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Effect of intensity and duration of anthropic noises on European mink locomotor activity and fecal cortisol metabolite levels

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Abstract

Human activities involving noise emission can affect wild animals. European mink was exposed to road noise and human voice playbacks to analyze how sound intensity level and duration of both noises altered the time that individuals were active and if their fecal cortisol metabolite (FCM) levels varied. A Hierarchical Analysis Cluster was performed to establish 2 mink groups with respect to both noise source type: short duration/low intensity (SL) and long duration/high intensity (LH). We performed general linear mixed models to evaluate the variation in locomotor activity duration (s) and FCM (nanogram per gram) levels, respectively. The results showed both road noise and human voices decreased locomotor activity duration in SL more sharply compared with LH, and human voices were the triggers that induced the most pronounced response to both exposure conditions. FCM (ng/g) levels increased in SL compared with LH during road noise while the opposite happened during human voices. Differences based on sex and age of individuals were observed. In conclusion, noise characteristics given by the sound type determined the variations in locomotor activity duration while noise exposure level determined the variations in FCM (ng/g) levels. Attention should be paid to noisy activities (e.g., recreational activities for visitors in protected natural areas) and loud groups of people to conserve wildlife, especially noise sensitive species.

Key words: anthropic disturbances, European mink, fecal cortisol metabolites, noise exposure, locomotor activity behavioral response.

Adequate behavioral and physiological responses to environmental noise may be essential for survival (Costanzo et al. 2021). In this sense, there is a growing concern about the indirect impacts of human activities on wildlife such as noise (Iglesias et al. 2012; Buxton et al. 2017a; Harding et al. 2019). Anthropic noise includes increases in the number of high-intensity noise events and high, homogeneous, and chronic background sound levels (Shannon et al.

2016). The responses of organisms to noise may vary as they depend on the perception and interpretation of noise (Francis and Barber 2013). A certain species may perceive noise as a threat and react with an anti-predatory response (Tyack et al. 2011) or may become distracted and ignore other environmental cues (Chan et al. 2010; Chan and Blumstein 2011), unable to discriminate acoustic signals from predators, prey, or conspecifics (Siemers and Schaub 2011).

For example, several studies showed that birds adjusted the pitch (Slabbekoorn and Peet 2003; Slabbekoorn and den Boer-Visser 2006), amplitude (Brumm 2004), or duration (Fuller et al. 2007) of their song to counteract noise. In addition, noise can cause sensory degradation and hinder intraspecific communication, leading to reduced reproductive success (Halfwerk et al. 2011). An example of this is the loss of acoustic space by male frogs to attract females due to traffic noise (Bee and Swanson 2007). Another example is a study by Iglesias-Merchan et al. (2016) on the influence of noise on the re-production of the black vulture *Aegypius monachus* showing that couples relocated their nesting sites to avoid noise intensities of >40 dB(A), thereby reducing the breeding habitat. It has also been shown that prolonged exposure to a chronic noise source can lead to habituation if an indirect benefit is obtained as a refuge from disturbance-sensitive predators (Francis et al. 2009; Brown et al. 2012; Shannon et al. 2014). This sometimes becomes a double-edged sword: proof of this is a study in which it was discovered, on the one hand, that hummingbird pollination benefited through reduced predation in noisy areas and, on the other hand, seed dispersion for dominant plants decreased because key dispersants (hummingbird predators) avoided noisy areas (Francis et al. 2012).

Roads are one of the most spatially extensive noisy alterations to the landscape (Riitters and Wickham 2003). Therefore, many studies focused on assessing the negative effects of roads on adjacent landscapes. However, roads contain other non-noise-related confounding effects such as habitat fragmentation, visual disturbances due to vehicle light, vehicle–animal collisions, and chemical pollution (McClure et al. 2013). Studies on ungulates showed how populations avoided proximity to all types of roads, both large and small (Gagnon et al. 2007; Fahrig and Rytwinski 2009; Rytwinski and Fahrig 2012). However, it cannot be demonstrated that this avoidance was due to noise and not to the other disturbances of roads previously mentioned such as vehicle–animal collisions (Glista et al. 2009). It is known that migratory birds avoid noisy environments such as roads, deviating from routes and reducing the duration of their stays at stopover sites (Wiącek et al. 2015). A study carried out during the migration of birds in autumn in Idaho (USA) showed that traffic noise, and no other factors, negatively affected these birds near roads, reducing the density of populations due to noise emitted by loudspeakers along a "ghost path" (McClure et al. 2013). Barrero et al. (2020) showed that some non-passerine birds adjust their vocal sexual display to anthropogenic noise, compromising their communication. Furthermore, it is important to note that these anthropic noises caused by roads can trigger not only a behavioral response, but also a physiological stress response (Blickley et al. 2012; Strasser and Heath 2013). For example, the proximity to roads is a factor that may increase the stress-related hormone levels in roe deer *Capreolus capreolus* (Zbyryt et al. 2018). Iglesias-Merchan et al. (2018) showed that stress-related hormone levels in roe deer were related to the acoustic escape distance which means the distance that individuals needed to travel to escape from road traffic noise-polluted areas based on sound pressure levels (SPLs).

Linked to road networks development, ecotourism impacts many animals in this Anthropocene epoch, and it may contribute to biodiversity loss (Hongjamrassilp and Blumstein 2021). The impact of ecotourism, recreational activities, and other human activities such as hunting and outdoor sports has been already investigated (Müllner et al. 2004; Barja et al. 2007; Balmford et al. 2009; Piñeiro et al. 2012; Tarjuelo et al. 2015; Casas et al. 2016). In fact, these human disturbances have been shown to affect spatial distribution, behavior patterns, habitat use, reproductive behavior, breeding, and

their survival (Baudains and Lloyd 2007; Sastre et al. 2009; French et al. 2011; Suraci et al. 2019). As in the case of road noise, effects of human recreational activities on the physiological condition of individuals have also been observed. For example, research on European marten showed how physiological stress were increased during times with greater tourist pressure in a natural park (Barja et al. 2007). Further studies showed how the pin-tailed sandgrouse *Pterocles alchata* (Casas et al. 2016) and little bustards *Tetrax tetrax* (Tarjuelo et al. 2015) changed their behavior patterns during the weekends, with more intense hunting activity. Moreover, an increase in cortisol metabolite levels was observed on the first days of the week in both bird species. In addition, these results coincided with studies of carnivores, such as wild cats, whose cortisol metabolite levels also increased in some areas where tourism intensity was higher within a natural park (Piñeiro et al. 2012).

One aspect of the physiological stress response consists of the activation of the corticotropic axis, with the subsequent secretion of glucocorticoids (corticosterone and/or cortisol) that can be quantified (Barja et al. 2011). The quantification of glucocorticoids can be carried out in plasma but is an invasive method that in itself generates stress in the animal due to the capture and handling necessary for the collection of the samples, which can generate biases in the results. Glucocorticoids can also be measured in saliva, urine (Möstl and Palme 2002), and fecal samples, a non-invasive method that does not require capture or management and reflects a glucocorticoid secretion pattern representative of longer periods of time (Barja et al. 2008, 2012; Zwijacz-Kozica et al. 2013; Navarro-Castilla and Barja 2019). Assessing the concentration of glucocorticoids in fecal samples not only provides interesting information on the ecology of the species, but also allows us to know if the welfare of animal is being compromised, especially in a captive context (Fernández-Lázaro et al. 2019).

There are already several studies that deal with the negative effects of road noise and human activities outside urban centers. However, studies assessing the effect of combination duration and intensity of noise exposure on the behavioral response and fecal cortisol metabolite (FCM) levels of mammals are practically unknown. Nevertheless, it is worth noting at this point that sound amplitude should not be confused with sound intensity (Popper et al. 2014). On the one hand, amplitude of a sound is quantified by means of sound pressure measurements at a point location and the SI unit is the pascal (Pa). On the other hand, sound intensity is defined to be the amount of energy passing through a given area per unit of time in a specified direction whose SI unit is watts per meter squared (w/m^2). However, sound intensity level (SIL) and SPL are commonly given in decibels (dBs), which is a relative unit of measurement on a logarithmic scale. SIL and SPL are related but they are different descriptors of a sound. In addition, the term intensity is often employed as synonym of loudness or amplitude above ambient values or background noise level (Francis and Barber 2013) among biologist in literature. In fact, when amplitude of a sound increases, its sound intensity increases, and it is also perceived as louder and vice versa. Therefore, we use just intensity to refer loudness in terms of SPL (in dB) in accordance with previous research regarding potential effects of noise on wildlife for >2 decades (Shannon et al. 2016).

This research aims to evaluate how 2 types of anthropic noise (road traffic noise and human voices) as well as their intensity and time of exposure influence the behavioral response and FCM levels of European mink *Mustela lutreola*. The hypotheses of this study were: (1) Exposure to anthropic noises would decrease mink locomotor activity and increase FCM (ng/g) levels (as in other mammals:

Shannon et al. 2014; Iglesias-Merchan et al. 2018); (2) human voices would cause a more noticeable decrease in mink locomotor activity than road traffic noise, since human voices would be considered as predator vocalizations by mesocarnivores (Clinchy et al. 2016) versus a simple source of disturbing noise of anthropic origin such as road traffic noise. However, FCM (ng/g) levels would increase during road traffic noise, since the distance to roads and noise level derived from traffic flow induces changes in glucocorticoid levels (Ising and Ising 2002; Bhattacharjee et al. 2015); (3) exposure to long-duration and high-intensity noises would result in a decrease of locomotor activity and an increase in FCM levels, since long-term noises can mask key acoustic signals, altering risk perception and generating uncertainty (Grade and Sieving 2016; Kleist et al. 2018); and (4) individual factors such as sex and age would be biased according to noise exposure in both locomotor activity duration response and FCM (ng/g) levels.

Materials and Methods

Study species and enclosures

Our research was carried out with 23 European mink (10 males: 4 subadults and 6 adults and 13 females: 6 subadults and 7 adults) who inhabit naturalized enclosures with logs and riparian vegetation. The enclosures have the following measurements: 40–60 m² (total area) of which $5 \times 3 \times 0.7$ m was stream water rafts. Enclosures were distributed in 4 corridors to which caregivers had access. All the individuals were born in captivity whose fathers were founding individuals (captured from the natural habitat) and their mothers were born in captivity. However, not all were siblings on the father's side, because the origins of the mink are from different breeding centers in Spain due to exchanges that occur between them due to the requirements of the captive breeding program.

This research was conducted in a breeding center of European mink located in Casarrubios del Monte (Spain) owned by Fundación para la Investigación en Etología y Biodiversidad (FIEB). FIEB is the largest breeding center of European mink in Spain, but also houses other animals from illegal trafficking and collaborations with researchers requiring housing for their study subjects. In this center the mink are fed once a day. During the days in which our study was conducted, they were fed in the afternoon so as not to interfere with the development of the experiment in order to avoid bias. All mink were fed the same kind of food, which was dead or living prey. The amount offered to each mink was determined by the weight of each mink.

Experimental design

We designed a playback experiment which consisted of recreating 2 situations: proximity to a road and proximity to outdoor recreation areas (i.e., human voices). For the experiment, we used two 4-min long audio files. Proximity to road was simulated by a recording of road traffic noise which was recorded from a bike bridge crossing over a two-lane highway in each direction located in Madrid, which had a speed limit of 80 km/h and an estimated traffic density of ~3,700 vehicles/h. Proximity to outdoor recreation areas was simulated by community noise recorded next to a school playground. As it was described by Ortiz-Jiménez et al (2021), spectral composition of the road traffic noise audio file was characterized by a dominant peak at a frequency of ~1 kHz. Most of road traffic noise energy is comprised <2.5 kHz and amplitude of the waveform is relatively constant. However, waveform of human voices audio file showed a

significantly oscillation along time, its acoustic energy spread up to 6 kHz and it also showed a variety of peaks located at 0.5, 1.0, 2.5, and 4.0 kHz. The playback experiment took place in every enclosure occupied by European mink. To do that, we established 2 cycles of 3 days for ambient sound (control), 3 days for road traffic noise, and 3 days for human voices. That was necessary to avoid forced habituation that may introduce bias in the experiment. The experiment lasted 18 days in total.

The playback system was calibrated every day and the equivalent continuous SPL (Leq, in decibels Z) was stated at 68 ± 0.5 dB(Z) at ~1.5 m from the loudspeaker during both playback treatments (i.e., road traffic noise and human voices). We selected Z-weighting instead of A-weighting because the first one is independent of human frequency perception. A professional sound level meter CESVA SC 420 class 1 was placed on a tripod at a height of 1.5 m in above ground in an empty enclosure to measure SPL from the loudspeaker during exposure to noise playbacks in neighboring enclosure. The frequency spectrum of field noise measurements ranged from 16 to 16,000 Hz. SPL is a logarithmic measure of the amplitude of sound pressure variations relative to a reference value (20×10^{-6} Pa in air). This way it was possible to verify that noise was inaudible at distances larger than 3 enclosures (because of sound attenuation due to walls between enclosures). Therefore, individuals may be exposed to different numbers of playback stimuli (i.e., they were potentially stressed during a different period of time) and also, they were exposed to different SPL each time depending on their position in the corridor. Thus, we decided to calculate sound exposure level (SEL) as an indicator of the total acoustic energy received in each enclosure instead of Leq. Leq is one of the most common indicators for noise measurements and it correlates with general annoyance in humans (Barti 2000; Ayres et al. 2010). Leq represents a value known as the equivalent continuous noise level measured at a receiver during a given time of measurement and it can be assessed directly with a professional sound-level meter. Nevertheless, SEL is a recommended metric to quantify the effects of exposure over time and over multiple and different noise events (Martin 2013). Indeed, SEL is considered a good predictor of injury, for instance, in marine mammals (Martin et al. 2019). In practice, SEL can be defined as a constant noise level for a time period of 1 s with the same energy as the actual acoustic energy received from the different events to be compared (Krylov 2001). Based on Leq measurement of a sound event, SEL can be calculated as:

$$SEL = Leq + 10 \ \log \ (t) \tag{1}$$

where t is duration of the sound event in seconds.

The sound-level meter was also calibrated (calibrators provide a 94 dB signal at the frequency of 1 kHz) before and after each measurement.

Behavioral data and fecal sample collection

Mink locomotor activity was evaluated to study the effect of noise exposure. Locomotor activity encompassed motor conducts like walking, running, jumping, swimming, and climbing, since other studies considered locomotor behaviors as an activity for solitary carnivores (Kavanau and Ramos 1972, 1975). We carried out the following steps to obtain the behavioral data. First, we enclosed the individuals in their nest box to be able to access the enclosures and place a SONY-branded loudspeaker (Personal Audio System SRS-XB2) connected to a SONY-branded digital voice recorder (Digital Dictation Machine ICD-PX370) on the ceiling centered. Second, we

released each mink and recorded its locomotor activity from a management window for 4 min using SONY Xperia Z5 mobile phone in HD quality. Only one researcher recorded the behavior of each mink, whereas another researcher recorded SPL at the same time in an empty enclosure while the loudspeaker was positioned at different distances. The recordings were not made at the same time and the order of recording of the experiment was done randomly. Third, we briefly enclosed again the individuals to remove the speaker and recorder and they were released immediately. Finally, 3 researchers generated a data matrix after viewing the videos of mink's locomotor activity using an individual focal sampling with a one-zero-time record with sampling intervals of 10 s (de Miguel and Barja 2015). The behavior was evaluated 3 times in each mink during the control (3 days of control), 3 times during the traffic noise (3 days of traffic noise), and 3 days during the noise of human voices.

To obtain the fecal sample, we took advantage of the first closure of the minks when we placed the speaker, to collect a fresh fecal sample every day 24 h after the previous day's test (just before starting the corresponding test the following day). We selected fresh feces based on the following characteristics: strong odor, no signs of dehydration, and the presence of a mucus layer (Barja et al. 2011; Horcajada-Sánchez et al. 2019). Then, they were frozen at −20 °C in individual airtight bags until hormonal analysis was performed.

Extraction and quantification of FCM

We used a heater to defrost and dry feces samples at 90 °C for 24 h. This drying allows tostabilize the weight of the feces by losing the water that they contain, which can vary between samples. The fecal samples from each 9-day cycle corresponding to each type of treatment (3 control samples, 3 traffic noise samples, and 3 human voice samples) were crushed and homogenized to obtain a mean concentration of glucocorticoids from each individual. Afterward, 0.5 g of sample was placed in assay tubes and 2.5 mL pure methanol and 2.5 mL of phosphate buffer saline were added. Then, the tubes were shaken for 16 h to obtain supernatants that then were centrifuged for 30 min at 4,000 rpm. Finally, the fecal sample extracts obtained were stored at −20°C for further analysis.

We carried out a quantification of FCM using a cortisol enzyme immunoassay kit (DEMEDITEC Diagnostics GmbH.D-24145 Kiel, Germany) using enzyme linked immunosorbent assay (ELISA) technique, which is based on the competition for feces sample antibody binding and antigen-marked with a horseradish cortisol peroxidase conjugate enzyme (Barja 2015). The evaluation of the cortisol metabolite levels in the fecal samples was made in a spectrophotometer (Microplate Reader, MR 600, Dynatech Product). This technique was validated in our laboratory for the target hormone in the European mink. Thus, the validation parameters were recovery and parallelism. Recovery rate of cortisol was >90% in the samples, and therefore the extracts did not contain substances that interfered with quantification. The parallelism tests indicated that the curves made were parallel to the standard curves. The intra- and inter-assay coefficients of variation were 8.5 and 10.9%, respectively. The following materials have been evaluated for cross reactivity (the percentage indicates cross reactivity at 50% displacement compared with cortisol): < 0.1% pregnenolone, < 0.01% estrone, < 0.1% estradiol, < 0.1% DHEA, 0.8% 17-Hydroxyprogesterone, 54.3% prednisolone, < 0.1% testosterone, 76% cortisone, 2.3% corticosterone, < 0.1% danazole, < 0.1% androstenedione, 100% prednisone, 37.5% 11-deoxycortisol, 0.4% estriol, < 0.1% dexamethasone, 0.5% 11-deoxycorticosterone, and < 0.1% progesterone. The lowest analytical detectable level of cortisol that can be distinguished from the Zero Calibrator is 3.79 ng/mL. We expressed FCM levels as a function of dry fecal mass (ng/g dry feces).

The results of the validation in the laboratory clearly supported that the used kits were correctly measuring cortisol metabolite levels in the collected fecal samples without specifically requiring an adrenocorticotropic hormone (ACTH) test. We did not perform ACTH challenge test to show the time passed for peak concentration in feces because the study aim was to consider the variation in FCM levels due to a continuous stressor (3 days each noise treatment and we collected the fresh feces after 24 h). Thus, we minimized unnecessary animal suffering using a noninvasive monitoring technique. In addition, the authors did not perform a biological ACTH validation because European mink is a protected species and the individuals (housed in FIEB) used in this study are participating in the *Ex situ* Conservation Program for European mink acting FIEB as a breeding and research center promoted by Ministerio de Transición Ecológica y Reto Demográfico of Spain.

Statistical analysis

A complete characterization of SEL is supposed to include the time period over which the summation is performed, the distribution of sound events within that period and changes in amplitude of the individual sound events (Popper et al. 2014). Therefore, we calculated SEL levels in each enclosure on the basis of the number of audible playback events (i.e., time period in our study case) from each enclosure and its amplitude. Second, a hierarchical cluster analysis was conducted to determine the number of groups of European mink based on the duration of total exposure time to audible playbacks and their corresponding SEL levels in order to study their differing behavioral characteristics and FCM levels. We adopted the minimum value of Akaike's information criterion as a typical indicator for determining the number of clusters (Dominguez-Ares et al. 2020; Alvarez et al. 2021).

As a result, 2 clusters were determined when mink were exposed to road traffic noise (Cluster #1 grouped 11 mink and Cluster #2 grouped 12 mink) and when they were exposed to human voices (Cluster #3 grouped 11 mink and Cluster #4 grouped 12 mink) (Figure 1). On average, a difference of ~3.9 dBZ in SEL values resulted between Cluster #1 ($M = 96.27$, *standar deviation* $= 1.11$) and Cluster #2 ($M = 100.15$, $SD = 1.10$) during exposure to road traffic noise. On the other hand, an average difference of ~4.0 dBZ in SEL values resulted between Cluster #3 ($M = 97.97$, $SD = 1.64$) and Cluster #4 ($M = 102.01$, $SD = 0.65$) during exposure to human voices. It must be noted that decibel is a relative unit of measurement on a logarithmic scale (in base 10) and it means that each 3 dB increase at a location implies that sound energy is doubled. Therefore, it can be considered that Clusters #1 and #3 correspond to a context in which the exposure to anthropic noises is short-lasting, and intensity is lower. In the same way, Clusters #2 and #4 correspond to a context in which the exposure to anthropic noises is long-lasting, and intensity is higher. Thus, we composed the variable called noise exposure with these clusters, and 3 categories were established for noise exposure: ambient sound, short duration—low intensity (SL), and long-duration—high intensity (LH).

Mixed generalized linear models (GLMMs) were performed to explain the variation in the response variables. We established Poisson distribution error with log link function for locomotor activity duration (because a count was made to record the behaviors and the values were transformed to seconds by multiplying by intervals of 10 s) and Normal distribution error with identity link function for FCM levels (ng/g). Both GLMMs proved the effect of sex (male/
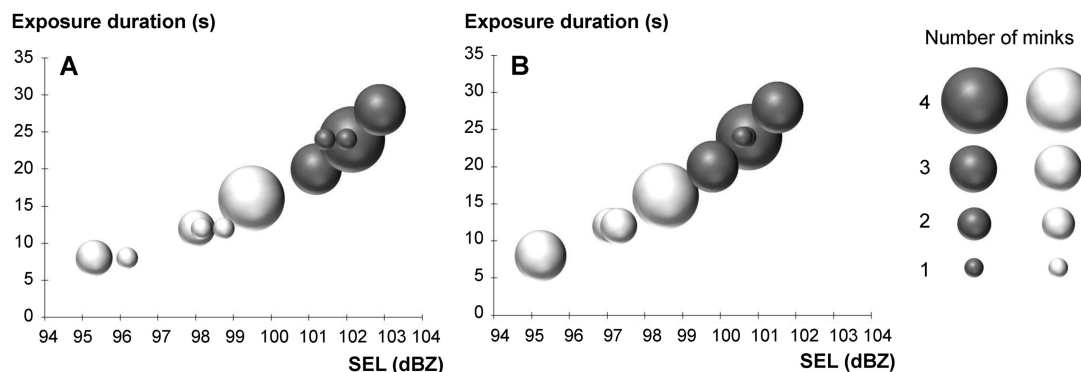
Figure 1. Cluster groups of European mink based on the duration of their total exposure to audible playback(s) and their corresponding SEL level (dBZ). (**A**) Clusters when mink were exposed to road traffic noise (white bubbles represent 11 mink grouped in Cluster #1 and gray bubbles 12 mink grouped in Cluster #2). (**B**) Clusters when mink were exposed to human voices (white bubbles represent 11 mink grouped in Cluster #3 and gray bubbles 12 mink grouped in Cluster #4).

female), age (subadult/adult), type of noise (control/road traffic noise/human voices), and noise exposure (SL/LH) as fixed factors and individuals as a random factor. The following interactions were also tested: sex*noise exposure, age*noise exposure, and type of noise*noise exposure. Results were considered significant at a probability value of $p < 0.05$. Software SPSS version 23.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Data are presented as mean $\pm$ standar error (*SE)*.

Results

Noise levels and total acoustic events

As a consequence of their facility's location in the corridor, European mink were exposed to different number of acoustic events at different intensities depending on the enclosure they occupied. Each enclosure was located in different positions in the corridor, so that some mink were able to perceive up to 6 acoustic events from up to 3 different enclosures on both sides of their enclosures (the loudspeaker was positioned once in each enclosure). Nevertheless, mink located at the end of a corridor perceived noise only from one side of their enclosure. This caused some mink to be exposed to different noise levels for longer than others. Consequently, this resulted in different SEL values for each enclosure (Figure 2).

In addition, spectral analysis of SPL measurements at a distance of 1.5 m from the loudspeaker to the sound level meter revealed a difference of 10 up to 20 dBZ in favor of human voices above road traffic noise between the frequencies of 4 and 10 kHz (Figure 3). On the contrary, road traffic noise remained at a higher level than human voices in frequencies <1 kHz. In this later case, maximum differences of ~15 dBZ were registered at low frequencies such as 125 and 16 Hz (infrasound).

Effect of the noise levels on locomotor activity

The results of GLMM showed that pure effects of sex, age, type of noise, and noise exposure explained the variations of locomotor activity duration (Table 1). Males (89.89 ± 8.39 s) spent more time on locomotor activity than females (40.61 ± 5.47 s). Likewise, adults (81.38 ± 6.95 s) carried out locomotor activity for longer than subadults (36.69 ± 6.38 s). The time of locomotor activity was longer during the ambient sound (117.33 ± 10.10 s), following by road traffic noise (46.74 ± 7.30 s), than human voices (25.22 ± 5.91 s). Locomotor activity lasted less time in mink

under SL exposure (26.89 ± 5.69 s) compared with those individuals under LH exposure (44.31 ± 7.37 s) and the ambient sound (117.33 ± 10.10 s).

In addition, variations in locomotor activity duration were explained by the interactions sex*noise exposure, age*noise exposure, and type of noise*noise exposure (Table 1). Sex*noise exposure interaction indicated that both males and females were less active when subjected to the experimental noises. Females that were exposed to SL were less active than those who were exposed to LH, whereas male locomotor activity duration did not suffer changes between males subjected to SL and those subjected to LH (Figure 4A). Furthermore, age*noise exposure interaction showed that both adults and subadults were less active when subject to the experimental noises. Subadults under SL decreased their time on locomotor activity compared with subadults under LH, whereas there were no differences in locomotor activity duration between adults subjected to SL and those subjected to LH (Figure 4B). Both during traffic noise and human voices, mink exposed to SL decreased their locomotor activity compared with mink exposed to LH. In addition, in both conditions (SL and LH), the response was more pronounced during human voices than during traffic noise, decreasing mink locomotor activity (Figure 4C).

Effect of noise levels on FCM levels

FCM (ng/g) levels were explained by the pure effects of sex according to GLMM (Table 1). FCM (ng/g) levels were higher in males (162.96 ± 17.59 ng/g) than in females (113.75 ± 10.73 ng/g). Likewise, the interactions of sex*noise exposure and type of noise*noise exposure also explained FCM levels (Table 1).

Males subject to SL exposure experimented higher FCM (ng/g) levels than those males subjected to LH exposure. Both groups showed higher FCM (ng/g) levels than basal levels (control). However, there were no differences between both group conditions (SL and LH) in females (Figure 5A). Type of noise*noise exposure indicated that individuals subjected to SL increased the FCM (ng/g) levels compared with those subjected to LH during road noise, whereas the opposite happened during human voices. Mink in group LH during road noise showed a decreased in FCM (ng/g) levels compared with their basal levels reflected during ambient sound. At the same time, human voices increased FCM levels compared with road noise in LH group while FCM (ng/g) levels were higher during road noise than during human voices in SL group (Figure 5B).
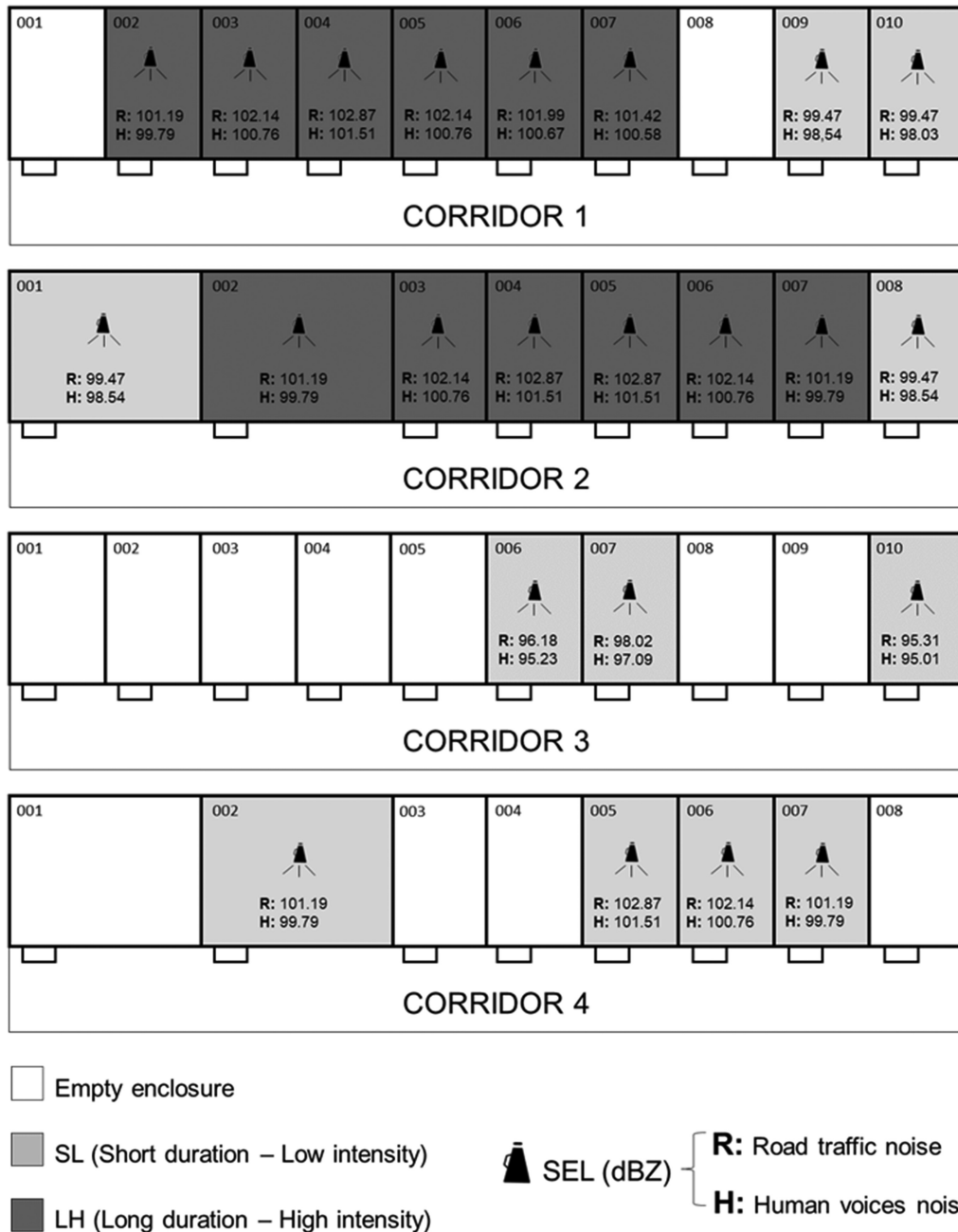
Figure 2. SEL in dBZ value received at each noise event according to the location of each enclosure in the corridor and depending on the type of sound.

Discussion

Our study showed that exposure to anthropic noise, as well as its duration and intensity, affected the behavior and physiological response of the European mink. First, the effect of noise exposure on locomotor activity was manifested in a decrease in this behavior, especially during exposure to human voices. Despite both noises (road noise and human voices) being played at the same volume, variations in their spectral composition were significant. Indeed, human voices were characterized by relatively greater energy content in frequencies between 4 and 10 kHz. This is a relevant result since SPL of human voices resulted between 10 and 20 dBZ higher than road traffic noise at a range of frequencies at which American mink (*Neovison vison*) showed the highest hearing sensitivity during an

experiment to determine the auditory brainstem responses in this species (Brandt et al. 2013). The fact that the locomotor activity was more durable in minks exposed to LH compared with those exposed to SL was probably due to habituation to long-term noise (Nedelec et al. 2016), because when a stressor is more predictable, it can become less stressful (Fletcher and Boonstra 2006). Human voices induced a decrease in locomotor activity more intensely than road traffic noise in both conditions of noise exposure possibly because mink could perceive humans as a potential predator (Beale and Monaghan 2004a, 2004b) and human infrastructures as perturbations that increase predation risk (Quinn et al. 2006). In addition, during both types of noise, SL condition caused a decrease in locomotor activity as it is more unpredictable because being a noise of
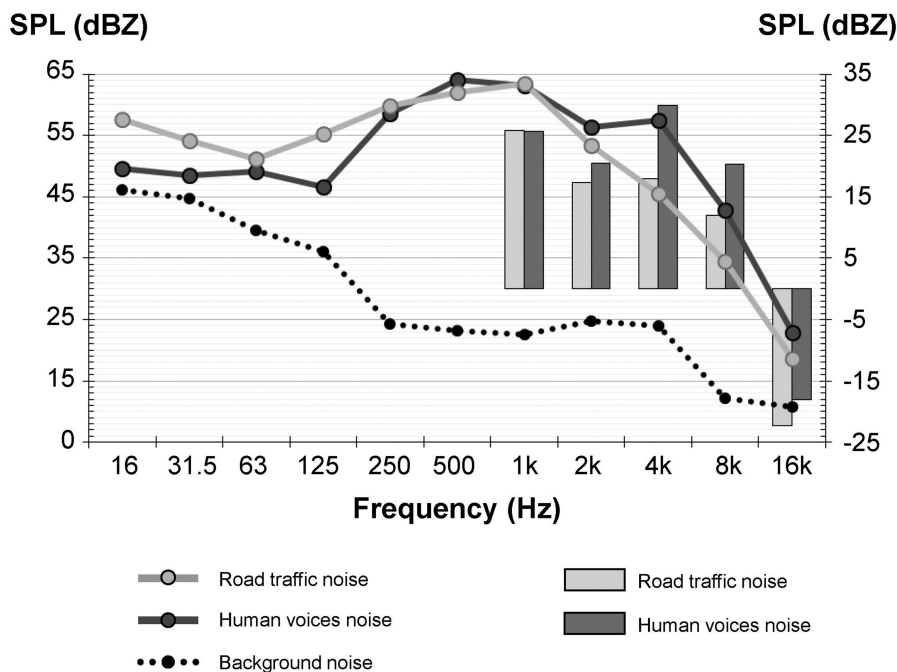
Figure 3. Spectral composition of ambient sound (background level), road traffic noise and human voices measured in dBZ (lines and markers, left axis) and its difference (in dBZ) above or below American mink's hearing threshold at the frequencies of 1, 2, 4, 8, and 16 kHz (bars, right axis).

Table 1 Results of the GLMM analyzing the effects of sex (male/female), age (subadult/adult), type of noise (road traffic noise/human voices) and noise exposure (SL/LH) as pure effects and sex*noise exposure, age*noise exposure, and type of noise*noise exposure interactions on mink locomotor activity duration (s) and FCM levels (ng/g dry feces)

	Factor	F	df1	df2	P-value	Estimate ± SE
Locomotor activity duration (s)	Corrected model	862.18	10	389	**0.000**	−0.91 ± 0.31
	Sex	9.85	1	78	**0.002**	1.31 ± 0.37
	Age	16.46	1	79	**0.001**	2.01 ± 0.37
	Type of noise	735.52	1	396	**0.001**	0.70 ± 0.03
	Noise exposure	61.02	1	396	**0.001**	0.09 ± 0.06
	Sex*noise exposure	153.99	2	396	**0.001**	0.07 ± 0.07
	Age*noise exposure	207.51	2	396	**0.001**	−0.63 ± 0.08
	Type of noise*noise exposure	26.23	1	396	**0.001**	−0.22 ± 0.04
FCM levels (ng/g dry feces)	Corrected model	2.40	10	126	**0.012**	149.97 ± 45.25
	Sex	6.99	1	126	**0.009**	39.01 ± 32.54
	Age	1.97	1	126	0.163	−37.11 ± 33.00
	Type of noise	0.001	1	126	0.976	−47.19 ± 32.10
	Noise exposure	1.51	1	126	0.222	−72.12 ± 44.43
	Sex*noise exposure	5.49	2	126	**0.005**	98.10 ± 47.72
	Age*noise exposure	0.10	2	126	0.902	8.93 ± 47.18
	Type of noise*noise exposure	4.27	1	126	**0.041**	95.78 ± 46.37

Bold means significance differences ($p < 0.05$).

short duration as mentioned above. However, the effect of individual factors (sex and age) must be taken into account, since females and subadults were less active both in a general context of noise and specifically in SL noise conditions. Males roam larger range areas than females (Palazón 1998; Garin et al. 2002) and, in addition, males probably are routinely exposed to noise during patrolling and could have habituated to both sounds conditions in terms of duration and intensity, since they have more potential to defend themselves from a predator than females (in those species whose body size is greater in males) as some studies indicate (Leutenegger and Kelly 1977; van Schaik and van Noordwijk 1989). The age bias could be due to a more cautious nature of younger individuals who

prioritize other behaviors such as concealment when they assess the risk of threat from a noisy stimulus (Ortiz-Jiménez et al. 2021).

Second, the effect of noise exposure on FCMs is not manifested in a general context of noise exposure but depends on the interaction between the type of noise and its duration and noise level. On the one hand, an increase in FCM (ng/g) levels in mink in the SL group compared with the LH group during road noise showed the importance of dealing with noise pollution for conservation, since habituation to roads can lead to road-kills showing a smaller road-effect zone. The road-effect zone is a well-known concept in Road Ecology Science. Road-effect zones are usually much greater than the coverage areas of road networks (Zong et al. 2003) and they are defined
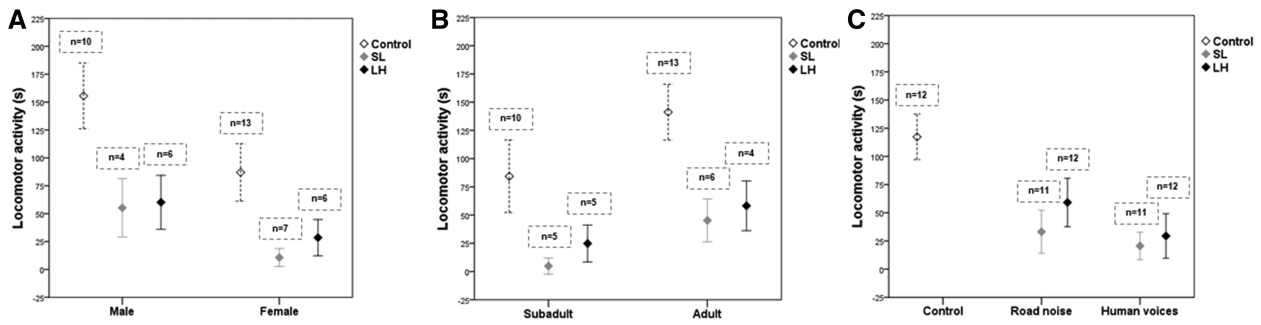
Figure 4. Mean locomotor activity (s) ± *SE* depending on: (**A**) sex and noise exposure; (**B**) age and noise exposure; (**C**) type of noise and noise exposure.
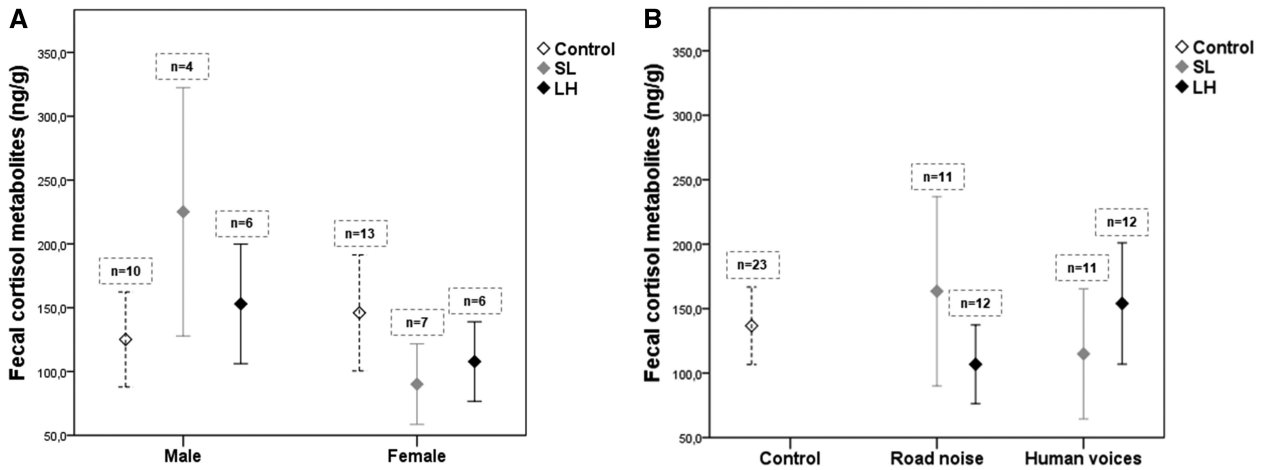

Figure 5. Mean FCM levels (ng/g) ± *SE* depending on: (**A**) sex and noise exposure; (**B**) type of noise and noise exposure.

as the extent where potentially ecological effects occur from the edge of a road (Eigenbrod et al. 2009). Road-effect zones can be species-specific but, depending on the ecological effect to be assessed, they can also be context-specific (Parsons et al. 2020). For instance, orography and design of roads can be crucial in terms of road traffic noise propagation through the potential habitat of sensitive species (Ortiz-Urbina et al. 2020). That mink in LH conditions reduce their FCM (ng/g) levels, even below basal levels, when road noise occurs may be due to habituation by masking of the danger due to the intensity of the sound (Bee and Swanson 2007). This could be the reason for the high unnatural mortality of this species in Spain whose death rate by roadkill was 8.3 individuals/year between the years 2000 and 2005 because roads are a common infrastructure crossing rivers or running parallel to rivers that are occupied by European mink (Palazón 2010). Moreover, from an acoustic point of view, this matter can be aggravated by the fact that water sounds with relatively greater energy in low-frequency ranges can act as an effective auditory masker of road traffic noise in roadsides (You et al. 2010). Even more in case of low traffic volume roads, this combination of factors may reduce the probability of detecting a passing vehicle and, therefore, increase the risk of wildlife road kills. Thus, prioritizing mitigation measures such as road fencing near streams becomes an option that deserves to be considered of particular importance in combination with attractive and safe underpasses (Plante et al. 2019) in conservation programs for European mink. On the other hand, it is possible that human voices induced an increase in FCM (ng/g) levels during LH conditions with respect to SL conditions due to an evaluation of predation risk by

the distance that prey detect the predator (Stankowich and Coss 2006). Because prey animals can react to human vocalizations in the same way as any other predator (Hettena et al. 2014), and humans can be considered a "super predator" for mesocarnivores (Clinchy et al. 2016). In our study, human voices generated significant audible (>20–30 dBZ) that would be within the hearing thresholds of American mink (Brandt et al. 2013) and the European mink's most sensitive range of frequencies. Therefore, a more intense human vocalization noise can be perceived as an immediate danger of predation by proximity (it is more intense because it is closer). Remaining inactive for the long period of time that a stalking predator vocalizes would result in increased physiological stress. In addition, human voices had more effect than road noise under LH conditions, probably because of 2 main reasons: humans are considered superpredators (Darimont et al. 2015; Suraci et al. 2019) and not a simple source of noise and disturbance (Frid and Dill 2002), but also because of spectral differences between road traffic noise and human voices at frequencies of between 6 and 10 kHz. Furthermore, in our study case, waveform of the road traffic noise signal is relatively constant, which makes its intensity to be more predictable while human voices signal fluctuates and shows several peaks at different frequencies (Ortiz-Jiménez et al. 2021). Therefore, alterations in signal regularity make unpredictable aversive events more stressful (de Boer et al. 1989). Even so, note that this startle effect detected in human voices could also occur in the case of roads which have an irregular traffic pattern or have a significant intermittent motorcycle effect (Buxton et al. 2020), something to consider when

characterizing the disturbance being studied for the management of areas intended for the species reintroductions.

It should be noted that individual factors such as sex also modulate the effect of anthropic noises on the concentration of FCMs. We observed a consistent result that males have higher basal concentrations of FCMs than females in many mammalian species such as the Western European house mouse *Mus musculus f. domesticus* (Touma et al. 2003) and the European pine marten (Barja et al. 2011). Some studies related dietary changes to different concentrations of FCMs, which could affect sex if individuals ate different foods (Cavigelli 1999; Foley et al. 2001). Nevertheless, in our study, all mink were fed the same diet, varying only the amount of food depending on the weight of each individual mink. It should be noted that males subject to SL increased their FCM (ng/g) levels more than those subject to LH probably because shorter noises can be more unpredictable than longer and more intense noises (de Boer et al. 1989). Being more predictable in LH, animals would be less active and therefore less exposed to a stress-inducing sound.

Our study supports research that show the great impact caused by roads (Iglesias et al. 2012; Pinto et al. 2020) and nature tourism that is often carried out in large groups (Müllner et al. 2004; Shutt et al. 2014) on mammals. In the case of the European mink (IUCN Critical Endangered Species; Maran et al. 2016), these disturbances can alter behavior strategies enabling them to survive in their natural habitat and hindering in situ conservation. Therefore, we consider it is necessary to pay attention to the ecological repercussions of recreation activities in natural areas (Buxton et al. 2017b), especially where in situ conservation programs are conducted. In this sense, researchers advocate the need of conservation management strategies aimed to reduce the negative impacts of ecotourism on biodiversity (Noriega et al. 2020). Given increasing pressures on national parks and wildlife an effective containment and management strategy may be to concentrate activities on a small number of trails and resistant sites where impacts tend to stabilize in order to limit their severity and spatial extent (Leung et al. 2001; Stronza et al. 2019). Since human voices have been less studied than road noise, it is a good chance to carry out new studies that provide valuable information to this line of research.

In conclusion, European mink modulated locomotor activity duration according to individual factors (sex and age) and external anthropic disturbances (type of sound and noise exposure). At the same time, FCM (ng/g) levels were modulated by sex as individual factor and type of noise and noise exposure as external factors. The major contribution of this study was to prove that type of noise is the prevailing factor in locomotor activity response of European mink while noise exposure is the prevailing factor in FCM (ng/g) levels. In the latter case, road noise had more effect on FCM (ng/g) levels during short duration—low intensity conditions and human voice had more effect during long duration exposure—high-intensity conditions. The results (a reduction of locomotor activity and an increment of FCM) show the importance of protecting reintroduction areas by restricting massive human presence (nature tourism, adventure sports, or hunting) especially during the breeding season, since this is a critical time for the species. Considering that the duration and intensity of sounds play an important role, the type of measures to be taken to protect these areas could be related to the reduction of sound pressure by establishing, time restrictions on access to strategic areas and a capacity limitation of persons and vehicles. In addition, for *ex situ* conservation, it is recommended that captive breeding centers remain silent near the enclosures, because the maintenance of high levels of stress for a long period can cause immune and reproductive suppression among other consequences.

Ethical Note

This research was guide by ASAB/ABS Guidelines for the Use of Animals in Research. Experimental design was devised in accordance with PREPARE guidelines and experimental stage was carried out in compliance with the ARRIVE guidelines. FIEB Foundation is registered as a zoo center and animal experimentation center covered by 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 center in the Ex situ Conservation Program for European mink acting as a breeding and research center promoted by Ministerio de Transición Ecológica y Reto Demográfico of Spain. It is important to point out that the experiment carried out did not have any lasting adverse effects on mink, because the reproductive success was maintained in FIEB.

Authors' Contributions

L.O.J.: conceptualization, investigation, methodology, collected data, hormonal analysis, statistical models, prepared the tables and figures, and writing-original draft. C.I.M.: conceptualization, methodology, made all the analyses of noise, prepared some tables and figures, and writing review and editing. A.I.M.S.: hormonal analysis. I.B.: conceptualization, investigation, methodology, hormonal analysis and validation, statistical analysis, writing review and editing, resources, supervision, and funding. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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