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ORIGINAL ARTICLE

Proctodeal extrusion as a defensive behavioral response in blister beetles (Coleoptera: Meloidae)

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Abstract

Defensive mechanisms in blister beetles (Coleoptera: Meloidae) include a wide variety of behavioral responses, chemical defense, and conspicuous external colorations. Although some of these mechanisms have been previously described, proctodeal extrusion, a defensive behavior involving the extrusion of inner abdominal membranes from the proctodeal region which appear intensely red or orange colored when the hemolymph is seen through them, has not been reported to date. Here, we tested the ability to display proctodeal extrusion in response to threat stimuli in wild populations of three blister beetle species inhabiting Central Spain: *Berberomeloe majalis* (Linnaeus, 1758), *Berberomeloe comunero* Sánchez-Vialas, García-París, Ruiz & Recuero, 2020, and *Physomeloe corallifer* (Germar, 1818). In addition, we observed and recorded various other defensive behaviors such as immobility, antennal threat display, autohemorrhage (reflex bleeding), defecation, and thanatosis (death feigning). The frequency at which proctodeal extrusion was observed differed among species, as did the stress intensity needed for extrusion and the probability of proctodeal extrusion in response to a particular threatening stimulus. Our findings indicate that, although proctodeal extrusion might be a widespread potential defensive mechanism in Meloidae, the ability to elicit it is not generalized across lineages. *Physomeloe* and *Berberomeloe* are endemic to the semi-arid Mediterranean region, and species adapted to such a climate would have developed strategies that limit hydric stress such as proctodeal extrusion, which mirrors the effect of autohemorrhage but without the fluid loss.

Keywords Arid zones · Character evolution · Iberian Peninsula · Mediterranean region · Proctodeal extrusion

Introduction

Blister beetles (Coleoptera: Meloidae) display a diverse array of active defensive behaviors in response to potential threats (Pinto 1975; Bologna and Marangoni 1986; Bologna 1991). Most responses in these beetles involve two main mechanisms: thanatosis (i.e., stop of all voluntary activity and adoption of a posture suggestive of death) and autohemorrhage (i.e., voluntary bleeding of hemolymph through articulating membranes) (Merriam-Webster 2020). Although a wide array of taxa (including vertebrates) (Humphreys and Ruxton 2018) display thanatosis, the autohemorrhage behavior is mostly limited to species whose hemolymph contain toxic or noxious compounds (Evans and Schmidt 1990), as in the case of species of Meloidae. Blister beetles are able to synthesize cantharidin, a potent systemic toxin that can degrade tissues (Bertaux et al. 1988; Muzzi et al. 2020), and serves as a powerful deterrent to invertebrate and vertebrate predators (Carrel and Eisner 1974).

Blister beetles also display passive defensive mechanisms involving conspicuous external colorations that are often considered aposematic (Bravo et al. 2017). In addition, many species of the family Meloidae present a brightly colored hemolymph (blood-red, dark orange, or yellow), which, combined with autohemorrhage and thanatosis, makes a dramatic effect on potential natural visual enemies, including humans (Percino-Daniel et al. 2013; Bravo et al. 2014).

During a series of field surveys conducted with the aim of analyzing the systematic position and conservation status

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of some species of Tenebrionoidea (López-Estrada et al. 2019; Rosas-Ramos et al. 2020), we observed a peculiar behavior in the endemic Iberian species *Physomeloe corallifer* (Germar, 1818). When handled, specimens of this taxon consistently extruded inner abdominal membranes from the proctodeal region (proctodeum: the embryonic posterior ectodermal part of the digestive tract, Merriam-Webster 2020; for a histological description of this region in a blister beetle, see Senarat et al. 2014), which appeared intensely red or orange colored when the hemolymph is seen through them. This behavior, called proctodeal extrusion, has not been previously recorded for Meloidae. We assessed whether other species of Meloidae that share a common general morphology with *Physomeloe* also display proctodeal extrusion in order to (1) compare the defensive responses of *P. corallifer* with those of other unrelated but morphologically similar species of Meloidae, and (2) identify stimuli, and intensities, that trigger proctodeal extrusion in selected Iberian species of Meloidae.

Methods

Experimental design

The ability to display proctodeal extrusion in response to threatening stimuli was tested in a wild population of three different blister beetle species that occur in Central Spain: *Berberomeloe majalis* (Linnaeus, 1758), *Berberomeloe comunero* Sánchez-Vialas, García-París, Ruiz & Recuero, 2020, and *P. corallifer*. Although phylogenetically unrelated (Bologna et al. 2008), species of the genera *Berberomeloe* and *Physomeloe* have a few characteristics in common, including large size (individuals in the study area ranged from 18.0 to 65.0 mm, from the head to the tip of the abdomen), a general body structure (sub-cylindrical body shape, with a very elongated abdomen, reduced elytra, and lack of wings), and a conspicuous coloration consisting of a general black body tegument, marked with bright red lines on the abdominal segments (*Berberomeloe*) or with red blotches on the pronotum (*Physomeloe*) (Fig. 1a, b, c). All three species have a high cantharidin content and thus are considered highly toxic (Bravo et al. 2014, 2017).

The study was conducted over four consecutive days in late spring of 2015 (5–8 May), and adult specimens were searched for daily between 12 and 18 p.m., coinciding with the maximum active period of the evaluated species. We studied a total of 30 specimens of *B. majalis*, 27 of *B. comunero*, and 28 of *P. corallifer*. Most specimens were found actively feeding, wandering around, or courting. Two specimens of *P. corallifer* were observed laying eggs; however, they were discarded from further analyses because oviposition might interfere with proctodeal extrusion.

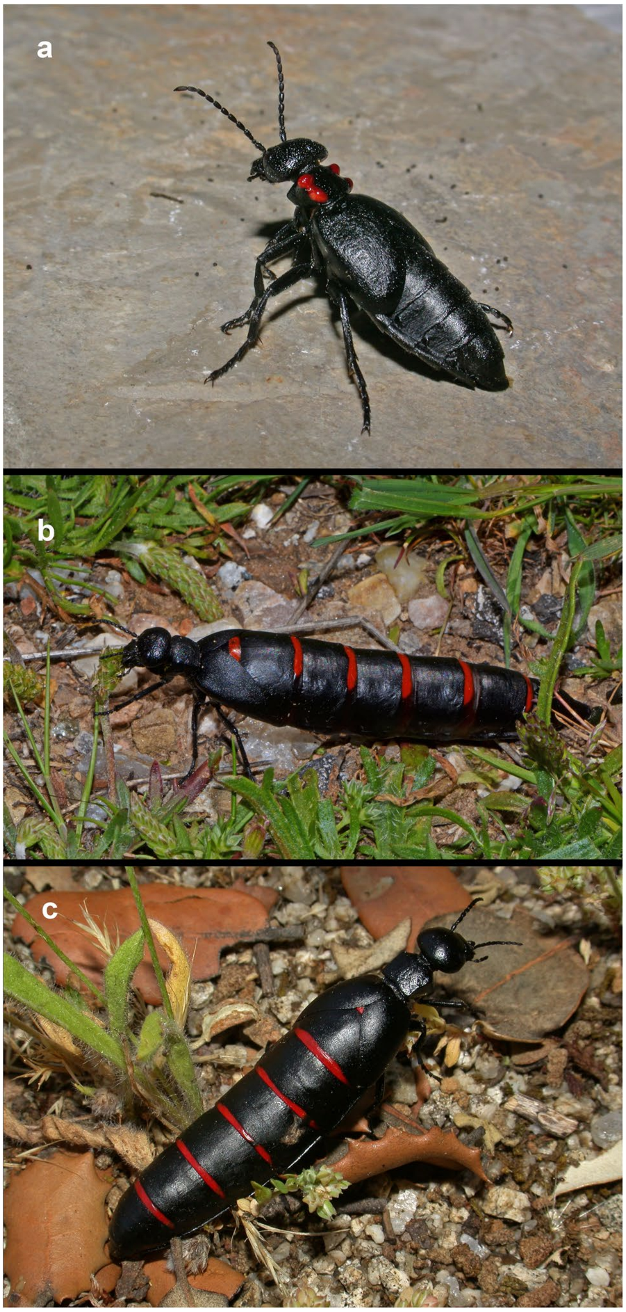

Fig. 1 Live specimens of the species of the family Meloidae analyzed in this study. **a** Specimen of *Physomeloe corallifer* alerted by the photographer (Casavieja, Ávila); note the conspicuous red tubercles on the sides of the pronotum. **b** Undisturbed specimen of *Berberomeloe comunero* feeding (2 km NE of Prádena del Rincón, Madrid). **c** Specimen of *Berberomeloe majalis* in an alert position (El Bonillo, Albacete). Photographs by MG-P

The areas selected for this study were known to host high densities of the studied species of *Physomeloe* and *Berberomeloe*, according to previous studies (García-París et al. 2003, 2006). *Berberomeloe majalis* (Fig. 1c) was observed at a single locality near the municipality of Illana

(Guadalajara). This area was at an elevation of 700 m and was located on the slope of a gypsum hill covered with sub-arid vegetation commonly associated with gypsum substrates. *Berberomeloe comunero* (Fig. 1b) was observed in the Sierra del Rincón (Madrid) at three geographically close localities (2 km NE of Prádena del Rincón, Puerto de la Puebla, and Puerto de la Hiruela). The search areas were mountain pastures near forests dominated by *Quercus pyrenaica* Willd. and *Pinus sylvestris* L, and from 1180 to 1420 m of elevation.

Observations of *P. corallifer* (Fig. 1a) were conducted in the Sierra de Gredos (Ávila) on the slopes of a granitic mountain chain on cropped areas (Niharra) and mountain pastures (Robledillo, Puerto de Menga, Puerto de Navalsauz, Miguel Muñoz, and Puerto del Pico) located between 1095 and 1400 m of elevation. These areas were surrounded by *Cytisus* Desf. scrub formations and forests comprised of *Q. pyrenaica* and *P. sylvestris*.

Experiments were performed in situ in the field, and none of the specimens was collected or moved from the study area. Five threatening stimuli belonging to different stress categories were applied sequentially to each specimen immediately after they were located: (1) "visual contact," consisting of making the presence of the observer evident by standing still less than 50 cm away from the specimen; (2) "one touch," lightly touching the dorsal surface of the beetle with the observer's finger; (3) "repeated touching," lightly touching the dorsal surface repeatedly three times; (4) "handling," grabbing the specimen between the fingers, exerting the minimum pressure necessary to keep the beetle still; and (5) "pressing," exerting additional pressure while handling the beetle. The behavioral responses elicited by applying each threatening stimulus were recorded. At the completion of each experiment, specimens were immediately released and briefly observed to determine if they resumed regular activities. Once released, most specimens immediately ran away; only a few remained still for some seconds before becoming active again.

Data analysis

Hypotheses on the association between species (*B. majalis*, *B. comunero*, and *P. corallifer*) and the proctodeal extrusion response (extrusion vs. no extrusion) were evaluated on contingency tables by using a chi-squared test with Monte Carlo estimation (5000 simulations). This test was also used to analyze the association between species and stress intensity, i.e., the number of threatening stimuli needed to elicit an extrusion response (from 1 to 5 stimuli). Also, adjusted residuals of contingency tables were used to explore, in greater detail, the patterns of extrusion and stress intensity among species. For both analyses, critical values of significance were corrected to avoid the problem of simultaneous testing when assessing the significance of the adjusted residuals (see Legendre and Legendre 2012).

Logistic regressions were fitted separately for each stimulus or stress factor type (visual contact, one touch, repeated touching, handling, or pressing) to analyze the effect of species (defined as the explanatory variable) on the presence/absence of the extrusion response (i.e., dependent variable). Bias reduction was required because some species never or always showed extrusion against a particular stimulus, such that the same value was recorded for all specimens in those categories. For this purpose, we used binomial bias-reduced generalized linear models (see Faraway 2016). Goodness-of-fit was examined by calculating the area under the receiver operating characteristic (ROC) curve (AUC) (see Hosmer and Lemeshow 2000; Faraway 2016).

Contingency table analyses were carried out using XlStat 2016 (Addinsoft 2016). Binomial bias-reduced generalized linear models were performed in R 3.6.0 (R Development Core Team 2016), and fitted using the "brglm" function in the "brglm" package (Kosmidis 2013).

Two independent sets of phylogenetic hypotheses were used to identify the relationships between the studied taxa. A first set, from Bologna et al. (2008) and Sánchez-Vialas et al. (2021), which included a wide range of genera within the subfamily Meloinae, was used to identify the relationship between *Physomeloe* and *Berberomeloe*. A second set, from Sánchez-Vialas et al. (2020), which included all species of *Berberomeloe*, was used to identify the relationship between *B. comunero* and *B. majalis*. These phylogenetic trees were used to discuss character evolution and propose possible evolutionary scenarios for proctodeal extrusion.

Results

Proctodeal extrusion was observed in specimens of all three species of blister beetles studied (Fig. 2a, b). However, differences in both the frequency and probability of the response against a threatening stimulus were evident. During the experiment, we also observed a series of other defensive responses (Online Resource 1) that included immobility, active escape, antennal threat display (Fig. 2c), autohemorrhage (Fig. 2d), defecation, and thanatosis (Fig. 2d). Specimens displayed these responses sequentially or, often, simultaneously. Proctodeal extrusion, when it occurred, started before either the autohemorrhage or defecation behavior, and remained active even after these behaviors began.

Significant differences in the total frequencies of proctodeal extrusion were observed among species in the contingency analysis (Monte Carlo method with 5000 simulations: $\chi 2 = 46.875$, d.f. $= 2$, $p = < 0.0001$). The observed frequency of proctodeal extrusion in *B. majalis* was significantly lower than expected, whereas in *P. corallifer*, it was higher than

Fig. 2 Defensive behavioral responses in *Berberomeloe* and *Physomeloe*. **a** Proctodeal extrusion in *Physomeloe corallifer*; note the rich red-colored inner abdominal membranes from the proctodeal region mimicking a hemolymph droplet (Villaviciosa de Odón, Madrid). **b** Proctodeal extrusion in *Berberomeloe comunero*, which occurs less frequently compared with extrusion in *P. corallifer*. **c** Antennal threat display in *Berberomeloe comunero*, in ventral view (2 km NE of Prádena del Rincón, Madrid). **d** Thanatosis and autohemorrhage in *Berberomeloe majalis* (9 km N Osuna, Sevilla); note the deep red hemolymph droplet, the translucent gular area, and the antennal threat display. Photographs by MG-P

expected. Observed and expected values in *B. comunero* did not differ (Table 1, Fig. 3).

Our analyses also revealed that species differed in the stress intensity needed for extrusion (Monte Carlo Method with 5000 simulations): $\chi 2 = 53.546$, d.f. = 2, $p = < 0.0001$). In this sense, *P. corallifer* tended to extrude with a lower number of threatening stimuli: the observed frequency of proctodeal extrusion was significantly higher than expected after two stimuli were applied (Table 1, Fig. 4). By contrast, *B. comunero* exhibited a significantly higher extrusion frequency after three or four stimuli; in the case of *B. majalis*, extrusion frequency was higher with five stimuli (Table 1, Fig. 4).

The GLMs revealed that the probability of proctodeal extrusion in response to a particular threatening stimulus or stress factor differed significantly among species. Although

Table 1 Frequency of proctodeal extrusion in *Berberomeloe majalis* (30 specimens), *Berberomeloe comunero* (27), and *Physomeloe corallifer* (28). Data on the observed and expected frequencies from the contingency table analysis (chi-squared test with Monte Carlo estimation)

	Berberomeloe majalis		*Berberomeloe comunero*		*Physomeloe corallifer*	
	Observed frequency	Expected frequency	Observed frequency	Expected frequency	Observed frequency	Expected frequency
Extrusion	5	19.059	21	17.153	28	17.788
No extrusion	25	10.941	6	9.847	0	10.212
1 stimulus	0	0.556	0	2.333	6	3.111
2 stimuli	0	2.407	4	10.111	22	13.481
3 stimuli	0	0.556	6	2.333	0	3.111
4 stimuli	4	1.389	11	5.833	0	7.778
5 stimuli	1	0.093	0	0.389	0	0.519

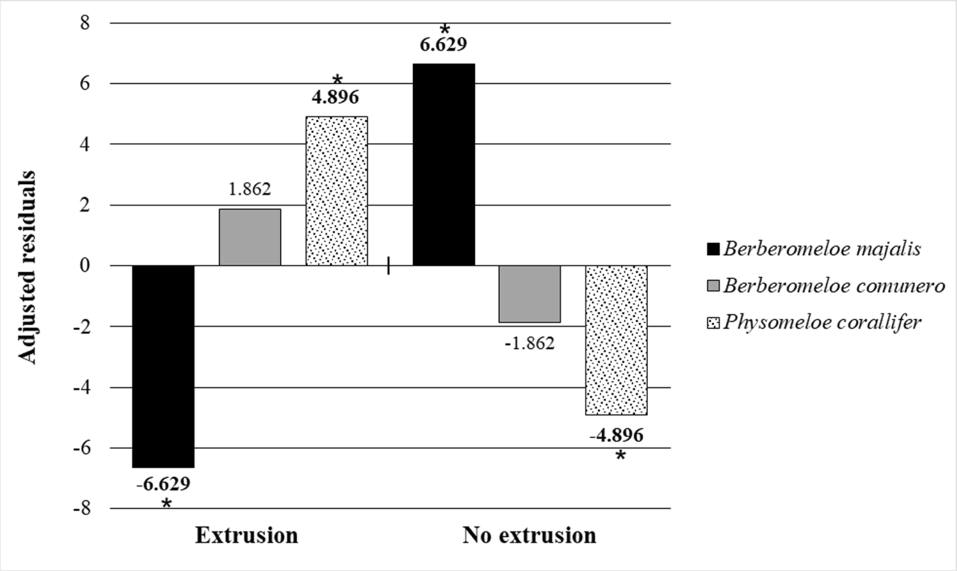

Fig. 3 Adjusted residuals for the analysis of proctodeal extrusion frequencies in the three Meloidae species analyzed: *Berberomeloe majalis* (30 specimens), *Berberomeloe comunero* (27), and *Physomeloe corallifer* (28). Columns marked with an asterisk (*) reflect significant values at $p < 0.05$. Black columns: *B. majalis*; gray columns: *B. comunero*; dotted columns: *P. corallifer*

marginally significant, *P. corallifer* exhibited a low probability of extrusion against visual contact; in both *B. comunero* and *B. majalis*, this probability was almost zero (Table 2, Fig. 5). After one touch, the extrusion rate was very high in *P. corallifer*, low in *B. comunero*, and almost zero in *B. majalis* (Table 2, Fig. 5). Similarly, the probability of extrusion after repeated touching was very high in *P. corallifer*, and still almost zero in *B. majalis*. In *B. comunero*, the extrusion probability was higher than that obtained for the one touch stimulus (Table 2, Fig. 5). After the handling stimulus, the probability of extrusion remained very high in *P. corallifer*, but increased in both *B. comunero* and *B. majalis* (Table 2, Fig. 5). The probability of the response against pressing was almost the same as that for handling in both *P. corallifer* and *B. comunero*, and only slightly higher than that after handling in *B. majalis* (Table 2, Fig. 5).

The Bayesian phylogenies showed that *Physomeloe* and *Berberomeloe* are unrelated taxa that have not shared a recent common ancestor since the Oligocene. *Physomeloe* is nested within the tribe Meloini (Sánchez-Vialas et al. 2021), while *Berberomeloe* is sister to a large clade consisting of species from tribes other than Meloini. Sánchez-Vialas et al. (2020) demonstrated that *B. comunero* and *B. majalis* are sister taxa.

Discussion

The repertoire of defensive behavioral responses observed in the studied species of *Physomeloe* and *Berberomeloe* included immobility, active escape, antennal threat display, proctodeal extrusion, autohemorrhage, defecation, and

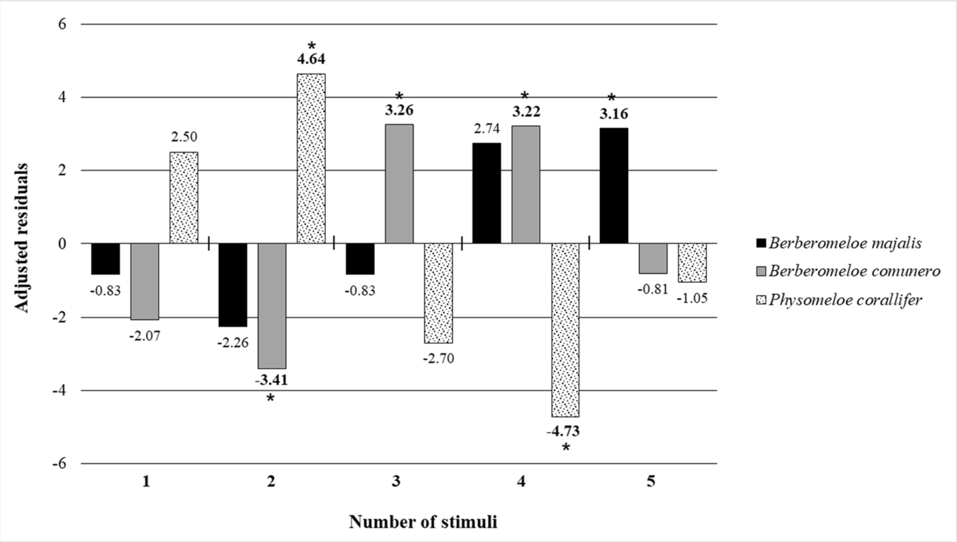

Fig. 4 Adjusted residuals for the analysis of proctodeal extrusion frequencies in *Berberomeloe majalis* (30 specimens), *Berberomeloe comunero* (27), and *Physomeloe corallifer* (28) after a different number of threatening stimuli (from 1 to 5). Columns marked with an asterisk (*) reflect significant values at $p < 0.05$. Black columns: *B. majalis*; gray columns: *B. comunero*; dotted columns: *P. corallifer*

Table 2 Proctodeal extrusion response in the analyzed species of blister beetles: *Berberomeloe majalis* (30 specimens), *Berberomeloe comunero* (27), and *Physomeloe corallifer* (28). Parameter estimates for the generalized linear models (GLM) assessing the effect of species (*B. comunero*, *B. majalis*, and *P. corallifer*) on extrusion response following the application of different types of threatening stimuli (visual contact, one touch, repeated touching, handling, and pressing). Reference coefficient is species (*B. comunero*) (<0.1; *$p<0.05$; **$p<0.01$; ***$p<0.001$)

Stimulus	Factor	Estimate	Std. Error	z value	p value	
Visual contact	Intercept	−4.007	1.453	−2.758	0.00582	**
	Species (*Berberomeloe majalis*)	−0.104	2.052	−0.05	0.95976	
	Species (*Physomeloe corallifer*)	2.766	1.522	1.817	0.06924	
One touch	Intercept	−1.653	0.524	−3.154	0.001608	**
	Species (*Berberomeloe majalis*)	−2.458	1.541	−1.595	0.110738	
	Species (*Physomeloe corallifer*)	5.696	1.544	3.690	0.000224	***
Repeated touching	Intercept	−0.511	0.398	−1.285	0.19878	
	Species (*Berberomeloe majalis*)	−3.600	1.503	−2.395	0.0166	*
	Species (*Physomeloe corallifer*)	4.554	1.505	3.025	0.00248	**
Handling	Intercept	1.006	0.435	2.314	0.0207	*
	Species (*Berberomeloe majalis*)	−2.779	0.676	−4.108	3.99e−05	***
	Species (*Physomeloe corallifer*)	3.038	1.516	2.004	0.045	*
Pressing	Intercept	1.006	0.435	2.314	0.0207	*
	Species (*Berberomeloe majalis*)	−2.540	0.646	−3.931	8.45e−05	***
	Species (*Physomeloe corallifer*)	3.038	1.516	2.004	0.045	*

thanatosis. Proctodeal extrusion occurred consistently in response to most of the stimuli in *P. corallifer*; however, intermediate and harsh stimuli were required to elicit this behavior, respectively, in *B. comunero* and *B. majalis*.

The defensive responses shown by these species of *Physomeloe* and *Berberomeloe*, with the exception of proctodeal extrusion and antennal threat display, have been reported in other lineages of Meloidae (Bologna 1991; Muzzi et al.

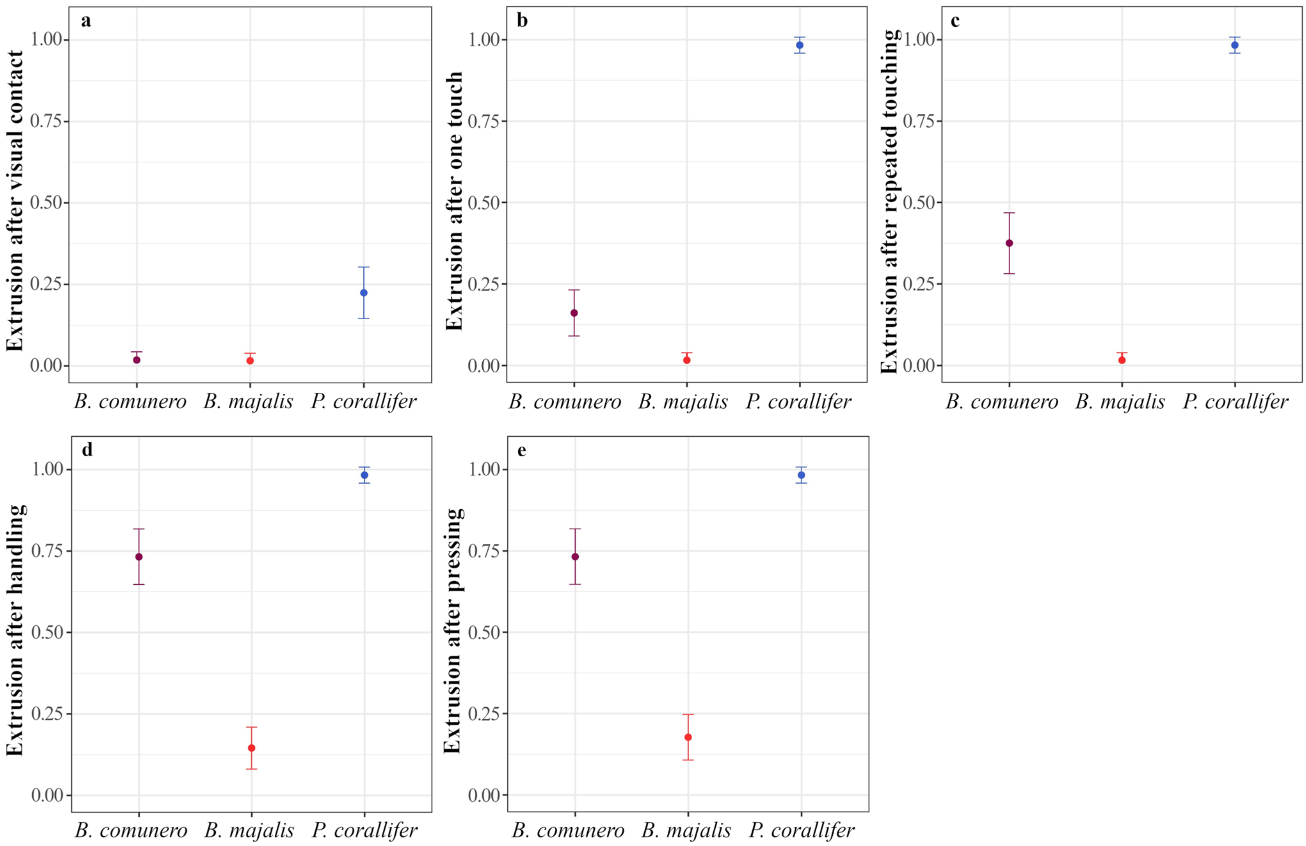

Fig. 5 Probability of proctodeal extrusion (estimated mean ± SE) in response to **a** visual contact, **b** one touch, **c** repeated touching, **d** handling, and **e** pressing in the three species analyzed: *Berberomeloe majalis* (30 specimens), *Berberomeloe comunero* (27), and *Physomeloe corallifer* (28) (purple dots: *B. comunero*; orange dots: *B. majalis*; blue dots: *P. corallifer*)

2020). The antennal threat display consists of directing the antennae straight and forward with the apices curved inwards, simulating long mandibles (see Fig. 2c). This behavioral response, plus immobility, active escape, proctodeal extrusion, autohemorrhage, and defecation, occurs in the three species studied here, and in other species of the genus *Berberomeloe* (e.g., *B. insignis* (Charpentier, 1818) and *B. payoyo* Sánchez-Vialas, García-París, Ruiz & Recuero, 2020) (Fig. 6a, b).

Adult specimens of different families of Coleoptera are known to evert glands or vesicles as part of their defensive strategy. Often the defensive value of everting glands relies on particular odors and visual cues, for example, in the case of the white odorant glands everted by *Ocypus olens* (Müller, 1764) and other species of Staphylinidae (Huth and Dettner 1990), or the lateral orange to red vesicles displayed by species of Malachiinae (Mirutenko 2013). Some adult Tenebrionidae can extrude the last abdominal segments while expelling a caustic fluid (Whitman et al. 1990). Larvae of some species of Chrysomelidae can telescopically extrude the normally invaginated ninth and tenth abdominal segments in order to deposit fecal material on their dorsal shields (Vencl et al. 1999). The use of proctodeal extrusion in Meloidae as a defense mechanism is likely not related to any of the aforementioned behaviors, but rather to responses related to autohemorrhage during thanatosis (Rogers and Simpson 2014; Humphreys and Ruxton 2018). The increase in internal pressure needed to expel hemolymph through articulations (Hollande 1911; Boldyrev 1928; Bateman and Fleming 2009) may also facilitate the extrusion of the internal abdominal membranes. Also, these membranes are transparent but appear to be colored due to the blood-red-colored hemolymph that can be seen through the membranes of these highly toxic species (Bravo et al. 2017).

Fig. 6 Coloration pattern and antennal threat display in other species of *Berberomeloe*. **a** Specimen of *B. insignis* (Tabernas, Almería) in thanatosis. Also shown are the typical conspicuous red markings on the head, red hemolymph droplets, proctodeal extrusion, and an extreme antennal threat display. **b** Specimen of *B. payoyo* (Malcocinado, Cádiz) in thanatosis. Also shown are the short orange stripes on the abdomen, orange hemolymph droplets, antennal threat display, and defecation. Photographs by MG-P

Berberomeloe and *Physomeloe* are phylogenetically unrelated taxa (Bologna et al. 2008; Sánchez-Vialas et al. 2021). Given the phylogenetic position of these genera, proctodeal extrusion may represent a homoplastic response elicited only in *Physomeloe* and *Berberomeloe*, or alternatively, a generalized trait that has been overlooked in all other genera of the subfamily. Our data indicate that proctodeal extrusion might not be as general of a response compared with the others, since its frequency differed markedly between the sister species *B. comunero* and *B. majalis*. Taken altogether, our observations imply that, although proctodeal extrusion might be a widespread potential defensive mechanism in Meloidae, the ability to elicit it is not generalized across lineages.

The most generalized defensive responses in Meloidae appear to be autohemorrhage and thanatosis (see Fig. 2d and Fig. 6a, b). During autohemorrhage, blister beetles release a large amount of colored hemolymph (Percino-Daniel et al. 2013), which is usually replaced after feeding (Bravo et al. 2017). Most species of Meloidae inhabit arid or semi-arid steppes, where the loss of hemolymph might pose a certain physiological risk (Nicolson 1994). All species of *Physomeloe* and *Berberomeloe* are endemic to the westernmost steppes of the generally semi-arid Mediterranean region, and they have been present in the area since, at least, the late Miocene, which is also when the lineage of *Berberomeloe* started its speciation processes (Sánchez-Vialas et al. 2020). Speciation events took place during the Messinian, one of the most arid periods in the history of the Mediterranean basin (Krijgsman et al. 1999; Bache et al. 2012). Under these conditions, species adapted to such a climate would likely develop strategies directed towards limiting hemolymph loss and, in turn, exposure to hydric stress.

Proctodeal extrusion mirrors the effect of hemolymph droplets but without its loss (see Fig. 2a). The use of morphological adaptations to simulate hemolymph droplets, thus avoiding its loss during a defensive behavior (Nicolson 1994), might be further supported by the particular morphology of *P. corallifer*. In this species, the usual transverse to subquadrate shape of the pronotum of the species of the tribe Meloini is replaced by a pronotum decorated with two pairs of red-colored lateral tubercles that permanently mimic hemolymph droplets (see Fig. 1a). Our data indicate that *P. corallifer* triggers the proctodeal extrusion response with minimal stimuli, possibly to avoid the use of autohemorrhage until faced with harsh stimuli. The presence of modified morphological structures, such as those shown by *P. corallifer*, is unique to the genus *Physomeloe*; however, red-colored blotches are occasionally present on the head and thorax of other species of Meloidae (such as *B. insignis*; see Fig. 6a) (García-París 1998; García-París et al. 2013). In this sense, it would be interesting to study the defense mechanisms of the only species of the tribe Meloini that has a pronotum marked with red, besides *P. corallifer*, namely *Meloe vlasovi* Semenov-Tian Shansky and Arnoldi, 1937, from central Asia (Bologna and Pinto 2002). The data presented here, in combination with phylogenetic analyses, represent a starting point with which to study the evolution of complex behavioral responses, which are often linked to particular morphological structures in clades with highly toxic species such as those belonging to *Berberomeloe* and *Physomeloe*.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00114-021-01728-y.

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Data availability Data are available as electronic supplementary material (Online Resource 1).

Code availability R code is available as electronic supplementary material (Online Resource 2).

Declarations

Ethics approval This study was conducted in accordance with applicable international, national, and/or institutional animal care guidelines.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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