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Learning from the environment: how predation changes the behavior of terrestrial Isopoda (Crustacea Oniscidea)

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Terrestrial isopods have adapted to predatory pressures by evolving a variety of behaviors, which arise from a combination of specific traits, such as volvation and tonic immobility. Evolutionarily, these behavioral adaptations have been shown to increase the fitness of the individuals of the species who show them because the probability of being predated is reduced due to the loss of interest by the predator towards the immobile prey and the increase of interest towards the other mobile ones. Even if some of these behaviors have been shown to have a genetic basis, there is limited knowledge about the effects of environmental influences and predator-induced learning abilities on the antipredatory strategies of invertebrates, and isopods in particular. Our study aimed to understand the degree to which "nature" and "nurture" (i.e. environmental and genetic factors) and their interactions influence these antipredatory behaviors. There might be a difference in the behavior of wild and captive isopods in their volvation frequency and duration of tonic immobility due to environmental factors (i.e. predation) that induce learning-related behavioral changes. Therefore, we tested this hypothesis. We applied the three types of stimuli, which aim to simulate the interaction of the predator with the isopod. All three species showed a significant difference, between individuals collected in the field (wild) and raised in the laboratory (captive), in the reaction to the stimulus that simulates the fall from a bird's beak or from the jaws of a lizard after a catch. Although volvation frequency was highly species- and stimulus-specific, the duration of tonic immobility and the delay in the response to each stimulus, when significant, was always higher in wild groups than captive ones. These substantial differences may reflect the evolutionary and ecological characteristics of each species and the importance of environmental pressures to shape the behavior of these invertebrates to optimize their life strategies.

KEY WORDS: Isopoda, stimuli, behavior, predation, captivity, nature, nurture.

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INTRODUCTION

Anti-predatory behavior has evolved by many prey species through evolution to face constant attacks by predators (Cooper & Blumstein 2015). Among animals, anti-predatory adaptations have evolved with different strategies, e.g. by avoiding attacks or detection, escaping after a caught, fighting against the predator, warding off the attack, etc. (Cooper & Blumstein 2015).

Terrestrial isopods (Crustacea Isopoda Oniscidea) are the preferred preys of a multitude of different predators such as arachnids (Řezáč & Pekár 2007), chilopods (Sunderland & Sutton 1980), opilionids (Santos & Gnaspini 2002), Hymenoptera (Dejean 1997), flatworms (Prasniski & Leal-Zanchet 2009), birds, amphibians and reptiles (Vitt et al. 2000; Van Sluys et al. 2001). Isopod's predators possess a wide variety of behaviors in the search and capture of their preys. Therefore, this group of invertebrates has adapted to these evolutionary pressures by developing a variety of antipredatory behaviors (Gorvett 1956; Deslippe et al. 1996).

According to the classification of Schmalfuss (1984), the species belonging to Isopoda can be divided into the following six eco-morphological categories on the basis of ethological and morphological adaptations: runners, clingers, rollers, spiny forms, creepers, and non-conformists. However, there are intermediate forms between each category and, for some species, this classification is often arbitrary.

Runners, clingers, rollers and spiny forms, show behavioral and morphological adaptations that reduce predatory attacks for their survival (Schmalfuss 1986). In the case of runners, clingers, rollers, their behavior is an adaptation to environmental pressures (mostly, predation) in their habitats (Schmalfuss 1977). Differently, spiny isopods show numerous spiny protuberances located dorsally in the tergites and protect themselves mainly through a morphological adaptation from their common predators such as birds, frogs, and lizards (Schmalfuss 1984).

Nevertheless, behavioral adaptations are more complex and still not fully investigated in terrestrial isopods. A very few studies have attempted to better understand when and how these behavioral adaptations take place and are shown by these species (Sokolowicz et al. 2008; Quadros et al. 2012; Tuf et al. 2016).

Contrary to runners, which run immediately and very quickly to hide in small ravines or micro-caves, threatened clingers remain motionless and stick firmly to a solid substrate for the duration of the attack. In this case, a bird or a lizard's jaw find very difficult to detach them. The species *Armadillidium granulatum* (Brandt 1833) is a typical example of clingers (Vandel 1960; Schmalfuss 1984).

Rollers, instead, have smooth tergites and, in a cross-section, a semi-circular shape, becoming circular once they fold back on themselves (Vandel 1962). The meaning of the volvation in these species is a unique strategy of defense against predators shown by a few invertebrates (e.g Isopoda and Chrysididae) and only seen in mammals such as armadillos, pangolins, and hedgehogs (Tuf et al. 2016) and in just a reptile (*Cordylus cataphractus*). Several Isopoda species of the Armadillidae, Armadillidiidae, Eubelidae and Tyliidae families belong to the rollers category (Schmalfuss 1984).

Moreover, once some isopods are in close contact with a predator, they can implement a kind of secondary anti-predatory strategy known as tonic immobility (Langerhans 2007; Miyatake et al. 2009; Humphreys & Ruxton 2018). This is a state of reversible physical immobility caused by muscular hypertension, during which the organism lacks responsiveness to external stimuli (Gallup 1974). Thanatosis is widespread as a passive antipredatory behavior used by a multitude of animals, including mammals such as opossums and

wild goats (Moore & Amstey 1962; Barratt 1965), reptiles (Santos et al. 2010), some chondrichthyes such as sharks, many arthropods such as Orthoptera (Nishino & Sakai 1996; Faisal & Matheson 2001; Honma et al. 2006), opilionids (Machado & Pomini 2008), Coleoptera (Miyatake et al. 2004), Hymenoptera (King & Leach 2006) and crustaceans (Holmes 1903; Saxena 1957; Bergey & Weis 2006; Scarton et al. 2009). This state is also called “apparent death” (Honma et al. 2006). Many experiments conducted on thanatosis mainly focused on the phylum of arthropods (Prohammer & Wade 1981; Honma et al. 2006; Miyatake et al. 2009; Nakayama & Miyatake 2010), where this behavior has been repeatedly tested and described as a defensive functional response and with an adaptive meaning in antipredatory. In this way, the probability of being predated is reduced due to the loss of interest by the predator towards the immobile prey (Miyatake et al. 2004) and the increase of interest towards the other mobile preys (Miyatake et al. 2009). A common feature that emerges from these studies is that there is a wide interspecific behavioral variability in the execution of the same thanatosis and this is reflected both in the degree of responsiveness and in the duration of the immobility status (Quadros et al. 2012).

Although some of these behaviors have been shown to have a genetic basis (Prohammer & Wade 1981; Miyatake et al. 2004), there is a limited knowledge about the effects of environmental influences and predator-induced learning abilities on the antipredatory strategies of invertebrates (Turner et al. 2006), and isopods in particular (Tuf et al. 2015).

Our study aimed to understand the influence of “nature and nurture” (i.e. environmental and genetic factors) and their possible interaction in these antipredatory behaviors (namely, response delay, volvation frequency, and duration of tonic-immobility). Here we test the hypothesis that environmental factors (in our experiment: a simulation of predation stimuli) reduce volvation frequency and duration of tonic immobility through learning-related (habituation) behavioral changes of wild individuals compared to those of captive animals. Therefore, our null hypothesis is that these behaviors have a genetic basis and there is no difference between wild and captive individuals.

MATERIALS AND METHODS

Target species and sampling

The species used for this study are *A. granulatum* (belonging to the clinger category), *Armadillidium vulgare* Latreille 1804, and *Armadillo officinalis* Duméril 1816 (both belonging to the rollers category). These species, whose some ecological aspects were studied (Messina et al. 2016b), are widespread in Sicily (Caruso et al. 1987). Our experiments involved two groups of individuals: those collected in the field (wild) and those grown in laboratory conditions (captive).

The sampling of wild individuals was performed on during random-walk, sight-based collection. The specimens of *A. officinalis* and *A. vulgare* were collected at the Monte Serra Park in Viagrande (Catania, Sicily, Italy) (37°37'10.1"N; 15°05'37.3"E); the specimens of *A. granulatum* were collected in a closed area at the “Pantanello” beach in Avola (Siracusa, Sicily, Italy) (36°54'23.3"N; 15°08'56.1"E). In all locations, predators of the target species, such as lizards (*Podarcis* spp.), ants and spiders, and insectivorous birds were common and exert a documented pressure on the wild isopods selected (Caruso et al. 1987).

The collected individuals of the wild group were kept in containers containing soil, litter and dry leaves. In the laboratory, they were positioned in a humid and well-ventilated place, not in direct contact with sunlight and left undisturbed in the 24 hr before the experiments, thus allowing them to have time to recover from the stress before being tested, in order to reduce any alterations of the results.

The individuals of the captive group were raised in the labs of the Biological, Geological and Environmental Sciences Department of the University of Catania, Sicily, Italy. No simulations of predator attacks or manipulations were applied to the captive individual tested since they hatched in the laboratory, to ensure that they had not experienced any threat from the environment before the experiment. To control for other factors that would influence the behavior of captive individuals, the captivity conditions (e.g. diet, temperature, etc.) have been set close to those these species usually experience in the wild conditions.

Eighty individuals per species were chosen randomly, 40 from the wild and 40 from the captive groups. We had 25 pregnant females out of 80 individuals in *A. vulgare* and 12 pregnant females out of 80 individuals in *A. officinalis*. No pregnant female was observed in the species *A. granulatum*. Each individual was isolated in a Petri dish with a 9-cm diameter. To each individual, three types of stimuli were applied by means of entomological forceps and the reaction time was recorded using a stopwatch. After each stimulation, we noted for each individual: its length (from the first cephalic segment to the last caudal segment), sex, and the possible presence of the marsupium in females (pregnant females) with a Leitz Wetzlar stereomicroscope equipped with a micrometric lens. A time interval of about 1 min was set between a stimulus and the next, so as to do not apply two successive stimuli in close temporal relation.

Types of stimuli

The three types of stimuli, which aimed to simulate the interaction of the predator with the isopods, were: (i) drop, (ii) squeeze, and (iii) touch.

The “drop” stimulus consisted in grasping the isopod with the entomological forceps and lifting it up to 10 cm in order to let it fall on the Petri dish, so as to simulate the fall from a bird’s beak or from a lizard’s jaws. This stimulus was applied three times before describing the individual as non-responsive (i.e. continuing with its usual motility and activity after the stimulus).

The “squeeze” stimulus consisted in applying light pressure with the tips of the entomological forceps on the pleon of the isopod, so as to simulate a bite, for instance by a lizard, or the capture by a predator, like a bird. This stimulus was applied 3 times before describing the individual as non-responsive.

The “touch” stimulus consisted in gently touching and moving the isopod with the tip of the entomological tweezers. This stimulus was applied to simulate the common behavior of some isopods that, while inspecting the litter, respond to either an accidental and unexpected touch of an element in the environment (biotic or abiotic) or a harmless touch of a predator with the voluntary or tonic immobility. Due to the higher frequency of this stimulus in nature, this stimulus was applied 5 times before describing the individual as non-responsive.

Data analysis

Because wild individuals may not only learn (i.e. “nurture” effect) from previous potential encounters with predator but may, also, be selected by predators, which could explain part of their genetic differences, we also tested whether our wild sample contained more “slow and dummy” individuals – more likely predated in nature – than those in the captive group, in which higher variability (from “dummy” to “clever”) in absence of natural selection might be present. Even if this is a possibility, it is very unlikely that all “dummy” isopods have been removed from the environment by predators. By plotting the immobility time vs their frequency distribution in a smoothed kernel density plot (package *sm* in R), we found no evidence that volvation and tonic immobility are related to the “smartness” or “ability” of each individual to perform them. In fact, the immobility time to stimuli for the wild vs the captive groups and the value distribution was almost identical (Supplementary Fig. S1). This showed that, in both groups, there is a good representation of both “dummy” and “clever” individuals.

In our analysis, we considered three species (*A. vulgare*, *A. officinalis*, *A. granulatum*) and three factors the status (wild, captivity) as the treatment, the sex (male, female, pregnant female), and the size. We applied three types of stimuli (drop, squeeze, touch) and studied three related responses: (i) duration of tonic immobility (measured in seconds), (ii) response delay (number of stimuli before a reaction; 0–3 for drop and squeeze; 0–5 for touch), (iii) volvation (frequency). We analyzed the results with intraspecific and interspecific comparisons with both non-parametric (because we did not detect their normal distribution after performing a Q-Q norm and a Shapiro-Wilk test) and factorial tests.

For the intraspecific analysis, we used a three-factor factorial analysis to evaluate the differences in the reaction to each stimulus due to status, sex, size and sex-size as co-variables (see below) for each species. We also evaluated the significance of the interactions among these three factors.

For this purpose, we built a three-factor ANCOVA model such as $y \sim a + b * c$, where y is the response (i.e. tonic immobility, response delay, and volvation), a is the factor “Status”, b is the factor “Sex” that may co-variate with c , the factor “Size”. The significance of the three factors for the response was alongside tested in the following combinations: status:sex, status:size, and status:sex*size.

We considered the variable of size as a potential covariate of sex because, in a preliminary analysis, the linear regression between an individual's size and the reaction to the stimuli was weak in all cases.

Then, for an easier visual interpretation of the results, we represented with boxplots the differences in the median and data distribution in percentiles between the wild and captive individuals of each species for the tonic immobility, the delay in the response and the volvation (testing their pairwise significance, at $\alpha = 0.05$ and $\alpha = 0.01$, with a non-parametric Mann-Whitney U-test). We also created a conditional density plot describing how the conditional distribution of our categorical factor – i.e. sex – changes over a numerical factor – i.e. response delay.

For the interspecific analysis, we run a non-parametric version of the analysis of variance (ANOVA), the Kruskal-Wallis rank sum test, to compare the differences among wild and captive individuals of the three species in: (i) the length of tonic immobility, and (ii) the delay in response.

All the statistical analyses were performed in R Studio (version 3.5.1, R Development Core Team 2018).

RESULTS

Intraspecific analysis of the reactions to the stimuli

The significance and the importance of the differences among wild and captive individuals per species to each stimulus, and the slope of the regression line (β_0) between the duration of tonic immobility and the size of animals are reported in Table 1.

We analyzed the differences between wild and captive individuals separately per each stimulus and species with a factorial analytical design (i.e. with all combinations and interactions of the factor effects):

Stimulus 1 (drop)

Armadillidium vulgare. There is no significant difference ($U = 826$, $P = 0.8$) between wild and captive individuals of this species in the length of tonic immobility when tested for this stimulus (Fig. 1A, Table 1). Captive individuals respond to this stimulus significantly later than wild ones ($U = 985$, $P = 0.01$) and exhibit a significantly higher volvation frequency (90 vs 65%, $U = 600$, $P < 0.01$). Moreover, pregnant females of wild individuals show a higher delay in the response, whereas in captivity have a faster response to this stimulus (Fig. 2a, Table 1). Status (treatment) and size alone, and the interaction between size and sex are

Table 1.

The length of tonic immobility, the delay in the response and the volvation frequency (as mean and SE values), and the slope (β_0) and R^2 of the linear regression between the size and the length of the tonic immobility is to the three different stimuli for the three target species (both wild and captive individuals). The statistical significance is reported when the P -value is $\leq \alpha$, with $\alpha = 0.01$ and 0.05 .

	Tonic immobility		Response delay		Volvation frequency	
	Mean	SE	Mean	SE	Mean	SE
Stimulus 1 (drop)						
<i>Armadillidium vulgare</i>			$P = 0.01$		$P < 0.01$	
Wild	32.49	8.51	1.20	0.10	0.65	0.08
Captive	37.62	9.01	0.93	0.04	0.90	0.05
<i>Armadillo officinalis</i>			$P < 0.05$		$P < 0.01$	
Wild	32.42	5.20	1.03	0.08	0.88	0.05
Captive	2.89	0.53	0.95	0.15	0.48	0.08
<i>Armadillidium granulatum</i>			$P < 0.01$		$P < 0.01$	
Wild	17.61	2.60	0.98	0.10	0.70	0.07
Captive	9.85	2.83	0.95	0.10	0.30	0.07
Stimulus 2 (squeeze)						
<i>Armadillidium vulgare</i>						
Wild	27.66	7.87	1.15	0.19	0.33	0.08
Captive	28.95	7.84	0.90	0.14	0.35	0.08
<i>Armadillo officinalis</i>			$P < 0.01$			
Wild	51.75	10.09	1.10	0.12	0.80	0.06
Captive	6.93	1.70	0.93	0.16	0.65	0.08
<i>Armadillidium granulatum</i>						
Wild	5.20	1.83	0.38	0.13	0.13	0.05
Captive	3.08	1.10	0.38	0.13	0.08	0.04

Stimulus 3 (touch)	<i>Armadillidium vulgare</i>							
	Wild	27.29	6.30	1.98	0.28	$P < 0.05$	0.25	0.07
	Captive	29.13	6.39	1.73	0.18		0.53	0.08
	<i>Armadillo officinalis</i>							
	Wild	31.06	7.59	1.73	0.19	$P < 0.05$	0.75	0.07
	Captive	4.27	0.77	1.68	0.23		0.60	0.08
	<i>Armadillidium granulatum</i>							
	Wild	12.29	2.42	0.93	0.20	$P < 0.05$	0.15	0.06
	Captive	8.38	2.24	1.15	0.22		0.30	0.07

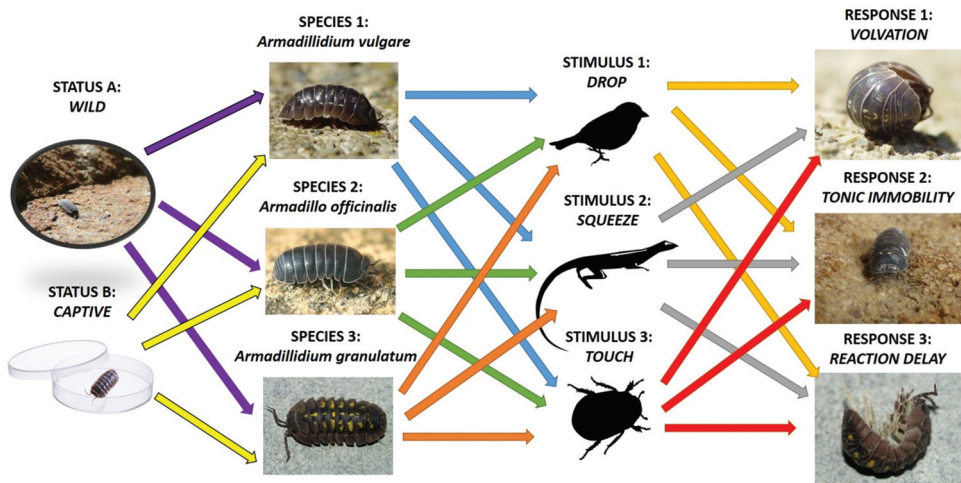


Fig. 1. — A schematization of the experiment, the species involved, the treatments considered and the responses to the stimuli applied in this study.

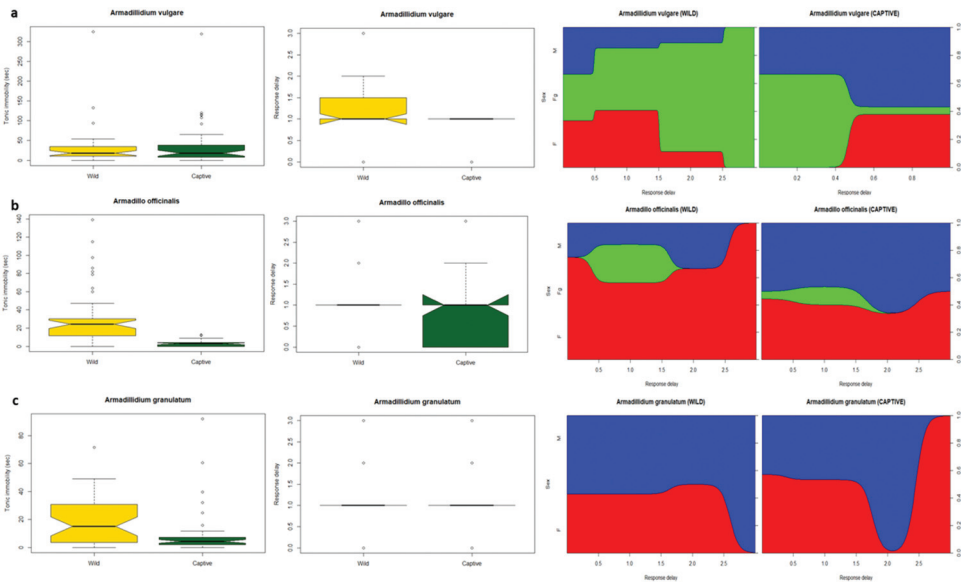


Fig. 2. — The duration of tonic immobility (in sec) (left panels), the response delay (in a 0–3 scale) (central panels), and the conditional density plot describing how the conditional distribution of sex changes over the response delay (right panel) to stimulus 1 of each species (a) *A. vulgare*, (b) *A. officinalis*, and (c) *A. granulatum*. Boxplots represents the median values (continuous line in the box), average value (x in the box), first (Q1) and third (Q3) quartiles, with whiskers of dispersion (the largest and smallest data elements that are \leq and \geq , respectively, to 1.5 times the interquartile range) and outliers. M = males, F = females, Fg = pregnant females.

significant factors that influence the response delay and volvation of this species ($F = 5\text{--}7.9$, $P < 0.01$).

Armadillo officinalis. Wild individuals of this species show tonic immobility to this stimulus significantly longer than those in captivity ($U = 1457$, $P < 0.01$) (Fig. 1B, Table 1). Wild individuals respond to this stimulus significantly later than those in captivity ($U = 993.5$, $P < 0.05$) and exhibit more volvation on average (88 vs 48%, $U = 1120$, $P < 0.01$). Females delay the response to this stimulus more than males and pregnant females, particularly in nature, (Fig. 2b). Only the status (treatment) shows a significant effect on tonic immobility and volvation frequency ($F = 17\text{--}31$, $P < 0.01$), whereas other factors, such as sex and size alone and the interactions do not have effects.

Armadillidium granulatum. Wild individuals show a tonic immobility to this stimulus longer ($U = 1079.5$, $P = 0.01$) than those in captivity. (Fig. 1C, Table 1). There is no significant difference between wild and captive individuals in the delay of response to the stimulus ($U = 829.5$, $P = 0.72$), whereas in captivity this species tends to do less volvation than in nature (70 vs 30%, $U = 1120$, $P < 0.01$). The differences in response delay are absent in a comparison between sexes of this species both in nature and in captivity (Fig. 2c). Status (treatment) and size are significant factors that influence tonic immobility and volvation frequency ($F = 4\text{--}15$, $P < 0.05$). No interaction between factors was significant for this species.

Stimulus 2 (squeeze)

Armadillidium vulgare. There is no statistically significant difference ($U = 814$, $P = 0.89$) between wild and captive individuals of this species in the duration of tonic immobility to this stimulus (Fig. 3a, Table 1). Similarly, there is no difference between individuals grown in nature and those raised in captivity in the delay of response to this stimulus ($U = 873$, $P = 0.46$) and in volvation frequency (33 vs 35%, $U = 780$, $P = 0.82$). Females have slightly delayed responses both in nature and in captivity than males and pregnant females (Fig. 3a). None of the factor considered (status, sex, and size) and their interactions exert a significant influence on the responses to this stimulus.

Armadillo officinalis. Wild individuals show a reaction time (tonic immobility) to this stimulus significantly longer than those born in captivity ($U = 1259$, $P < 0.01$) (Fig. 3b, Table 1). There is, instead, no significant difference between wild and captive individuals both in the response to this stimulus ($U = 680$, $P = 0.21$) (Fig. 3B, Table 1) and in volvation frequency (80 vs 65%, $U = 920$, $P = 0.14$). In captivity, non-pregnant females respond earlier than males and pregnant females (Fig. 3b). Only the status (treatment) is a significant factor that influence tonic immobility ($F = 19.51$, $P < 0.01$). No interaction between factors was significant for this species.

Armadillidium granulatum. There is no statistically significant difference between wild and captive individuals in the length of tonic immobility to this stimulus ($U = 800$, $P = 1$), in the delay of response to this stimulus ($U = 787$, $P = 0.87$) and in volvation frequency (12 vs 7%, $U = 840$, $P = 0.46$) (Fig. 3c, Table 1). Significant differences are also absent between sexes (Fig. 3c). None of the factor considered

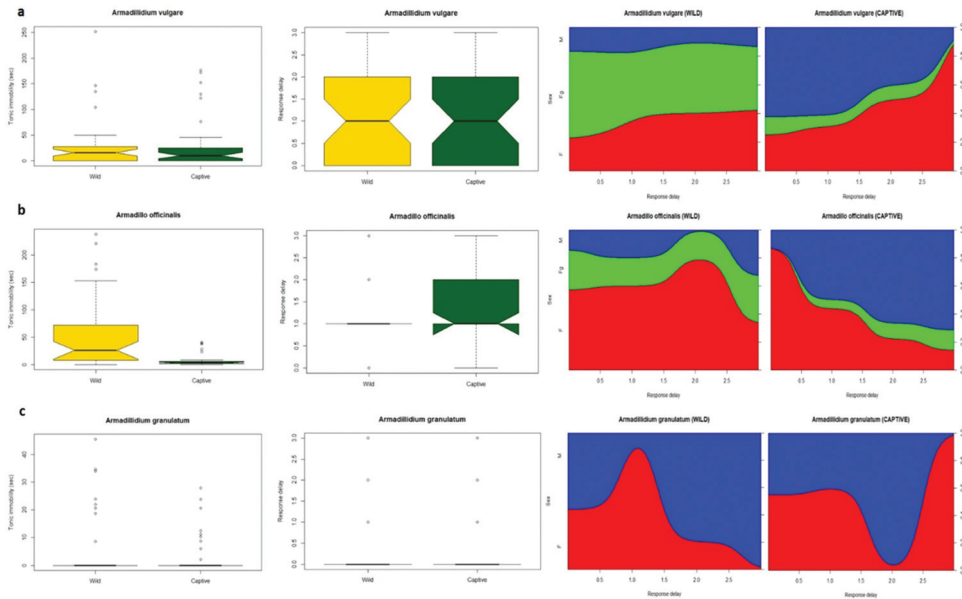


Fig. 3. — The duration of tonic immobility (in sec) (left panels), the response delay (in a 0–3 scale) (central panels), and the conditional density plot describing how the conditional distribution of sex changes over the response delay (right panel) to stimulus 2 of each species (a) *A. vulgare*, (b) *A. officinalis*, and (c) *A. granulatum*. Boxplots represents the median values (continuous line in the box), average value (\bar{x} in the box), first (Q1) and third (Q3) quartiles, with whiskers of dispersion (the largest and smallest data elements that are \leq and \geq , respectively, to 1.5 times the interquartile range) and outliers. M = males, F = females, Fg = pregnant females.

(status, sex, and size) and their interactions exert a significant influence on the responses to this stimulus.

Stimulus 3 (touch)

Armadillidium vulgare. There is no statistically significant difference between wild and captive individuals of this species in the tonic immobility to this stimulus ($U = 765$, $P = 0.74$) (Fig. 4a, Table 1). Similarly, there is no significant difference between wild individuals and those raised in captivity in the delay of response ($U = 836.4$, $P = 0.72$), while volvation is moderately more frequent in captivity (52.5 vs 25%, $U = 580$, $P = 0.01$). Pregnant females respond earlier than males and females to this stimulus in captivity (Fig. 2a). Only the status (treatment) is a significant factor that influences the volvation frequency ($F = 6.62$, $P < 0.05$). No interaction between factors was significant for the response of this species.

Armadillo officinalis. Wild individuals of this species show a length of tonic immobility to this stimulus significantly longer than those born in captivity ($U = 1255.5$, $P < 0.01$) (Fig. 4b, Table 1). There is, instead, no significant difference between wild and captive individuals both in the response to the stimulus ($U = 909.5$, $P = 0.28$) and in volvation

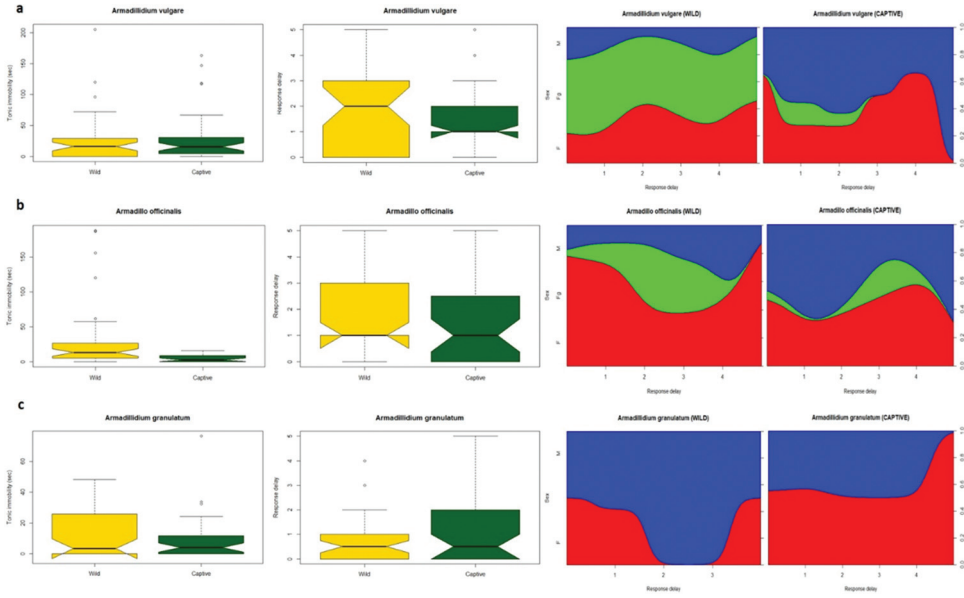


Fig. 4. — The duration of tonic immobility (in sec) (left panels), the response delay (in a 0–3 scale) (central panels), and the conditional density plot describing how the conditional distribution of sex changes over the response delay (right panel) to stimulus 3 of each species (a) *A. vulgare*, (b) *A. officinalis*, and (c) *A. granulatum*. Boxplots represents the median values (continuous line in the box), average value (x in the box), first (Q1) and third (Q3) quartiles, with whiskers of dispersion (the largest and smallest data elements that are \leq and \geq , respectively, to 1.5 times the interquartile range) and outliers. M = males, F = females, Fg = pregnant females.

frequency (75 vs 60%, $U = 920$, $P = 0.16$). Pregnant females show less late responses to this stimulus in nature than non-pregnant females and males; no difference in captivity (Fig. 4b). Only the status (treatment) is a significant factor in the tonic immobility to this stimulus ($F = 12.03$, $P < 0.01$). No interaction between factors was significant for this species.

Armadillidium granulatum. There is no statistically significant difference between wild individuals and those raised in captivity in tonic immobility ($U = 873.5$, $P = 0.46$) and in response delay to stimulus ($U = 745$, $P = 0.57$) (Fig. 4c, Table 1), where differences in volvation are moderately significant (15 vs 30%, $U = 680$, $P < 0.05$). Differences are absent among the sexes (Fig. 4c). None of the factor considered (status, sex, and size) and their interactions exert a significant influence on the responses to this stimulus.

Interspecific analysis of the reactions to the stimuli

The non-parametric analysis of variance, the Kruskal-Wallis test to check the differences in the duration of tonic immobility among the three target species in each status (wild and captive) to stimulus 1 (drop), shows no dissimilarity among the three species in the wild ($\chi^2 = 4.56$, $P = 0.1$). A significantly longer reaction for *A. vulgare*

than other species is evident in captivity ($\chi^2 = 42.59$, $P < 0.01$). The duration is significantly higher for *A. officinalis* in the wild ($\chi^2 = 29.26$, $P < 0.01$) and *A. vulgare* in captivity ($\chi^2 = 18.68$, $P < 0.01$) to stimulus 2 (squeeze). Tonic immobility is also significantly longer for *A. officinalis* in the wild ($\chi^2 = 5.88$, $P = 0.05$) and *A. vulgare* in captivity ($\chi^2 = 25.21$, $P < 0.01$) to stimulus 3 (touch).

Moreover, no significant difference is in the delay of response among the three target species both in wild ($\chi^2 = 3.92$, $P = 0.14$) and in captive ($\chi^2 = 4.53$, $P = 0.10$) conditions to stimulus 1 (drop). The delay in response is significantly longer for both *A. officinalis* and *A. vulgare* in the wild ($\chi^2 = 19.59$, $P < 0.01$) and captivity ($\chi^2 = 24.44$, $P < 0.01$) to stimulus 2 (squeeze), and significantly longer for *A. vulgare* in the wild ($\chi^2 = 12.39$, $P < 0.01$) but not differences are in captivity ($\chi^2 = 5.45$, $P = 0.07$) to stimulus 3 (touch).

DISCUSSION

Terrestrial isopods represent ecological (Messina et al. 2016a) and evolutionary (Cazzolla Gatti et al. 2018) indicators, as well as model animals for some peculiar physiological characteristics and behaviors (Dixie et al. 2015). Some typical characteristics of ethological interest, i.e. thanatosis and volvation, were the topic of this research study. We hypothesized that environmental influences, predation pressures in particular, and not only genetic factors, play an important role in shaping these behaviors. To test this idea, we compared wild and captive individuals of three different species that naturally exhibit thanatosis (as tonic immobility) and volvation (“rollers”) as antipredatory strategies.

Our results reveal several and, sometimes, contrasting behaviors that, in most cases, confirmed our hypothesis. All three species showed a significant difference, between individuals collected in the field (wild) and raised in the laboratory (captive), in the reaction to stimulus 1 (drop), which simulates the fall from a bird’s beak or from the jaws of a lizard after a catch. Volvation frequency was significantly higher in captive individuals of *A. vulgare* and in wild individuals of *A. officinalis* and *A. granulatum*. At the same time, the duration of tonic immobility was significantly higher in wild individuals of *A. officinalis* and *A. granulatum*, but no differences emerged for *A. vulgare*. The response delay was moderately higher in captive individuals of *A. vulgare* and *A. officinalis*. This allows us to speculate that *A. vulgare* behavior may be differently influenced by ecological pressures (such as predation) with respect to *A. officinalis* and *A. granulatum*. However, further studies are needed to clarify the reasons of these differential predatory pressures. In general, the reaction to the “drop” stimulus seems to confirm our hypothesis of an environmental influence on thanatosis and volvation. However, whereas *A. vulgare* is more sensitive to predatory pressures in captivity, increasing the volvation frequency although delaying the response to the stimulus without changing the duration of tonic immobility, *A. officinalis* and *A. granulatum* show higher sensitiveness to this stimulus (with longer thanatosis and more frequent volvation) in the wild. These substantial differences may reflect the evolutionary and ecological characteristics of these species. However, more studies on these species are needed to provide an answer to these speculations. Moreover, it is well-known that *A. vulgare* is able to withstand much drier conditions than other woodlice, including *A. officinalis* and *A. granulatum* (Nichols et al. 1971), and this eco-physiological adaptation may be reflected in this species behavior.

On the other hand, very few differences emerged in the reaction to stimulus 2 (squeeze), which simulates a bite by a lizard or the capture by a predator like a bird. Only *A. officinalis* showed longer tonic immobility in the wild (but no delayed response and volvation). A similar behavioral difference was manifested in the wild by this species to stimulus 3 (touch), which simulates the response to an accidental and unexpected touch of an element in the environment. The other two species (*A. vulgare* and *A. granulatum*) showed a moderately higher volvation frequency in captivity to this stimulus 3 (although no differences are evident in thanatosis and response delay).

Therefore, we have evidence that *A. officinalis* exhibits longer tonic immobility in the wild for all the stimuli, including the “squeeze” and “touch” toward which the other species show no relevant differences in the duration of thanatosis between natural and laboratory conditions.

We suggest that a reason for the higher duration of thanatosis of *A. officinalis* to all stimulus in the wild may be due to the nature of this species' main predators (Castilla et al. 2008) and/or to species specific adaptations. In fact, the limitation of movements, which can represent a useful adaptation of preys to reduce capture rates (Castilla et al. 2008; Steinberg et al. 2014), is well documented but a better understanding the reasons why *A. officinalis* shows an higher duration of thanatosis compared to the other two species requires further studies and more detailed eco-evolutionary information on this species.

Although no difference between sexes of both wild and captive individuals was detected for *A. granulatum* except for the tonic immobility to stimulus 1 (drop), females and pregnant females of *A. vulgare* and *A. officinalis* showed different and, often, opposite behaviors in comparison to males in natural and controlled environments. This is an expected evidence of the differentiated life and reproductive strategies that exert differential adaptive pressures on the two sexes and, particularly, on the females when are pregnant and need to protect their pouch for brooding (the marsupium).

The comparison among the three target species evidenced the specific differences in the reaction to the stimuli. In fact, for both response delay and duration of tonic immobility, the reaction to the stimuli of *A. officinalis* and *A. vulgare* was longer than that of *A. granulatum*. *A. vulgare* showed both a more delayed response and a longer thanatosis with respect to the other two species, particularly in captivity. This, besides highlighting the importance of genetic differences among the species, could be also motivated by the associated ecological reason that *A. vulgare* is a highly gregarious species (Beauché & Richard 2013) and a delayed but longer immobility may represent a selective strategy to decrease the probability to be predated in large assemblages when other individuals of the same or other species are moving (Lima & Dill 1990; Tuf et al. 2015).

Finally, we showed that the size of the isopods has no influence to the reaction to the stimuli and this removes the possible bias due to the dimension of the individuals collected in the wild and those raised in laboratory conditions. In this study, we provided evidence that environmental pressures, such as predation, are important evolutionary forces to address the differential behavior of some invertebrate species. The differences in ethological adaptations among the species show signs of their evolutionary differentiation (Cazzolla Gatti 2016a, 2016b). However, the trade-off between displaying and not-displaying some specific behaviors by some individual may depend on the habituation to a specific stimulus. We showed that developmental environment, species-specific adaptations, and sex/reproductive state may influence the response to these stimuli. For instance, individuals that are more frequently

subject to harmless stimuli in nature may reduce their sensitivity threshold and exhibit a higher reaction in controlled conditions where they are never or seldom stimulated.

Together with the relevant genetic and evolutionary differences among the woodlice species considered in this study, an evidence emerged in previous studies and from the interspecific comparison in our research, our intraspecific analysis between wild and captive groups highlights the importance of environmental pressures to shape the learning-related ethology of these invertebrates, besides their instinctive behavior, to optimize their life strategies.

This evidence should motivate researchers in animal ecology and zoology to dedicate more attention to the ethology of invertebrates, because ecological and evolutionary patterns can be strongly driven not only by genetic and phylogenetic causes but, also, by behavioral adaptations to the environment. On the other hand, as in the case of vertebrates, ethology may provide more clues about the ecology and evolution of invertebrate than expected.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

ETHICAL STANDARD

The authors declare that the animals used in this study have been not harmed by the experiments and have been subject to ethical treatments, although all of them were invertebrates.

AUTHOR CONTRIBUTION

R. Cazzolla Gatti and G. Messina contributed equally to the manuscript. R. Cazzolla Gatti analyzed the data and performed the statistics. R. Cazzolla Gatti and G. Messina wrote the manuscript; G. Messina designed the data acquisition and conceived the research approach; L. A. Ursino, F. Tiralongo, and G. Messina collected the specimens and the data; B.M. Lombardo supervised the data collection and the study implementation. All authors provided a critical revision to the final manuscript.

SUPPLEMENTARY MATERIAL

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