.6) a	671.1 (83.8) b
Ovipositor inside hole	5.636	< 0.001	541.2 (73.7) a	285.0 (51.0) b	266.1 (34.0) b	461.4 (59.6) a	280.1 (42.4) b
Oviposition	1.035	0.392	425.7 (40.4)	452.6 (47.6)	366.6 (41.8)	358.2 (46.7)	461.4 (57.7)
Total sequence (s)	7.516	< 0.001	2741.2 (200.1) a	1837.6 (144.1) b	1686.7 (137.1) b	2450.3 (163.1) a	1919.7 (167.6) b
	χ^2_4	<i>P</i>					
Number of eggs	3.066	0.547	3.1 (0.2)	2.9 (0.3)	3.1 (0.3)	2.4 (0.2)	2.9 (0.2)

Proportion of females completing sequence	4.183	0.382	25/30	27/30	29/30	25/30	27/30
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Table 3. Pearson's correlation coefficients between durations of behaviors of the oviposition sequence of *M. aeneus* on oilseed rape ($df = 131$ in each case)

	Walking with ovipositor	Resting	Biting	Ovipositor inside hole	Oviposition
Walking	0.598 ***	0.052	- 0.012	0.149	0.011
Walking with ovipositor	—	0.044	0.021	0.107	- 0.032
Resting	—	—	0.066	0.044	- 0.071
Biting	—	—	—	0.700 ***	0.039
Ovipositor inside hole	—	—	—	—	0.043

No symbol: $P > 0.05$; *** $P < 0.001$

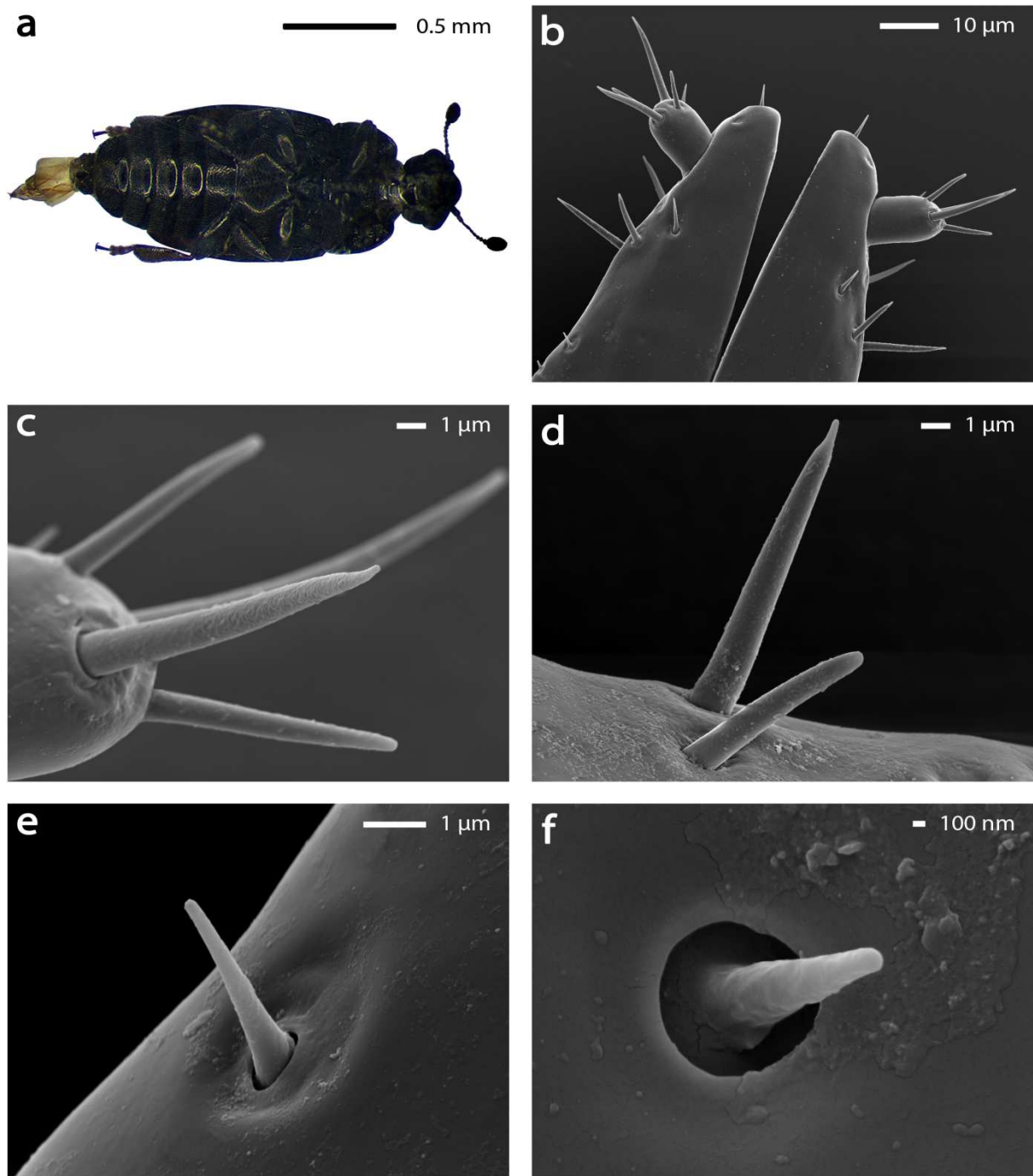


Fig. 1 Stereomicroscope observation: **a** - pollen beetle female (*M. aeneus*) with fully extruded ovipositor (x 4). SEM observation of the ovipositor: **b** - distal end of the ovipositor (gonostyloid) (x 1,400); **c** - Stylus borne by the gonostylus (x 7,000); **d** - trichoid sensilla (x 7,000); **e** - basiconic sensillum (x 15,000); **f** - basiconic sensillum (x 30,000)

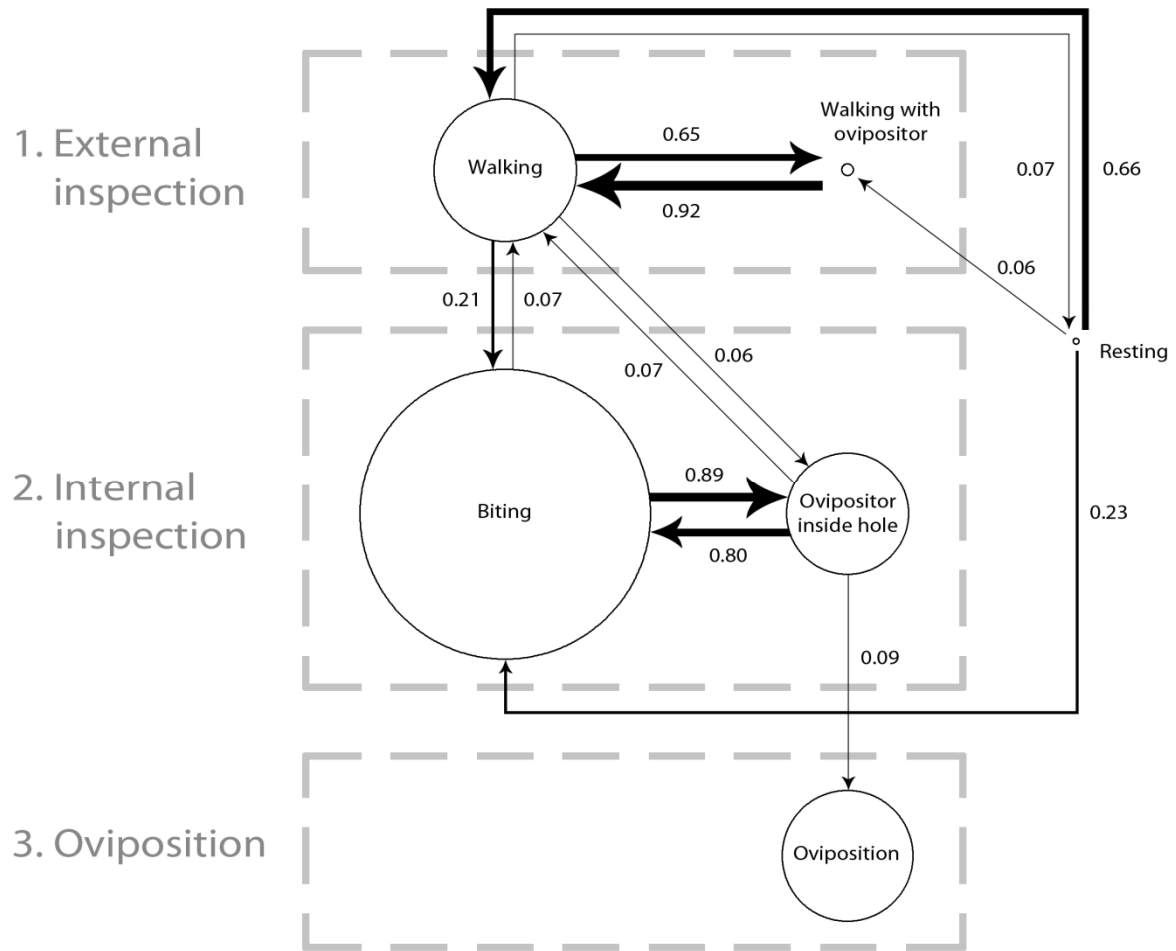


Fig. 2 Transitional diagram of behaviors of the oviposition sequence of the pollen beetle (*M. aeneus*) on oilseed rape (*B. napus*). Size of circles is proportional to the mean duration of the corresponding behavior. Size of arrows is proportional to the corresponding transition rate between the two linked behaviors. Transition rates ≥ 0.05 are represented

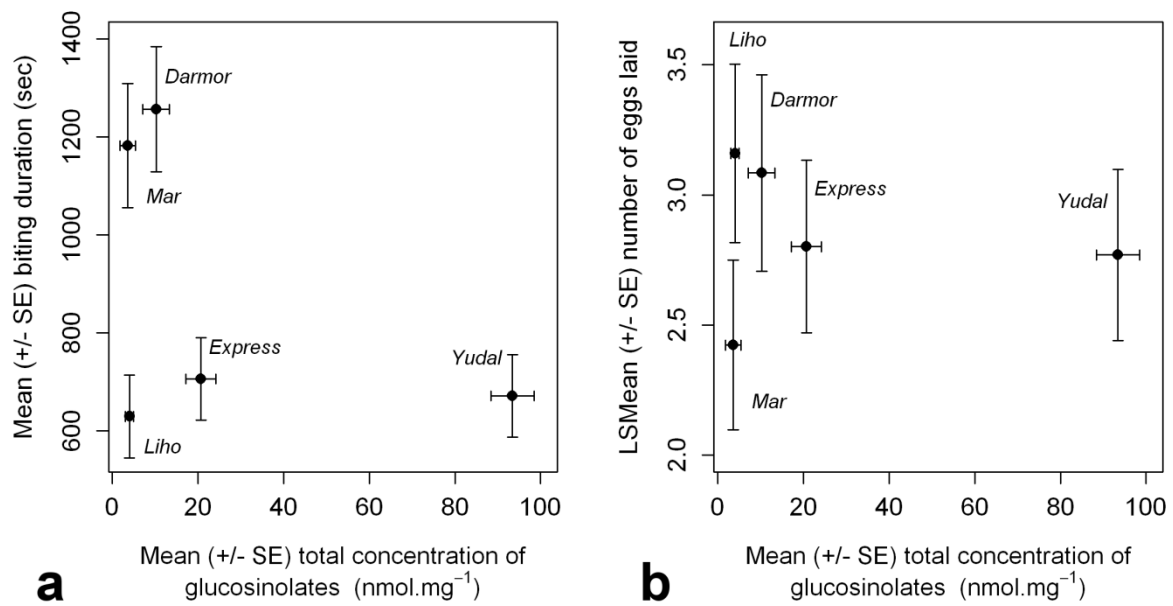


Fig. 3 Relationships between the mean total concentration of glucosinolates in the perianth of five oilseed rape (*B. napus*) genotypes and **a** - the mean biting duration of ovipositing pollen beetle (*M. aeneus*) females ($r^2 = 0.18$); **b** - the mean number of eggs laid ($r^2 = 0.03$).

Horizontal bars: N = 4 for 'Mar', N = 5 for other genotypes; vertical bars: N = 25 for 'Darmor' and 'Mar', 27 for 'Express' and 'Yudal', 29 for 'Liho'. Data on glucosinolate concentration come from Hervé et al. (2014a)