



Surrounded by challenges: The simulated presence of competitors and predators modulates perianal secretion marking behaviour in the European mink (*Mustela lutreola*)

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ABSTRACT

Animals face a variety of daily challenges to their reproduction and survival that can detect in time through to the environment cues. By using an individual focal sampling, we evaluated the variations in the time devoted to the perianal secretion marking behaviour (PSMB) of European mink when they were exposed to the simulated presence of conspecifics and two potential predators. Model results indicated that males and adult individuals dedicated more time to PSMB than females and subadults. The presence of conspecifics increased PSMB time only in adult and males, probably as an intrasexual territorial competence response. The heightened decrease in PSMB time in presence of a dog suggests an innate response due to the detection of volatile substances from faeces of carnivorous. In addition, simulated conspecific presence increased PSMB in absence of odours (control) and with owl faeces. However, when facing dog faeces, the simulated conspecific presence had no effects on PSMB. Thus, minks seem to prioritize the imminent risk of predation to avoid being detected. The stimulation of PSMB in captivity by simulated cues from conspecifics and potential predators could be useful to facilitate the reintroduction of individuals into nature, as well as their adaptation and survival.

1. Introduction

Early detection of competitors and predators increases the chances of survival of species in the wild (Navarro-Castilla and Barja 2014a, b; Sánchez-González et al. 2017). Both are biotic processes based on interactions between species that influence the distribution and abundance of species in time and space and determine the composition and structure of entire communities (Chase et al. 2002; Huitu et al. 2004). Animals detect the presence of both competitors and predators by means of cues present in their environment (Hughes et al. 2010; Garvey et al. 2016; Suraci et al. 2016; Navarro-Castilla et al. 2018). In mammals, these cues are typically visual, acoustic, or olfactory (Garvey et al. 2020). Thus, the visual cues of a predator or competitor consist in the manifestation of its silhouette in face of the prey or congener (Clarke et al. 2012; McLean and Bonter, 2013) and acoustic signals consist of vocalizations or sounds emitted by the predator or by members of the same species (alarm vocalizations) (Wisenden et al., 2008; MacLean and Bonter, 2013). Olfactory cues are indirect signals that manifest through traces of odour on rubbed surfaces, odorous secretions, urine, and

faeces, especially in the case of carnivores, as they carry out territorial marking behaviours with communicative purposes (Fendt, 2006; Barja, 2009; Piñeiro and Barja, 2012; Allen et al. 2015 a,b). Thus, olfactory communication makes it possible to individually recognize congeners, determine sex and age, identify social status, identify reproductive status, and defend territories (Barja et al. 2008; Barja et al. 2011; Kean et al. 2011, 2015; Apps et al. 2019). The defence of territory through marking behaviour allows carnivores to communicate with their conspecifics (Hutchings and White, 2000; Barja et al. 2011, Navarro-Castilla et al., 2014a) and, at the same time, alert prey to their presence (Hughes et al. 2009, 2012; Navarro-Castilla et al. 2019). This is possible due to volatile compounds present in the anal secretions, urine, and scats (Dickman and Doncaster, 1984; Dickman, 1992; Martín et al. 2010, Navarro-Castilla and Barja, 2014a).

Scent communication plays a fundamental role in solitary carnivores and even more so in those that are twilight or nocturnal animals when visual cues are more difficult to detect (Gorman and Trowbridge, 1989; Gould and Overdorff, 2002; Barja et al. 2008; Janda et al. 2019). It is therefore logical that marking behaviour plays an important role in both

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partner choice and intra-sexual competition (Gosling and Roberts, 2001). Similarly, odorous cues help to avoid other individuals spatially and/or temporarily to evade costly aggressive encounters (Gosling and McKay, 1990; Harrington et al. 2009). Many species of mustelids use different types of scent marking (Kruuk et al. 1984; Hutchings and White, 2000; Barja, 2005; Barja et al. 2011; Sittenthaler et al. 2020). Nevertheless, the marking by secretions of anal glands, consisting of organosulfur compounds, is highlighted, as they are common to all mustelids (Hutchings and White, 2000; Harrington et al. 2009). These secretions can be deposited with or without faeces and their volatile components are qualitatively and quantitatively different depending upon the sex of the individual and the species (Brinck et al. 1983; Hayes et al. 2006). Perianal secretion marking behaviour (PSMB) has been studied in a wide variety of mustelid species, include the European mink (*Mustela lutreola*) (Brinck et al. 1983). This behaviour consists of wiping the anal glands on a surface or object. These glands are located in two anal sacs and to do this, mustelids need to contract the muscles around the pouches (Brinck et al. 1978). In the European mink, the scene visible to the naked eye is the dragging of the anus over a surface as if the individual were walking seated (Ortiz-Jiménez and Barja, unpublished data).

Despite the available literature evaluating behavioural responses to visual and olfactory cues from competitors and predators, little is known about perianal secretion marking behaviour in response to cues from conspecific competitors and predators occurring together. The main objective of this study was to determine whether the presence of other competing congeners (manifested by a visual cue) and, predators (manifested by olfactory cues) both separately and in combination modify the PSMB in the European mink. Additionally, sex and age of individuals was considered to study PSMB variations. According to these objectives, our hypothesis were: i) the simulated presence of a conspecific would increase PSMB as in other species that increase their scent marking behaviour in defence of the territory or in mate selection (Allen et al. 2015a, 2016); ii) scent traces of predators would decrease PSMB since individuals would prefer to avoid detection in order not to be preyed upon (Roberts et al. 2001); iii) predation risk inhibits PSMB in the simulated presence of conspecific competitors when both threats would be combined because the bet on survival and not competition would prevail (Gurevitch et al. 2000); iv) we would observe a bias in PSMB in favour of males and adults, as in other species (Woodmansee et al. 1991; Janda et al. 2019).

2. Materials and methods

2.1. Subjects and housing

The study was performed in the same 24 European minks (10 males: 4 subadults and 6 adults; 14 females: 7 subadults and 7 adults) from the Foundation for Research in Ethology and Biodiversity (FIEB) breeding centre, located in Casarrubios del Monte (Toledo, Castilla La Mancha, Spain) that were used in other study (Ortiz-Jiménez et al. 2021). The subadult range corresponded to ages up to one year old and the adult range corresponded to 1 year and above (Mañas et al. 2016). They were born in captivity and housed in individual naturalized pens (riparian vegetation and individual pool) of an area between 40 and 60 m². All pens were in a covered corridor where caregivers and researchers had access to each individual's nest box and to a management window located in the pen doors to minimize contact between animals and humans. In addition, pens have visual connection windows which allow viewing without physical contact between individuals, and connection hatches between pens on the side walls. These windows were completely closed during the study to avoid vision and scent proximity bias.

The study was carried out before feeding the animals to avoid caregivers interfering the experiment and prevent minks being stimulated with other outside odours. European minks were fed once a day in the afternoon, through the control window, according to the latest

recorded weight and the calorie index of each food item for each individual. Diet composition was based on raw food (trout, quail, chicken), cooked food (eggs), and live prey (mice and rats). The live prey came from the foundation's vivarium.

2.2. Experiments and behavioural measurements

The experiment consisted of simulating the presence of conspecific (visual cue) and predators (olfactory cues) and lasted 18 days (between January and February) divided in two periods (first: without mirror; second: with mirror to simulated presence of a conspecific through the reflection of each mink). Each period lasted nine days and in each one a simulation lasting six days took place. During the first period each individual was exposed to three days without the mirror (control: no visual cue), and three days with owl faeces and three days for dog faeces (olfactory cues without visual cue). During the second period the European mink were exposed to three days with a mirror (control: visual cue without odours) and to three days for each faeces predator keeping the mirror presence. The order of exposition to the simulations was randomly determined by Microsoft excel.

We recorded European mink behaviour for four minutes using a SONY Xperia Z5 mobile phone in HD quality. In order not to interfere with the experiment, we recorded through the feeding and management window. An individual focal sampling with a one-zero time record at sampling intervals of 10 s were used to register the perianal secretion marking behaviour (de Miguel and Barja, 2015). PSMB was considered when minks scooted their anus across the ground.

2.3. Simulation of conspecific and predators' evidence

To introduce the visual cue and the olfactory cues, European mink were locked up in their respective nest boxes and, just before starting the behavioural record, they were released. We used a round mirror (20.3 × 20.3 × 1.5 cm) in order to simulate the presence of conspecific when the individuals were reflected. It had an integrated support which was placed on the floor one meter from the exit hole of the nest box. The mirror was placed in a vertical position with its edge to the nest box, so that individuals would only see their reflection when they stood in front of the mirror. We previously performed an ad libitum test to verify that the minks did not show a neophobic behaviour to the mirror. In addition, we observed that when the minks approached the edge of the mirror, they acted as if it were any object (they smelled it, licked it or scratched it on the sides with their legs). Instead, when they approached the mirror and looked reflected, they reacted differently (keeping distance, flinching, hiding quickly, and showing aggressive behaviour: muscle tension, raised tail, bristling hairs and dental exposure).

Fresh faeces of Eurasian eagle-owl (*Bubo bubo*) and dog (*Canis lupus familiaris*) were used to provide olfactory cues. We chose these potential predators based on a study that showed that a large part of the deaths of European mink after a reintroduction were due to predation by raptors (18.2%) and dogs (18.2%) among others (Maran et al. 2009). Owl feces were collected by scratching the ground with a spatula. The identification of fresh feces was carried out by the soft consistency, the ease of detaching from the ground and the strong odor given off. Freshly collected dog faeces had a mucus layer and a strong odour without signs of dehydration, easy to select them for the experiment (Barja et al., 2007; Navarro-Castilla and Barja, 2014b). We froze the faeces at - 20 °C until required for the experiments. Freezing fresh faeces is important to ensure a minimal loss of volatile compounds. Since volatile compounds can vary depending on the individual, sex, age, and season, we mixed and homogenized them to avoid bias (Hayes et al. 2006; Scordato and Drea, 2007; Martín et al. 2010). Just before the start of the experiment, we placed the faeces one meter from the exit hole of the nest box within each pen in the morning. We distributed 7 g of faeces per pen in sandy areas free of vegetation and replaced the previous day's faeces by new fresh faeces each day of predator simulation. To do this, the surface was

cleaned removing the remains and underneath of the previous day before placing the new faeces. This methodology was used in other study (Ortiz-Jiménez et al. 2021).

2.4. Ethical statement

The FIEB is registered as a zoo centre and animal experimentation centre covered by the Consejería de Agricultura y Servicios Periféricos de Castilla la Mancha with registration code: ES450410000053. This registration carries the implications of housing and handling animals according to animal welfare criteria. Furthermore, FIEB is a participating centre in *Ex situ* Conservation Program for European mink acting as a breeding and research centre promoted by the Ministerio para la Transición Ecológica y Reto Demográfico of Spain.

Universidad Autónoma de Madrid and FIEB approved the experimental protocol of this study. ARRIVE guidelines and regulations were rigorously considered in the development of our methodology. The stressors put on the minks were those that they experience in natural environment. We collected owl and dog faeces from dewormed animals housed in FIEB for provide the odour cues. The mirror was properly disinfected before being placed in the pens to comply with aseptic measures.

2.5. Statistical analysis

We transformed into seconds the variable perianal secretion marking behaviour recorded in counts since each YES/NO recording took place in 10-second intervals. Each PSMB event was transformed in 10s which is how long each interval last. Since the data did not show a normal distribution and these were still multiple counts of 10, we used a Mixed Generalized Linear Model (GLMM) with a Poisson distribution as a multifactorial analysis to analyse the effect of the fixed factors (sex, age, conspecific's presence simulation, and simulated predation risk) on the response variable "perianal secretion marking behaviour (s)". We include "individual" variable as a random factor. For this purpose, the following categories were established: sex (male/female), age (sub-adult/adult), conspecific's presence simulation (without mirror/with mirror), and simulated predation risk (control/owl faeces/dog faeces). We also studied the effects of the following interactions: sex*conspecific's presence simulation, sex*simulated predation risk, age*conspecific's presence simulation, age*simulated predation risk, and conspecific's presence simulation*simulated predation risk. We established a probability value of $p < 0.05$ to consider the results as significant. For statistical analysis we used the software SPSS 23.0 for Windows (SPSS Inc, Chicago, IL, USA). Data were presented as means and standard errors.

3. Results

Perianal secretion marking behaviour (PSMB) was explained by the pure effect of sex, conspecific's presence simulation, or simulated predation risk factors as well as by the interaction of sex*conspecific's presence simulation, age*conspecific's presence simulation, age*simulated predation risk, and conspecific's presence simulation*simulated predation risk according to GLMM (Table 1). Males (6.85 ± 0.89 s) carried out PSMB for longer than females (2.32 ± 0.46 s). Similarly, adults (4.61 ± 0.63 s) spent more time on PSMB than subadults (3.75 ± 0.71 s). Regarding PSMB during exposure to visually simulated conspecific presence, European minks performed PSMB for longer when facing the simulated presence of a conspecific (5.34 ± 0.79 s) than in the absence of conspecific visual simulation (3.15 ± 0.52 s). In relation to olfactory cues from predators, dog faeces (3.54 ± 0.65 s) did not lead differences in PSMB compared to controls (4.04 ± 0.85 s), while we found a significant increase in PSMB when individuals were exposed to owl faeces (5.07 ± 0.93 s) (Fig. 1).

The interaction between sex and period showed that males markedly

Table 1

Results of the GLMM analyzing the effect of the factors (sex, age, conspecific's presence simulation and simulated predation risk) and their interactions on perianal secretion marking behaviour (s) of European mink.

Factor	F	df1	df2	p
Intercept	34.19	13	104	0.001
Sex	7.36	1	17	0.015
Age	0.21	1	17	0.654
Conspecific's presence simulation	23.77	1	410	0.001
Simulated predation risk	11.81	2	410	0.001
Sex*Conspecific's presence simulation	97.7	1	410	0.001
Sex*Simulated predation risk	1.87	2	410	0.156
Age*Conspecific's presence simulation	12.72	1	410	0.001
Age*Simulated predation risk	12.52	2	410	0.001
Conspecific's presence simulation*Simulated predation risk	12.39	2	410	0.001

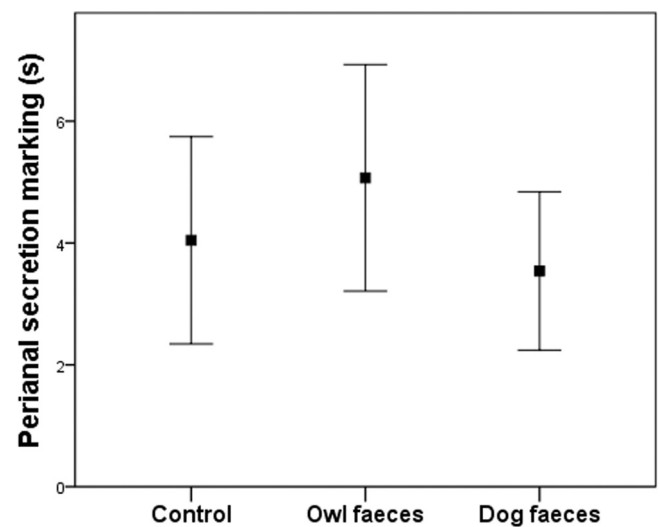


Fig. 1. Mean time (s) spent on perianal secretion marking behaviour by European mink depending on odour cues. Error bars represent standard error of means.

increased PSMB compared to females when in the presence of a conspecific (Fig. 2a). Similarly, the interaction between age and conspecific's presence simulation showed that adults increased the time spent on PSMB in presence of a conspecific while subadults do not make changes in the duration of their marking behaviour (Fig. 2b). Furthermore, the interaction between age and simulated predation risk revealed a decrease in the time spent on PSMB by adults when they were exposed to dog faeces, which was similar to PSMB time by subadults during the control (Fig. 3a). Finally, the interaction between simulated conspecific's presence*simulated predation risk showed that under the exposure to dog faeces, the simulated conspecific presence had no effects on PSMB. However, the simulated conspecific presence significantly increased PSMB in absence of odours (control) and with owl faeces (Fig. 3b).

4. Discussion

Our study showed that the European mink performs perianal secretion marking behaviour (PSMB), as known in other mustelids (Begg et al. 2003; Lampa et al. 2015; Noonan et al. 2019), that may be influenced by threat signals, i.e., visual simulated cues of intraspecific competition and chemical signs reflecting predation risk. We detected differences between sex and between age groups of individuals in the time spent on PSMB and its variation with both threats (conspecific and predation signals). Thus, males performed PSMB for longer than females; this is in

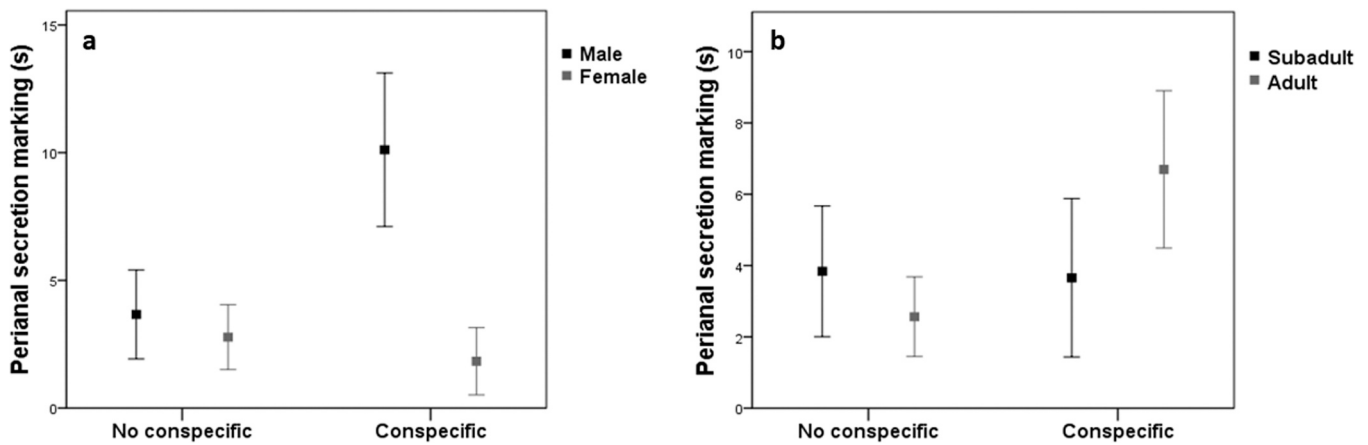


Fig. 2. Mean time (s) spent on perianal secretion marking behaviour during conspecific's presence simulation by European mink plotted by: a) sex; b) age. Error bars represent standard error of means.

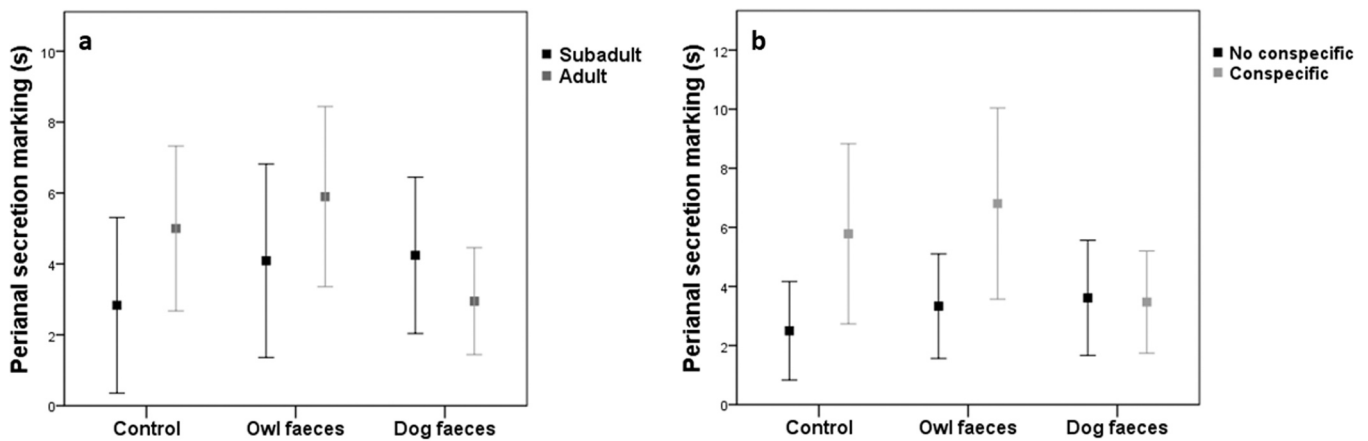


Fig. 3. Mean time (s) spent on perianal secretion marking behaviour during predation risk simulation (odour cues) by European mink plotted by: a) age; b) conspecific status. Error bars represent standard error of means.

accordance with the overall trend found in a large number of mammalian species where males may show up to 2–10-fold marking rates than females (Barja et al., 2004; Pochron et al. 2005; Jordan, 2007; Müller and Manser, 2008; Janda et al. 2019). Taking as an example the Eurasian otter, Lampa et al. (2015) detected higher PSMB rates in males than in females. Moreover, considering the ecology of the European mink, males patrol larger home-range areas than females do (Palazón and Ruiz-Olmo, 1998; Mañas et al., 2001; Garin et al. 2002). If territories are larger, more active marking behaviour can be more efficient. Likewise, PSMB was more evident in adults than subadults, presumably due to increased dominance (Erlinge et al. 1982; Kruuk et al. 1984; de Monte and Roeder, 1993) and self-advertisement to attract mates (Müller and Manser, 2008). A study on fallow deer (*Dama dama*) determined that scent marking was the only one of a set of variables with which they evaluated dominance, which was influenced by age, with adults having a higher duration of marking (Komers et al. 1997). It was also observed that adult wolves (*Canis lupus*) had more than twice as many volatile aromatic compounds in their faeces as did young individuals (Martín et al. 2010). Since glandular secretions in mammals are mainly controlled by sex hormones (Müller-Schwarze, 2006; Johnston and del Barco-Trillo, 2009) and individuals who reproduce are those who have reached sexual maturity, the duration in the marking behaviour is accentuated with age. An example of this is a study on spotted hyena (*Crocuta crocuta*) that showed that the frequency of marking increased significantly with the onset of puberty (Woodmansee et al. 1991). This trend has also been observed in other species of mammals (golden

tamarin, *Leontopithecus rosalia*: Kleiman and Mack., 1980; koala, *Phascolarctos cinerus*: Smith, 1980; aardwolf, *Proteles cristata*: Sliwa, 1996; domestic dog: Cudd-Wirant et al. 2007; and brown bears, *Ursus arctos*: Clapham et al., 2014).

The presence of a conspecific increased the time that the individuals dedicated to the PSMB probably as a strategic response of intraspecific competition or to communicate with potential mates (Allen et al. 2016). Since the study was not carried out during the breeding season, we consider the intraspecific competition as the most plausible hypothesis. However, since it is a simulation and not the presence of a real conspecific, it may be that the reaction of the minks was not due to a matter of competition but rather to a new object in the facility. The visual capacity of the European mink is unknown, so it is not possible to firmly affirm that individuals perceived the mirror as a conspecific (Ortiz-Jiménez et al., 2021). Owl faeces caused an increase compared to the control (an absence of introduced predatory olfactory cues) while dog faeces decreased this behaviour compared to owl faeces. Studies show that some animals as for example primates behave differently depending on whether the predator is aerial or terrestrial (Fichtel and Kappeler, 2002; Barros et al. 2004), so this result could explain the decrease in PSMB of mink due to a higher probability of being eaten by a terrestrial predator than by an aerial one. In fact, a study assessing the main causes of death in European mink after reintroductions determined that the main predators of the European mink were other carnivorous mammals of larger size (59.1%) such as the fox (*Vulpes vulpes*) and domestic dog, although there were cases of predation by birds of prey

(18.2%) (Maran et al. 2009). Another explanation is that the ability to recognize volatile faeces from carnivorous mammalian faeces increased (Kats and Dill, 1998; Navarro-Castilla and Barja 2014a). This is made possible by metabolites of meat-derived sulphur molecules found in the faeces of carnivores that trigger an innate response in prey (Nolte et al. 1994).

The interactions sex*conspecific's presence simulation and age*conspecific's presence simulation revealed that males and adults significantly increased PSMB compared to females and subadults when conspecific presence was simulated, whereas in the absence of conspecifics, differences in PSMB between both genders were not so marked. Therefore, the result suggests that adult and males, already sexually mature, devote more time to this behaviour as an intra-specific competition, for which they compete with other males and subadults for access to females and/or territory in the same way that occurs in other mustelids, such as European marten (Barja et al. 2011). At the same time, it is possible that females and young individuals avoided interacting with larger and more dominant conspecifics to avoid costly encounters in the non-breeding season, such as was found in the American mink (Zschille et al. 2010). Furthermore, during the control and in the presence of owl faeces, adults of European mink spent more time on PSMB, while in the presence of dog faeces they decreased their time dedicated to PSMB. This can be due to the high risk of predation by a terrestrial predator for which the costs are greater to ensure the survival (not being detected) than the benefits of increasing their PSMB to ensure, for example, the defence of territory (Sih et al. 1985). Thus, owl faeces caused adults to increase their time on PSMB. This result suggests that they did not interpret owl faeces as a predator but as a novelty within their territory. Environmental modifications, especially with scents, may encourage an increase in marking behaviour (de Monte and Roeder, 1993). Moreover, European mink increased their PSMB time both in the control and during exposure to owl faeces in the presence of conspecifics. Since there is no risk of being preyed upon, according to their interpretation of odours, they are dedicated to marking for longer for inter- and intra-sexual competition. This may be possible not only because individuals interpret owl faeces as novel odours but because they consider them to be of low predation risk, since the evaluation not only interprets whether there is risk, but also the strength or intensity of that risk (Chase et al. 2002; Sánchez-González et al. 2017). In contrast, the predominant effect of the faeces of a larger carnivorous mammal such as the dog on the presence of a conspecific when both situations happened together shows an avoidance response to protect themselves rather than competing with other individuals. That is to say, European mink assess the risk of both types of threat and prioritize the imminent risk of predation as a trade-off. An example of this is a study that showed that cheetahs (*Acinonyx jubatus*) decreased intraspecific scent communication when they were exposed to scent marks by predators (Cornhill and Kerley, 2020).

Finally, since captive individuals included in this study were never exposed to real predation risk, the obtained results must reflect that the modulation of the PSMB in the presence of a potential terrestrial predator seems to be an innate response. However, the same did not occur with owl faeces, either because the individuals would have lost the anti-predatory behaviour that was previously adaptive during ontogeny or generations (Berger, 1998, Coss, 1999, Foster, 1999, Griffin et al., 2000) or because the response to aerial predators is learned and does not derive from an innate response. We would like to emphasize that the marking behaviour is also a good indicator of animal welfare (Zala et al. 2004), so it could be interesting to stimulate it by simulating odours in ex situ conservation centres. Recreating common situations from the natural environment can help to improve the successful reintroduction of minks in their natural habitats by facilitating their adaptation and possible survival.

5. Conclusions

Overall, this research concludes that the simulated conspecific presence significantly increases the time devoted to PSMB in males and adults. In addition, the simulated conspecific presence significantly increased PSMB in absence of odours (control) and with owl faeces. However, under the exposure to dog faeces, the anti-predatory response prevails over the competitive response and the simulated conspecific presence had no effects on PSMB.

Although it has been observed that a visual cue prevails over the olfactory cue as a trigger for different behaviours performed by different species (Rubene et al., 2019), this fact can change depending on the context that takes place. In our study, when simulating two threat scenarios, it is the olfactory cue that prevails when the visual cue is presented at the same time, since the first one comes from a predator and the second from a conspecific competitor. Therefore, the threat risk is greater in the case of predation. Based on these considerations, it would be interesting to carry out a new research in which the same scenario shown in this study is presented as opposed to another scenario in which the visual cue comes from a predator and the olfactory cue from a conspecific competitor (Ward and Mehner, 2010). In this way, the relationship between the type of cue (direct/indirect), the mode of cue (visual/olfactory) and the context in which they take place can be clarified more precisely.

Author contributions

Isabel Barja confectioned and designed the study. Material preparation and data collection was performed by Lorena Ortiz-Jiménez. Both authors carried out the statistical analysis. The first draft of the manuscript was written by Lorena Ortiz-Jiménez. Isabel Barja commented on previous versions of the manuscript. Both authors read and approved the final manuscript.

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The experiments carried out in this research as well as the study subjects are part of Lorena Ortiz-Jiménez's doctoral thesis.

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