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Front cover: A vocalising White-Spotted Tree Frog *Boana albopunctata*, see article on page 34. (© Gerson Muzzi)

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Dichotomy in morphology of the same genetic lineage of green turtles

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Morphological studies of marine turtles are important to provide insight into changes in their developmental environment. This study aimed to determine green turtles' *Chelonia mydas* morphological differences within the same genetic lineage in the eastern Mediterranean MED3 management unit and to find the best conversion equations between carapace size. A total of 106 adult green turtles (curved carapace length [CCL] range 79–105 cm) were measured on the five major nesting beaches of the eastern Mediterranean during 2020 and 2021. Morphological differences were tested with PERMANOVA and the relationship among body sizes was tested by linear regression. In the eastern Mediterranean green turtles, the mean CCL and SCL (straight carapace length) were 88.5 cm and 83.5 cm, respectively. There were no statistically significant differences in any of the examined morphological characteristics of green turtles collected from five nesting beaches. In the clustering analysis, however, it was found that all the turtles fell into two distinct groups: larger (> 95.2 cm) and smaller (< 85.2 cm) turtles. As well, the conversion equations between CCL and SCL showed a high coefficient of determination ($R^2 = 0.938$). We suggest that the conversion equations may be applied to all green turtles belonging to this population and nesting in the eastern Mediterranean.

Keywords: Chelonia mydas, morphology, conversion equation, K-means, eastern Mediterranean

INTRODUCTION

Through morphological studies (van Dam & Diez, 1998), it is possible to obtain basic information on topics such as animal development, evolution, biomechanics, behaviour, ecology, and physiology. Marine turtles provide great opportunities to study morphological variations because of their global distribution and because they move across very different ecological zones (Tiwari & Bjorndal, 2000). For instance, local conditions such as food availability and nutrient uptake rates affect food stock dynamics. This could affect the growth rate and hence the carapace length of marine turtles (Chaloupka et al., 2004). Further, the phenotypic variation of a species can be used to characterise the populations (Glen et al., 2003). For example, the relationship between size and weight of the body form can be used to describe the degree of differentiation of different populations of marine turtles (Figueroa & Alvarado, 1990; van Dam & Diez, 1998). Many researchers have indicated that green turtles differ between regions in carapace size (Figueroa & Alvarado, 1990), skull morphology (Kamezaki & Matsui, 1995), and flipper size (Wyneken et al., 1999). Similarly, it was stated that morphometric scaling varies among life stages of the loggerhead turtle in the western north Atlantic (Marn et al., 2015). In addition to these, inter-regional variation in carapace shape of the green turtle between Atlantic, eastern Pacific, and western Pacific genetic lineages was investigated (Álvarez-Varas et al., 2019), and at least three distinct morphotypes are proposed. Similar research was also conducted on foraging grounds (Álvarez-Varas et al., 2021), and all body traits (carapace, plastron, head and flipper) of south-central/western Pacific genetic lineage turtles showed variations between foraging grounds (south-west Atlantic and eastern Pacific).

In addition to determining inter-regional variations, carapace size and tail length are used to distinguish between sexes in both adults (Godley et al., 2002) and hatchlings (Sönmez et al., 2016). Also, carapace sizes help us determine growth rates in adult turtles (Omeyer et al., 2018). Hatchlings' morphology can give us clues about their locomotor performance and their survival, as larger hatchlings are associated with faster crawling and swimming speeds (Ischer et al., 2009). Growth and proportional increases in carapace width versus length in post-hatchlings may provide clues about higher morphological defenses and the ability to escape from predators (Salmon et al., 2016). Also, we can use morphology to understand how nest relocation affects the hatchling phenotype, e.g. nest relocation may cause scute abnormalities (Sönmez et al., 2011; Sönmez, 2018). Morphology can be used to estimate the reproductive output or clutch size of nesting turtles, as larger females

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are associated with larger clutch size (Broderick et al., 2003), and assess longitudinal and temporal trends in nesting populations (Tiwari & Bjorndal, 2000; Sönmez, 2019).

Carapace size is measured at nesting beaches to determine the minimum size at sexual maturity, reveal the body size relationship with reproductive output, and monitoring nesting female size for a certain nesting beach (Bolten, 1999). It is also measured on foraging grounds to determine the frequency of turtle size classes and monitor their growth rate (Bolten, 1999). Thus, information on habitat quality and physiological conditions of marine turtles could be obtained by analysing the growth rates (Bolten, 1999) hence the carapace size. There are two types of linear measurements for carapace size, which are straight line measurements (taken with calipers) and curved measurements (taken with a flexible tape measure). Straight line measurements are more reliable because curved measurements tend to be less accurate and precise due to irregularities in the surface of the carapace and epibionts (Bjorndal & Bolten, 1988; Bolten, 1999). During field studies, carapace sizes are commonly measured, but the lack of size data in both foraging and nesting populations causes a significant gap, particularly in Mediterranean green turtle populations (Casale et al., 2018). Moreover, there are no equations to convert between the carapace size of the green turtle for the Mediterranean population. This equation can be useful for finding the missing measurement when one of the measurements is unavailable. It may therefore be helpful for comparative studies with other populations.Furthermore, the minimum carapace sizes to help categorise stranding green turtles as adults or sub-adults (Türkozan et al., 2013; Casale et al., 2018) are limited for the Mediterranean. In a recent study on the Samandağ beach, it was reported that the carapace size of the nesting green turtles decreased over the years and the minimum curved carapace length (CCL) was 72 cm (Sönmez, 2019). There is no study in which nesting beaches are represented separately, and a common evaluation is carried out for the eastern Mediterranean population.

The green turtle, which has a global distribution in tropical and subtropical waters, has genetically differentiated groups in the Mediterranean due to strong natal homing (Bowen & Karl, 2007). According to the International Union for Conservation of Nature (IUCN) (Seminoff, 2004), the population of green turtles in the Mediterranean is Endangered (EN). It is also one of the 17 most important management units (MUs) (Wallace et al., 2010) because of the strength of the threats in the area and the risk of extinction. Based on a study of mitochondrial short tandem repeats (mtSTR), Tikochinski et al. (2018) suggest that the Mediterranean green turtle population consists of at least 4 MUs. These MUs are Akamas, Alagadi, Israel, and Turkey. A recent study, Karaman et al. (2022) extended the above-mentioned study and proposed a minimum of 3 MUs for the Mediterranean population. The MUs are MED1 (Akamas and Akdeniz), MED2 (Alagadi), and MED3 (North and South Karpaz, Israel, Samandağ, Akyatan, Sugözü, Kazanlı, Alata, and Davultepe). The nesting beaches of Alata, Kazanlı, Akyatan, Sugözü, and Samandağ, which are the main focus of this study, are located in MED3, with these beaches accounting for about 78 % of all nests in the Mediterranean (Casale et al., 2018). Therefore, this study will fill the following knowledge gaps by determining in MED3 MU: a) carapace size ranges of nesting green turtles and morphological similarities and differences between nesting beaches, b) obtaining the best conversion equations between carapace dimensions and c) identification of the presence of polymorphy.

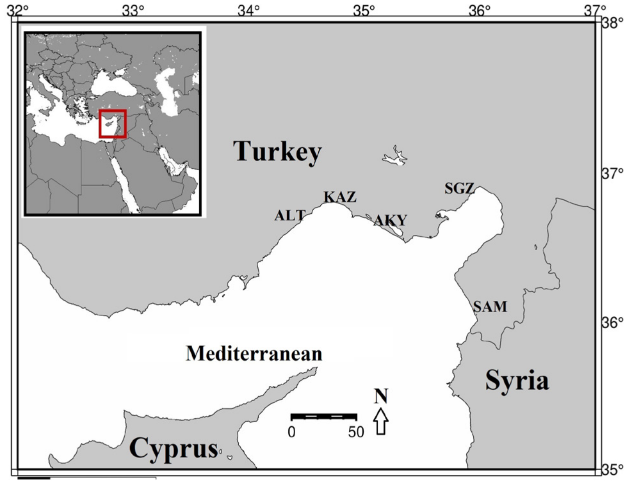

Figure 1. Map depicting the nesting beaches of *Chelonia mydas* in the eastern Mediterranean where carapace sizes were measured (ALT: Alata, KAZ: Kazanlı, AKY: Akyatan, SGZ: Sugözü, SAM: Samandağ)

METHODS

The study area includes Alata, Kazanlı, Akyatan, Sugözü, and Samandağ beaches (Fig. 1), which are major nesting beaches for *C. mydas* in the MED3 MU (Karaman et al., 2022). The average number of nests for the recent five years on these beaches ranges between 125 and 365 (Casale et al., 2018). Night patrols collected samples from each nesting beach during the conservation studies 2020 and 2021 nesting seasons. Samples were collected from the last week of June to the second week of July, and each beach was visited a total of six times during the two years. Five people patrolled the beach at night to observe female nesting turtles. Turtles were tagged and measured after they laid their eggs. The metal tags were placed on the trailing edge of the left fore flipper, as recommended by Balazs (1999). In each turtle, curved carapace length (CCL) and width (CCW) and straight carapace length (SCL) and width (SCW) were measured according to Bolten (1999) and Sönmez (2019). Three carapace measurements were carried out by an observer at each nesting event for each female, and a mean was calculated. A flexible tape measure and a mechanical caliper (Haglöf Mantax Blue, accurate to the nearest mm) were used to obtain the CCL and SCL, respectively.

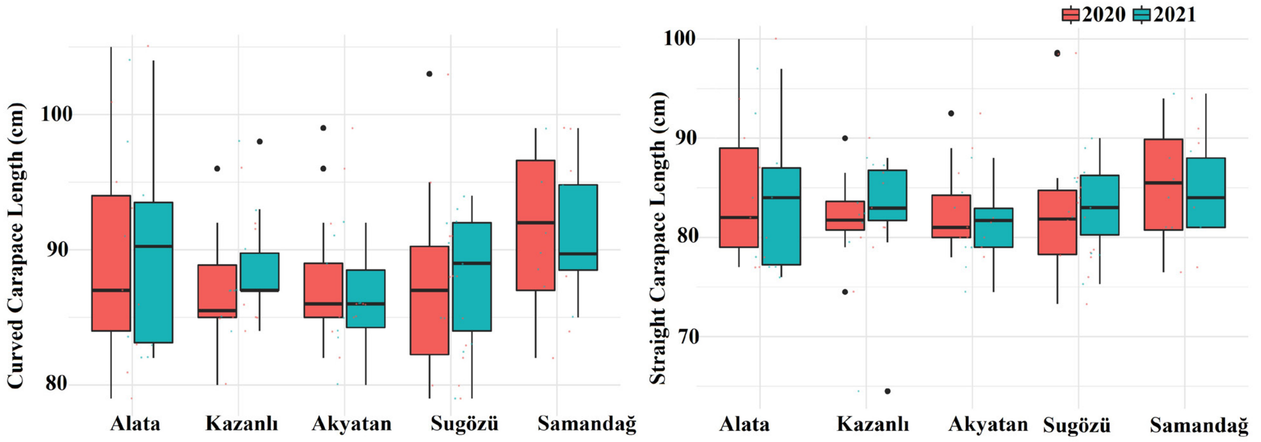

Figure 2. Boxplot of CCL and SCL by year and nesting beach, with standard deviation, median and data points. Whiskers showing highest and lowest observations. Black dots show statistical outlier value.

Data analyses

The study's methodology and models are designed around three primary topics: (i) modelling the relationships between morphological traits and nesting beaches, (ii) identifying the linear relationship between body measurements, and (iii) multivariate estimation of morphological fit using a morphological trait data set. Before evaluating the data set, exploratory data analysis was performed using descriptive statistics to examine its structure. A boxplot was then drawn to visually evaluate how CCL and SCL changed over the years and in relation to nesting beaches. The independent t-test was carried out to examine how the four body measurements changed by year (for 2020 and 2021) on each nesting beach, and along the entire beach.

Then, The Permutational Multivariate Difference-based ANOVA (PERMANOVA), the main test (and pairwise comparisons) were used to determine whether morphological traits differed substantially between nesting beaches (Anderson & Walsh, 2013). The pairwise PERMANOVA was utilised to compare beaches that showed significant differences in PERMANOVA (p-value 0.05).

In the study, we utilised linear regression analysis to estimate the relationship between body sizes, which was specified in the equation:

$$Y_i = \beta_0 + \beta_1 X_i + \varepsilon_i$$

where, Y is vector of dependent variable, *X* is vector of independent variable, β_0, β_1 are model parameters, and ε_i is error term.

The analysis was based on a model that transformed the dependent variable's variation into a linear function of the independent variable. The β_1 term is the regression coefficient, which describes the empirical relationship between dependent and independent variable, whereas ε denotes the random error of, which encompasses environmental variation (Khadivi-Khub, 2014).

The non-hierarchical K-means clustering algorithm was employed in this study to determine whether or not subjects have a polymorphic structure in terms of dimensions and to group them into appropriate clusters (Wagstaff, 2012). The silhouette coefficient was calculated to determine the efficacy of the K-Means algorithm's cluster separation (Kodinariya & Makwana, 2013). The aim here is to reduce the variation within the group, if there is a variation on the basis of size, and to decompose it so that each group has homogeneous variance within itself.

The statistical analyses were performed with R version 4.1.0 (R Core Team 2021) using the R packages of ade4 (Dray & Dufour, 2007), pairwiseAdonis (Arbizu, 2019), vegan (Oksanen et al., 2020), AppliedPredictiveModeling (Khun & Johnson, 2018), and summarytools (Comtois, 2021).

RESULTS

A total of 106 green turtles were measured in four body dimensions. The resulting descriptive statistics in terms of each nesting beach are shown in Table 1. The mean CCL and CCW were 88.5 ± 5.8 cm (79–105) and 79.1 ± 5.5 cm (70–102), respectively. The mean SCL and SCW were 83.5 ± 5.5 cm (73.3–100) and 64.6 ± 4 cm (57.6–77.2), respectively. The boxplot that was drawn to visually evaluate CCL and SCL changed over the years in terms of nesting beaches is shown in Figure 2.

The independent samples t-test showed that there was no statistical difference between years in terms of the four body dimensions on each nesting beach, and in total ($p > 0.05$). Multivariate statistics (PERMANOVA) identified no significant differences in variables between nesting beaches ($p > 0.05$). Also, it confirmed that the four body dimensions showed no variation between the years according to the nesting beaches ($p > 0.05$). The PERMANOVA pairwise test showed no significant differences for all body sizes in the pairwise comparison of nesting beaches ($p > 0.05$).

Through linear regression the CCL and SCL dimensions showed significant relationships ($R^2 = 0.938$, df = 102, $p = 0.0002$). The conversion equation between them:

$$CCL = 1.03 + 1.04 * SCL \quad \text{(Fig. 3a).}$$

Table 1. The descriptive statistic of morphological data on each nesting beach (CCL: Curved carapace length, CCW: Curved carapace width, SCL: Straight carapace length, SCW: Straight carapace width)

		CCL		CCW		SCL		SCW	
Nesting beach	**n**	**Mean (Sd)**	**Min-Max**	**Mean (Sd)**	**Min-Max**	**Mean (Sd)**	**Min-Max**	**Mean (Sd)**	**Min-Max**
Alata	22	87.8 (4.2)	80–98	78.5 (3.6)	73–87	83.1 (3.5)	74.5–90	64.4 (2.8)	59.5–70
Kazanlı	21	87.1 (4.5)	80–99	77.2 (4.5)	72–90	81.9 (4.2)	74.5–92.5	63.1 (3.9)	58–72
Akyatan	21	89.8 (7.7)	79–105	80.8 (8.1)	70.5–102	84.4 (7.1)	76–100	65.2 (4.5)	58.7–73.5
Sugözü	17	91.2 (5.4)	82–99	80.6 (5.1)	73–89	85.1 (5.4)	76.5–94.5	64.5 (3.4)	60–71
Samandağ	25	87.3 (5.8)	79–103	78.3 (4.7)	70–92	83.1 (6.2)	73.3–98.6	65.3 (4.7)	57.6–77.2
Total	**106**	**88.5 (5.8)**	**79–105**	**79.1 (5.5)**	**70–102**	**83.5 (5.5)**	**73.3–100**	**64.6 (4)**	**57.6–77.2**

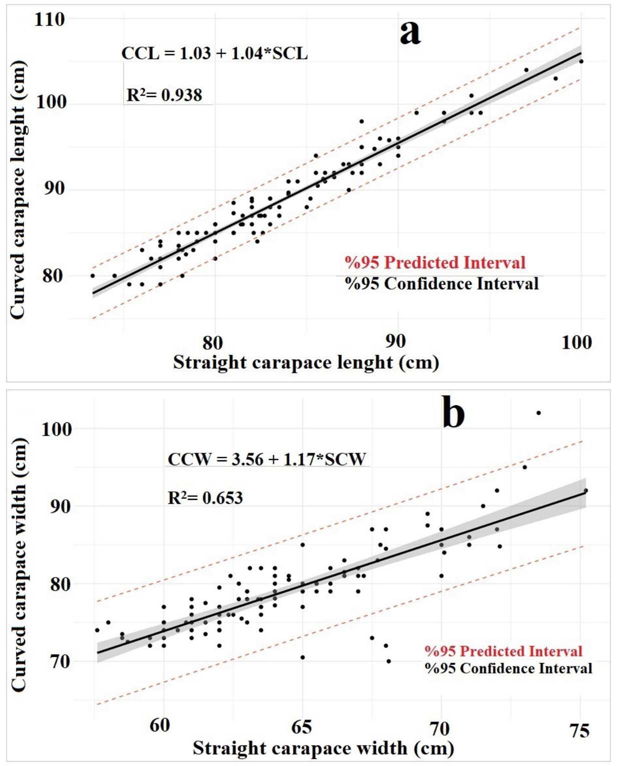

Figure 3. The significant relationships between CCL and SCL, CCW and SCW. The red dots represent the 95 % predicted interval and the grey dots represent the 95 % confidence interval. Conversion equation with the coefficient of determination in each relation is shown on the left of Figure.

According to these conversion equations, there is a 1.04 cm increase in CCL for every 1 cm increase in SCL. The CCW and SCW dimensions showed significant relationships ($R^2 = 0.653$, df = 102, p = 0.0002). The conversion equation between them:

$$CCW = 3.56 + 1.17 * SCW$$ (Fig. 3b).

According to these conversion equations, there is a 1.17 cm increase in CCW for every 1 cm increase in SCW.

Two clusters were found based on four body dimensions for all nesting beaches (Fig. 4). The within-cluster sum of squares by the cluster was 60.8 %. The first cluster (69.1 %) included individuals with smaller (< 85.2 cm) body dimensions, and the second cluster (33.1 %) had larger (> 95.2 cm) body dimensions (see Table 2 for details).

DISCUSSION

Our results showed that the mean CCL and SCL on the nesting beaches of the eastern Mediterranean were 88.5 cm and 83.5 cm, respectively. The minimum CCL was 79 cm and the SCL was 73.3 cm, both of which were recorded for turtles nesting on Samandağ beach. The PERMANOVA tests showed no clear difference between nesting beaches, and they overlapped each other. The nesting beaches in the present study are included in the same MU (MED3) with the Israel, north and south Karpaz, and Davultepe beaches. There is limited study on the morphological data for the other MUs. Only one study has reported the green turtle CCL size of 91.5 cm for Alagadi (MED2) (Broderick et al., 2003). The CCL data for nesting beaches within the same MU (MED3) presented in this study is shown in Supplementary Table S1.

The smallest nesting turtle was recorded on the Samandağ beach as 72 cm in previous years (Sönmez, 2019). In the present study, the lowest CCL recorded on the Samandağ beach is also the lowest CCL recorded in the same MU and around the world (see Supplementary Table S1 for details). Why is the Samandağ population smaller than other populations in the same MU? This may be due to the fact that the CCL size of the Samandağ population tends to decrease over the years (Sönmez, 2019). Also, the size of the Samandağ population may be affected by the recruitment of new females. The CCL size of the green turtle nesting in Cyprus decreases over time due to the recruitment of neophytes (Stokes et al., 2014). Another reason may be the high mortality rate of nesting turtles as a result of anthropogenic or natural effects. Sönmez (2018) reported that the CCL size of the stranded green turtle on the Samandağ beach increased after 2012. It is recommended to investigate the long-term morphological tendency or differences of other nesting beaches within MED3 MU.

The size data reported in several reports from various nesting beaches in different regions or oceans recorded larger mean CCL and SCL values than that of the Mediterranean nesting population (see Supplementary Table S1 for details). Previously, it was stated that the Mediterranean population of green turtles was smaller than the Atlantic and Pacific populations (Erhart, 1982). Mediterranean green turtles probably colonised from North Atlantic green turtles at the Younger Dryas Event, a global cooling event 10,000 years ago (Encalada et al., 1996). The observed size differences may be due to a number of factors in addition to genetic differentiation, as follows:

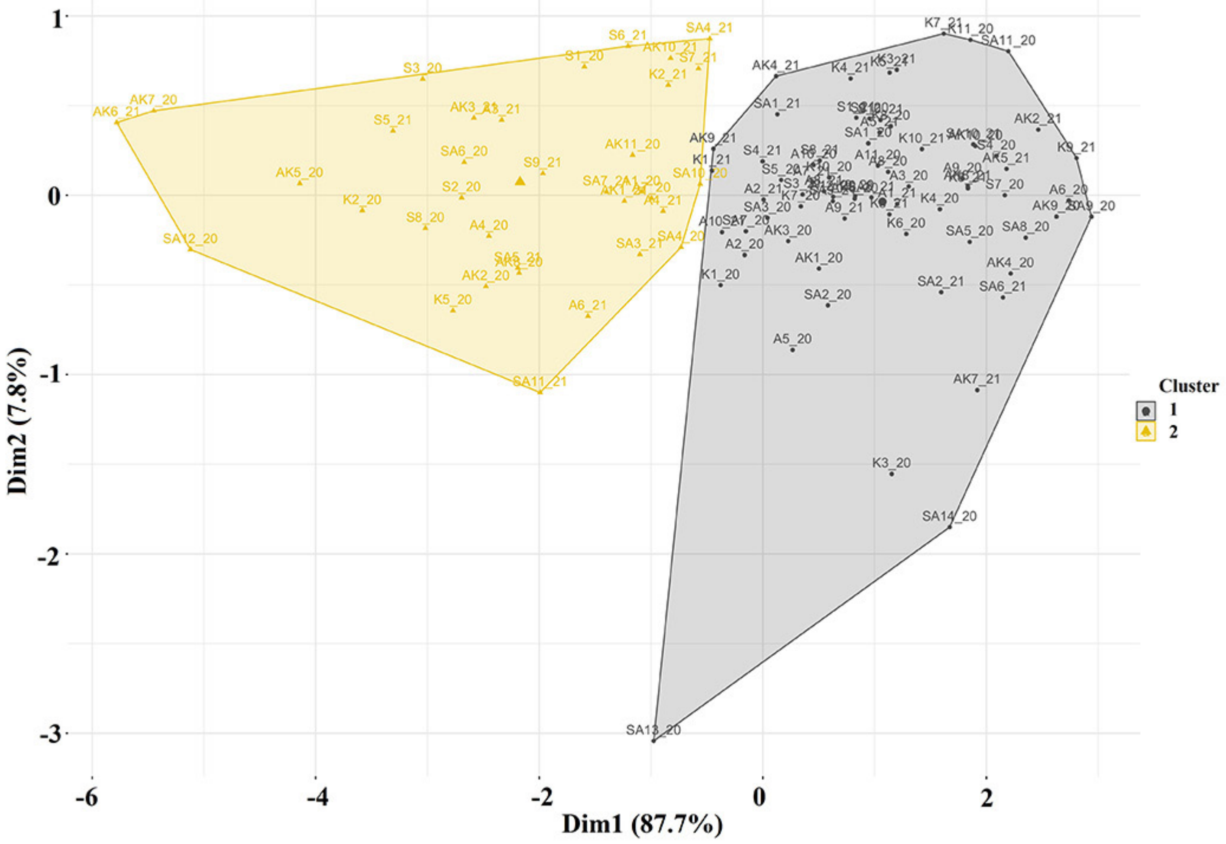

Figure 4. Two clusters based on morphological data for five nesting beaches. The grey cluster indicates first (smaller, <85.2 cm) and the yellow cluster indicates second (larger, > 95.2 cm). Figure includes nest numbers and nesting beach short names of each specimen. Paying attention to the nesting beach of each sample, it can be seen that each nesting beach is represented in both clusters.

Table 2. Descriptive statistics of each morphological dimension represented under two clusters as a result of non-hierarchical K-means clustering algorithm (CCL: Curved carapace length, CCW: Curved carapace width, SCL: Straight carapace length, SCW: Straight carapace width)

	Cluster 1			Cluster 2		
	n	**mean (±sd)**	**min-max**	**n**	**mean (±sd)**	**min-max**
CCL	71	85.2 (±3.01)	79–92	35	95.2 (±3.94)	90–105
CCW	71	76.0 (±2.69)	81–76	35	85.0 (±4.74)	79–102
SCL	71	80.3 (±2.85)	73.3–86.5	35	89.6 (±4.13)	84–100
SCW	71	62.7 (±2.92)	57.6–77.2	35	68.3 (±3.16)	62.4–75.2

(i) Food abundance; the Atlantic system has a richer nutrient level than the Mediterranean system (Tiwari & Bjorndal, 2000). Even within the same population, females experiencing higher food availability are larger (Marn et al., 2017). It has been stated that Atlantic loggerhead turtles have better feeding conditions and are larger than Mediterranean loggerhead turtles (Marn et al., 2019). Therefore, it may be considered that low food availability may have a size-reducing effect on the Mediterranean green turtle.

(ii) growth rate and maturation; marine turtle growth rate is connected with size (i.e. SCL or CCL), and green turtles may have different growth rates in different regions (i.e. the Atlantic and Pacific Oceans) (Bjorndal et al., 2000; Omeyer et al., 2018). The rate of growth among green turtle populations with the same mtDNA haplotype may vary depending on environmental conditions (Chaloupka et al., 2004). Marn et al. (2019) noted that Mediterranean loggerhead turtles grow and mature faster than their Atlantic counterparts due to faster assimilation, but reach a smaller ultimate size due to lower food availability and higher somatic maintenance. Also, it was stated that Mediterranean loggerheads are sexually mature at a smaller size due to the lower cumulative investment to maturation (Marn et al., 2019). Depending on environmental conditions such as food availability and nutrient uptake rates, green turtles may have a smaller ultimate size due to faster maturity and growth.

The conversion equations for green turtles in the eastern Mediterranean were first reported by this study. Although there is a conversion equation between CCL and SCL for the loggerhead turtle in the Mediterranean (Casale et al., 2017), its absence has been noted as a deficiency for the green turtle in the Mediterranean (Casale et al., 2018). There are not many conversion equations for green turtles globally. (Supplementary Table S2). Moreover, the existing conversion equations are specific to a type of feeding ground or are made of stranded individuals, that is, to research such as the relationship between age and size or sexual dimorphism. Moreover, some of them also cover different life stages (Supplementary Table S2). In contrast, our analysis is based on nesting female data. The coefficient of determination (R^2) in our study is similar to that of other studies, only higher than the value found by the authors in Ascension Island (Supplementary Table S2). Because growth rate decreases as carapace size increases (Bjorndal & Bolten, 1988; Patricio et al., 2014; Colman et al., 2015; Omeyer et al., 2018), conversion equations incorporating different life stages may potentially increase data scatter and model uncertainty. In addition, we should not forget the morphological scale between life stages. There are no morphological scaling studies on green turtles. However, it was stated that although morphometric scaling in loggerhead turtles differs between life stages, a common model can be used for all life stages (Marn et al., 2015). Considering a conversion equation that will cover the entire Mediterranean and all life stages, the effect of different growth rates in different regions (Bjorndal et al., 2000; Omeyer et al., 2018) and different life stages (Bjorndal & Bolten, 1988; Patrício et al., 2014; Colman et al., 2015) should not be ignored. Therefore, we suggest that our conversion equations can be applied to just all green turtles belonging to this MED3 population and nesting in the eastern Mediterranean.

MED3 management unit, including the five beaches analysed in this study, is important for genetic variability because it contains a set of partially connected populations (especially the Samandağ and Alata hubs of connectivity) (Karaman et al., 2022). Samandağ nesting beach connects Alata, north Karpaz, and Israel to the nesting beaches of Akyatan, Kazanlı, Sugözü, and Davultepe (Karaman et al., 2022). The fact that these five beaches have connection hubs for genetic diversity as well as display morphological compatibility, reveals the need for special conservation and management plans.In this context, future body sizes may decrease due to the effects of temperature on the developmental and physiological processes of marine turtles as a result of global climate change (Ohlberger, 2013; Marshall et al., 2020). Currently, some populations exhibit decreases in body size (Sönmez, 2019; Le Gouvello et al., 2020; Mortimer et al., 2022). Considering the relationship between reproductive output and morphology, which is that larger females have greater reproductive output (Broderick et al., 2003), changes in annual nesting activities with the body size may help contribute to better estimates of the population size structure and abundance (i.e. hatchling recruitment) (Wu et al., 2022). Therefore, it is recommended that macro-ecological models (Wu et al., 2022) of size dependent reproductive output should be "reveal" for the MED3 population, which will play a role in revealing the future conservation and management plan.

Although the five nesting beaches are not separated in terms of morphological characteristics and are clustered together, they are divided into two clusters that correspond to the sizes of turtles (smaller and larger). Therefore, if a morphological study is designed in the future, sampling from any beach might be sufficient. However, because of the dichotomy, it could be necessary to sample both size groups, because there are individuals representing each nesting beach in each cluster (see Fig. 4). So, why are they separated into two different clusters even though they are in the same MU and overlap in clusters?

First, this MED3 MU population may have size-dependent habitat use, i.e. a polymorphic foraging strategy. Polymorphic foraging strategies in green turtles have been studied using different methods such as satellite transmitters (Richardson et al., 2013), stable isotope analysis (Hatase et al., 2006), and stomach content (Jiménez et al., 2017). Also, size-dependent post-nesting habitat use for the green turtle has been reported in the Galapagos using satellite tracking data (Seminoff et al., 2008), and it has been suggested that smaller turtles may prefer neritic waters. Similar polymorphic foraging strategies have been reported for loggerhead turtles in Japan (Hatase et al., 2002). Larger females (> 95.2 cm) may benefit from offshore ocean habitats, and smaller females (< 85.2 cm) may benefit from neritic habitats in this MED3 MU population. In MED3 MU the unpublished data indicates that two foraging strategies depend on size, smaller and larger size females prefer neritic and pelagic feeding strategies, respectively (Yalçın Özdilek, unpublished data).

Second, the migration routes of the two clusters may differ. Shorter migrations may result in smaller sizes in turtles due to a similar maturity age and lower growth rate (Casale et al., 2011). Cluster 1, with a smaller body, may use different foraging habitats as a neritic area, with shorter migration routes.

Lastly, the growth rates of the two clusters may differ. The growth rate among populations of green turtles with the same mtDNA haplotype may vary depending on environmental conditions such as food availability and nutrient uptake rates in the foraging ground (Chaloupka et al., 2004). In juvenile loggerhead turtles, the neritic feeding strategy would have a greater growth advantage, while the oceanic feeding strategy provides slower growth but a safer life cycle (Peckham et al., 2011). It is known that nutrient richness in different habitats affects the growth of marine turtles (Bjorndal, 1985) and when food availability increases, turtles grow larger (Stubbs et al., 2020). Food stock dynamics subject to local environmental stochasticity can lead to differences in the CCL size of green turtles (Chaloupka et al., 2004).

In a mechanistic model based on Dynamic Energy Budget (DEB), it was found that the allocation of energy to the growth of green turtles occurs more slowly at lower temperatures (Stubbs et al., 2020). It was also stated in this modelling that CCL length was mostly affected by the presence of food, whereas the effect of temperature would not be discernible (Stubbs et al., 2020). Similarly, the growth rate of loggerhead turtles was primarily affected by temperature and also positively correlated with available food (Marn et al., 2017).

In conclusion, the adult females nesting on the five major nesting beaches of the eastern Mediterranean have a mean CCL of 88.5 cm and a mean SCL of 83.5 cm. The minimum recorded CCL and SCL are 79 cm and 73 cm, respectively. Nesting populations were not morphologically separated and clustered. Also, the conversion equations that can be used between CCL and SCL were first obtained in the Mediterranean. These are the first published conversion equations for carapace size for eastern Mediterranean green turtles. Adult females from the five nesting beaches were divided into two clusters, larger and smaller. Presumably, although these two clusters are the same MU (Karaman et al., 2022), they may use different foraging grounds. The fact that these five beaches are hubs of genetic diversity connections (Karaman et al., 2022) and that they are morphologically similar shows how important it is to find special ways to protect and manage them.

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Acoustic monitoring of anuran communities in road noise disturbed soundscapes

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Vocalisation is the main form of communication in frogs. These signals have different social structures and functions. Road noise has increased over the past few decades to the point where it can mask acoustic signals with impacts for animal communication. Anurans are sensitive to noise masking, but few studies have investigated how noise impacts their vocal behaviour. Here, we compared noise levels and activity, as well as calling activity and richness of aurally-identified species, between two sites; one near and one far from a road. We also assessed the potential of noise masking. Noise was significantly higher at the site near the road and during the day at both sites, while vocalisations were more frequent at the far site and during the night. Species richness and composition was the same at both sites, however, *Boana albopunctata*, *B. cipoensis*, and *Scinax curicica* had greater vocal activity at the far site while *S. squalirostris*, *Leptodactylus jolyi*, and *Dendropsophus minutus* had greater vocal activity at the near site. Traffic noise was found to overlap with the frequencies occupied by vocalisations. Since many natural areas around the world are bordered by roads, we emphasise the importance of establishing regulations for the control and monitoring of road noise.

Keywords: Anurans, vocalisation, passive acoustic monitoring, noise pollution

INTRODUCTION

Amphibians are facing a global extinction crisis and represent the most threatened taxon of the world (IUCN, 2021). It has become clear that it is very important to understand the factors contributing to amphibian declines (Houlahan et al., 2000; Stuart et al., 2004). According to Fahrig & Rytwinsky (2009), anurans are among the taxa most threatened by roads, mainly due to roadkill, and studies have found lower species richness and/or abundance in noisier environments (Gibbs & Shriver, 2005; Colino-Rabanal & Lizana, 2012; Grace & Noss, 2018). This finding may be due to animals moving away from noisy places, either temporarily or permanently. On the other hand, some anuran species can persist at noisy areas by adjusting their vocal behaviour to improve communication in the presence of noise (Brumm, 2013). Some such documented vocal adjustments are: avoidance of vocalising during noisy times of the day; increase call amplitude (Lombard effect); and increase or decrease call frequencies, number of notes, syllables and calling rate (Schwartz & Bee, 2013, Leon et al., 2019). However, it is unclear if these adjustments are sufficient to overcome acoustic interference imposed by traffic noise and its negative consequences (Parris et al., 2009). Furthermore, the production of sounds by anurans involves high energetic costs (Taigen & Wells, 1985), and so to change call characteristics to effectively communicate in noisy areas can be even more costly and bring negative consequences for species fitness (Bucher et al., 1982; Taigen et al., 1985; Prestwich et al., 1989; Wells & Taigen, 1989; Emerson, 2001).

Vocalisation is the main communication mechanism in frogs (Morais et al., 2012; Forti et al., 2015; Heard et al., 2015; Köehler et al., 2017). Many species are capable of producing sounds with different functions that vary depending on the social context (Duellman & Trueb, 1986; Narins et al., 2006; Wells & Schwarts, 2007; Toledo et al., 2015). For frogs, acoustic signals are important for species recognition (Gambale et al., 2014; Guerra et al., 2017), for sexual partner attraction (Haddad & Cardoso, 1992; Lingnau et al., 2004; Costa & Toledo, 2013), for warning about the presence of predators and for the defense and maintenance of breeding sites (Toledo et al., 2015).

Anurans can be severely affected by noise, as it can mask their acoustic signals and prevent individuals from effectively receiving and interpreting the sounds of interest (Barber et al., 2010; Dowling et al., 2012; Kunc & Schmidt, 2019; Leon et al., 2019). One of the characteristics of road traffic noise is that it has more spectral energy (amplitude) at low frequencies, below 1000 Hz (Bee & Swanson, 2007; Cho & Mun, 2008; Cunnington & Fahrig, 2010). Thus, low frequency signals, such as those produced by many frog species, are expected to experience greater masking than species with higher frequency call types (Legett et al., 2020). The masking effect consists of temporal and spectral overlap (total or partial) between sounds and occurs, for example, when noise is emitted at the same time as the vocalisations of animals and occupies the same frequencies and amplitudes (or higher). Studies with frog species report behavioural changes (Sun & Narins, 2005; Vargas-Salinas et al., 2014; Zhang et al., 2015, Nelson et al., 2017; Medeiros et al., 2017; Higham et al., 2021), physiological (Tennessen et al., 2014; Kaiser et al., 2015) and serious consequences during reproduction (Wollerman & Wiley 2002; Bee & Swanson, 2007; Grace et al., 2017; Simmons & Narins, 2018; Schou et al., 2021).

In the present study we analysed calling activity of two anuran communities in a noise disturbed soundscape at an important site of the Espinhaço Mountain Range in Brazil, that houses endemic species. Anuran species were acoustically monitored at two sites, one near (50 metres) and another far (500 metres) from a paved road, to test the following hypothesis: (1) Areas near and far from the road differ in background noise and anuran calling activity. We predict higher noise levels and lower calling activity at the site near the road. (2) Anuran species richness differ between the sites. We expect greater species richness at the site far from the road.

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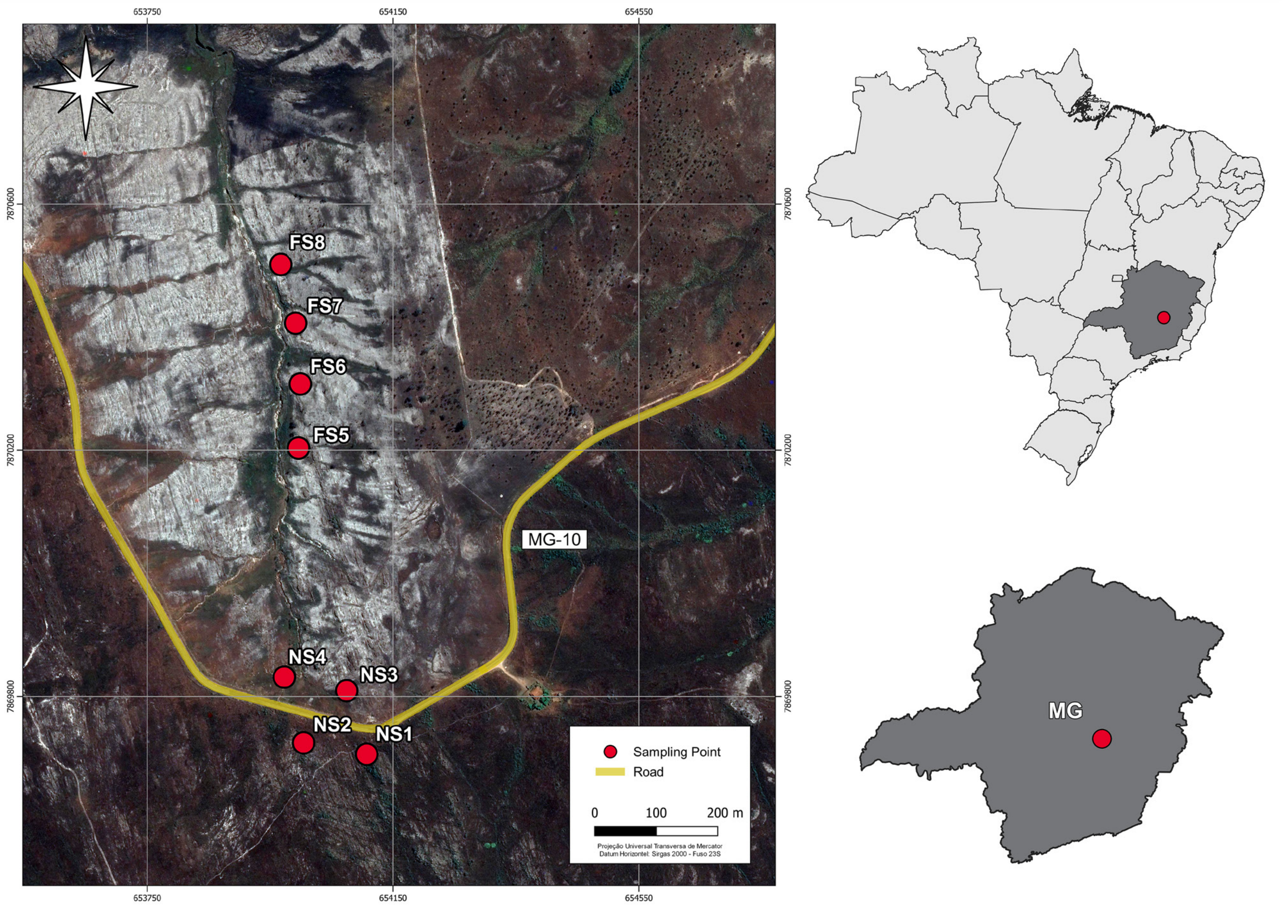

Figure 1. Sampling points located at the near site (NS1-NS4) and the far site (FS5-FS8) from road MG-10, in an area of campo rupestre (rupestrian grassland) at Alto do Palácio, municipality of Morro do Pilar, Minas Gerais, Brazil.

MATERIALS & METHODS

Study area

Data were collected in an area of rupestrian field, one of the most endangered ecosystems of the world (Pieretti et al., 2015), located in the municipality of Morro do Pilar, southern Espinhaço Mountain Range (EMR), Minas Gerais State, south-east Brazil (19° 15' S, 43° 31' W). The EMR is the second largest mountain range in south America, with a length of 1000 kilometres (Giulietti et al., 1987; Eterovick et al., 2020). The south portion of EMR is considered an area of high richness and endemism of both flora and fauna, including anuran species (Leite et al., 2012). Furthermore, it is located between the Cerrado and Atlantic Forest domains, two global biodiversity hotspots (Myers et al., 2000; Mittermeier et al., 2005). Although recognised as a priority area for conservation in Minas Gerais (Drummond et al., 2005), agriculture and mining activities are increasing in southern EMR, as is tourism, causing accelerated modification of the environment, including the expansion of roads and increased road traffic (Eterovick et al., 2005; Lopes et al., 2012). As tourism and human settlement increase in the vicinities of Parque Nacional da Serra do Cipó (PNSC), which is occurring in southern EMR, new barriers can appear for the migration of species susceptible to disturbances in the area as a whole. According to the Köppen-Geiger classification, the climate of the study area is 'subtropical highland' (de Sá Jr. et al., 2011) with two well defined seasons - a dry season from April to September and a rainy season from October to March. Higher precipitation rates are concentrated between November and February (de Sá Jr. et al., 2011).

Data collection

The study was conducted from January to March during the 2018 rainy season. Data were collected using passive acoustic monitoring sensors (Song Metre II, Wildlife Acoustics, Inc., Massachusetts, USA). Four sensors were installed at sampling points at a location within the Morro da Pedreira Environmental Protection Area around the PNSC. The site, henceforth called 'near site', is approximately 50 metres from the MG-10 state highway at km 121, with two sensors installed on the right bank of the internal road and two on the left bank. The MG-10 highway is an access road between the municipalities of Santana do Riacho and Conceição do Mato Dentro, an area known as Alto Palácio, which has intense tourist activity, especially during school holidays (January) and long holidays, such as Carnival (February). For comparison purposes, another four sensors were installed in a quieter area, approximately 500 m away from the road, in a location henceforth called 'far site' (Fig. 1). The far site was chosen because it is easily accessible and has floristic and physical characteristics similar to the near site with the presence of streams and temporary ponds, which tend to be full during the rainy season and used by anurans as breeding sites. The sensors were configured to record soundscapes 24 hours a day, two consecutive days a month, always in the first weekend of the month, totaling 1,152 hours of recordings. At each site the sensors were installed 1.5 metres above the ground and spaced 100 metres apart at each sampling site. The distance between the near and far sites was approximately 500 metres. The sensors were programmed to record at a sampling rate of 44.1 kHz, 16-bit, in stereo channels, using two waterproof omnidirectional microphones with a flat frequency response between 0.020 – 20 kHz, sensitivity of -36 ± 4 dB and gain of 36 dB.

We conducted noise level measurements for 15 minutes at each recording point using a Z-weighted B&K 2270 sound level metre to verify differences between the studied sites. Noise measurements were done at every sampling point during three periods of the day: (1): 0600–0900 - intense road traffic; (2): 1600–2000 - moderate road traffic, and (3): 2300–0500 low road traffic. These time intervals were chosen based on previous analysis of the soundscape recorded with the same type of sensor, which permitted us to identify periods of more and less intense vehicular traffic. We excluded from the recordings all animal sounds close to the microphone using BZ5503 software. The standard sound pollution measurement of Equivalent Sound Levels (Leq) was then extracted from the recordings (Rossing, 2007).

Data analysis

I. Anuran calling and noise

Data were subsampled by analysing one minute every ten minutes of the recordings (Pieretti et al., 2015). We used Arbimon software (Sieve Analytics) to automatically detect anuran vocalisations present in every recorded file. The automatic detection was performed after we created a species-specific model of vocalisation for each anuran species detected in the recordings. For these models we selected species with vocalisations that had the possibility of registration in at least 50 Regions of Interest (ROIs). To produce conservative models, we determined an acceptable average of only 5 % false positive detections (Aide et al., 2013). Arbimon detected the presence or absence of each species in every one-minute analysed per recording. All species automatically detected by the Randon Forest approach (RF) were checked and adjusted manually. Contrary to vocalisations, the road traffic noise presented high spectral variation, making it impossible to automatically detect the different sources of noise recorded. Therefore, we manually detected the presence or absence of noise in every one-minute analysed per recording.

II. Spectral analysis

Thirty advertisement calls of each anuran species were randomly selected from the recordings to perform spectral characterisation in Raven pro 1.5 software. We measured the following acoustic parametres: minimum, maximum, and peak frequencies, bandwidth and duration. In order to determine the potential for masking by noise we conducted the same analysis for 30 randomly selected noisy events emitted by road traffic.

III. Statistical Analysis

We performed the Kruskal-Wallis test to assess differences in noise levels between diurnal and nocturnal periods and vocalisation detections among months. We used Student's t-test to assess differences between noise levels at the near and far sites. The Mann-Whitney test was applied to assess differences between the near and far sites in relation to detections of noise and vocalisations. Analyses were done using R software (R Core Team, 2022). Results were considered significant when P < 0.05.

RESULTS

Characterisation of road noise

The 'near site' had noise levels measuring 1 to 18 dB (Z) higher than the far site (t = 3.45, df = 22, P < 0.001, Table 1). Noise levels at both sites were higher during 0600–0900 hours (near: H = 7.63, df = 2, P < 0.05; far: H = 7.81, df = 2, P < 0.05).

The main sources of road noise identified in this study were car, truck, and motorcycle traffic. Manual detection of these noise sources confirmed the results of the noise level measurements, since vehicle traffic noise was detected significantly more at the near site (U = 12535, Z = 14.4, P < 0,001). Vehicle traffic at the near site was significantly lower in March (H = 18.69, df = 2, P < 0.001).

Table 1. Noise levels- Leq dB (Z)- recorded at the sites near and far from the road MG-10, municipality of Morro do Pilar, Alto Palácio, Minas Gerais, Brazil.

Sampling point	0600–0900	1600–2000	2300–0500
Near 1	44	46.9	34.5
Near 2	41.7	46.2	34.2
Near 3	47	39.4	34.5
Near 4	43.5	41.2	33
Far 1	39.6	32.3	31
Far 2	39.5	30.1	29.9
Far 3	40.4	28.7	28.1
Far 4	38.6	34.2	30.9

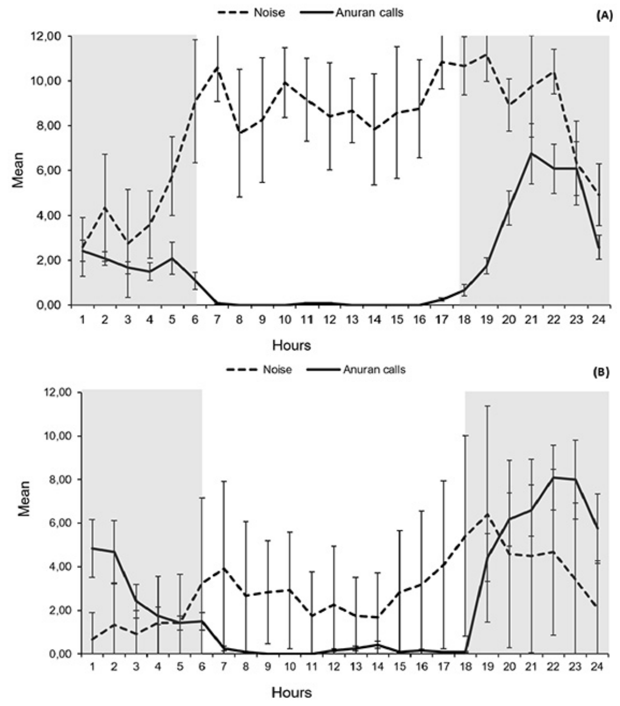

Figure 2. Means for road noise and anuran calls detected per hour from January to March of 2018 at the site near **(A)** and the site far **(B)** from road MG-10, at Alto do Palácio, Municipality of Morro do Pilar, Minas Gerais, Brazil. The white area represents the diurnal period (0600–1759) and grey the nocturnal period (1800–0559).

There was no significant difference in vehicle traffic among the sampled months for the far site (H = 2.70, df = 2, P = 0.25). Traffic at the near site was higher in the diurnal period (0600–1759) than in the nocturne period (1800–0559) (U = 6093, Z = 6.04, P < 0.001, Fig. 2), while there was no significant difference in vehicle traffic noise between the diurnal and nocturnal periods for the far site (U = 9837, Z = 0.75, P = 0.22, Fig. 2).

Noise of the passage of trucks had the longest duration (27.10 s ± 10.32 s) while the noise produced by motorcycle traffic had the highest maximum frequencies (13.17 kHz ± 4.17 kHz). The spectral characteristics of each type of noise are presented in Table 2.

Table 2. Spectral characteristics of the different categories of noise recorded at road MG-10, at Alto do Palácio, municipality of Morro do Pilar, Minas Gerais, Brazil. Values are represented by mean ± SD.

Noise source (N=30)	Duration (s)	Peak frequency (Hz)	Maximum frequency (Hz)	Minimum frequency (Hz)	Bandwidth (Hz)
Car	7.8 ± 4.7	5.7 ± 21.8	7363.6 ± 3013.7	0.0 ± 0.0	7363.6 ± 3013.7
Truck	27.1 ± 10.3	14.3 ± 32.6	12054.6 ± 3215.2	0.0 ± 0.0	12054.6 ± 3215.2
Motorcycle	15.5 ± 6.7	2.8 ± 15.7	13172.9 ± 4173.2	0.0 ± 0.0	13172.9 ± 4173.2

Anuran calling activity

We identified vocalisations of seven anuran species belonging to three families at both the near and far sites: Hylidae *Boana albopunctata*, *B. cipoensis*, *Dendropsophus minutus*, *Scinax squalirostris*, *S. curicica,* Brachycephalidae *Ischnocnema juipoca*, and Leptodactylidae *Leptodactylus jolyi*. Species composition did not differ between the two sites.

We detected more anuran vocalisations at the far than at the near site (U = 11167, Z = 3.28, P < 0.001, N far = 162, N near = 174). The species *B. albopunctata* (t = -7.31, P < 0.05), *B. cipoensis* (t = -3.96, P < 0.05) and *S. curicica* (t = -2.86, P < 0.05) had greater calling activity at the far site. In contrast, the species *D. minutus* (t = 4.24, P < 0,05), *S. squalirostris* (t = 4.13, P < 0,05), and *L. jolyi* (t = 3.11, P < 0,05) had significantly greater calling activity at the near site. The calling activity of *I. juipoca* did not differ significantly between sites (t = 1.02, P = 0.15).

The calling activity of species differed among the sampled months. We registered more calling activity in February at both the near and far sites (near: H = 10.30, df = 2, P < 0.05; far: H = 11.61, df = 2, P < 0.05). All species had greater calling activity during the nocturnal period at both sites (near: U = 1, Z = 3, P < 0.001; far: U = 2.5, Z = 2.81, P < 0.001).

We registered more vehicle traffic noise than anuran vocalisations in the nocturnal period (U = 4462, Z = 8.8, P < 0.001, Fig. 2) at the near site, while significantly more vocalisations than road noise (U = 79.94, Z = 4.8, P < 0.001) were recorded nocturnally at the far site.

Potential of noise masking

All of the recorded anuran species emitted vocalisations within the frequency ranges occupied by the noises produced by vehicle traffic (Fig. 2). Vocalisations of *B. albopunctata* had the lowest minimum and peak frequencies among the registered species. Table 3 presents the spectral characterisations of the detected anuran calls.

Table 3. Spectral characteristics of anuran vocalisations recorded at the site near and the site far from road MG-10, at Alto do Palácio, municipality of Morro do Pilar, Minas Gerais, Brazil. Values are presented in mean ± SD. N = 30 sampled vocalisation for each species.

Species	Duration (s)	Peak freq. (Hz)	Max. Freq. (Hz)	Min. Freq.(Hz)	Bandwidth (Hz)
B. albopunctata	0.55 ± 0.10	1929.3 ± 152.7	3175.6 ± 414.8	849.6 ± 290.5	2326.0 ± 675.1
B. cipoensis	0.06 ± 0.01	4002.2 ± 167.3	4509.7 ± 153.7	3414.1 ± 152.2	1095.5 ± 142.7
D. minutus	0.04 ± 0.01	4203.3 ± 1130.8	5525.4 ± 125.8	2168.1 ± 121.3	3357.2 ± 177
S. curicica	0.78 ± 0.08	3327.5 ± 384.8	3989.8 ± 164.5	1163.7 ± 100.5	2826 ± 188
S. squalirostris	0.34 ± 0.03	4062.6 ± 121.9	10539.2 ± 1236.3	2262.9 ± 349.6	8276.2 ± 1291.6
I. juipoca	0.53 ± 0.14	3353.4 ± 158.2	4106.7 ± 170.2	2498.7 ± 206.4	1608 ± 255
L. jolyi	0.03 ± 0.01	2156.2 ± 99.8	2692.5 ± 78.4	1263.4 ± 79.8	1429.1 ± 97.9

DISCUSSION

We provide the first assessment of the anuran vocal activity using passive acoustic monitoring at the study area, which is recognised for high species richness and endemism and a priority for conservation. It is also the type locality for the treefrog *S. curicica* (Pugliese et al., 2004). Our results showed that road noise was loudest and most frequent at the location close to the road. Because it has the ability to compromise the amount of information that can be extracted from a signal (Bee & Swanson, 2007; Duarte et al., 2019), recorded road noise has the potential to mask anuran vocalisations. Although road traffic noise was greater during the day and anuran vocalisations were concentrated at night, we found more noise than anuran vocalisations in the nocturnal period at the site near the road, while the opposite was observed at the site far from the road. This finding is evidence that noise is competing for acoustic space with anuran vocalisations at the site near the road.

Noise can affect species richness and abundance and change the composition of animal communities, including those of anurans (Francis & Barber, 2013; Duarte et al., 2015, Alvarez-Berríos et al., 2016; Potvin, 2016; Grace & Noss, 2018). As noise can compete with animal vocalisations for acoustic niches, it is expected that species that are more sensitive to noise (such as those that vocalise at lower frequencies, for example) will avoid noisy areas (Duarte et al., 2015). We did not find any differences in species richness and composition between the two sampled sites. Anuran species generally have restricted ranges and poor dispersal capacity in comparison to birds, which limits their ability to move from areas with high noise disturbance (Alvarez-Berríos et al., 2016). Moreover, some anuran species can tolerate a high degree of habitat modification (Herrera-Montes & Aide, 2011). The factors described above can explain the similar species richness and composition between the two sites. However, such similarity also suggests that anuran responses to road noise can be related to other factors, such as a difference in abundance or changes in calling rate and spectral and/or temporal characteristics of vocalisations.

Anuran species respond to anthropogenic noise using distinct strategies (Sun & Narins, 2005; Halfwerk et al., 2016; Caorsi et al., 2017; Lima et al., 2022). For example, they can change both temporal and spectral parameters of their calls, such as calling rate, amplitude, duration, and frequency (Schwartz & Bee, 2013). Overall, we detected more call activity at the location far from the road than at the location close to the road, mainly from the species *B. albopunctata*, *B. cipoensis* and *S. curicica*, the last two endemic to Espinhaço Mountain Range. This result can be interpreted as a response of frogs to road noise, which is penetrating and occupies the same frequency bands as frog vocalisations. Thus, with less acoustic space available at the site close to the road, the species could be vocalising less, compared to the community inhabiting the site far from the road (Vargas-Salinas et al., 2014). Studies have shown that species tend to expend less energy calling where there is interference from traffic noise (Sun & Narins, 2005; Cunnington & Fahrig, 2010; Love & Bee, 2010), which may be the case for *B. albopunctata*, a species very susceptible to masking due to its lower call frequency, and *B. cipoensis* and *S. curicica*. Vocalising is one of the most energetically expensive activities for frogs (Taigen & Wells, 1985; Bradbury & Verenhcamp, 2011), and so males should call more when there is an effective distance of transmission of calls (Wells & Schwartz, 2007). Lengagne (2008) also found that noise triggered a decrease in male calling activity. Grace & Noss (2018) found that traffic noise caused a significant reduction in anuran vocalisations, but the effect was absent when the traffic noise was digitally altered to remove frequencies that overlap with anuran vocalisations. On the other hand, the higher calling activity found at the site far from the road can simply be a result of a greater abundance of individuals at this site, which is difficult to estimate using passive acoustic monitoring (Duarte et al., 2015; 2019).

The species *D. minutus*, *S. squalirostris*, and *L. jolyi* emitted more vocalisations at the site near the road. Narins (2013) reports that responses to noise may vary between species. While some anurans decrease their calling rate with the presence of noise, others can increase their calling rate in response to an identical stimulus. Increasing calling rate in a noisy environment can be interpreted as an attempt to increase the efficiency of the communication and guarantee that the signal will be received by the receptor. However, the costs associated with this behavioural change can be high since calling activity is energetically demanding (Taigen & Wells, 1985; Lukanov et al., 2014; Yeo & Sheridan, 2019). On the other hand, higher rates of vocalisation at the site near the road may be a result of a greater abundance of individuals, although this seems improbable since many studies have found lower abundances of anurans near roads (Colino-Rabanal & Lizana, 2012; Eigenbrod et al., 2009; Fahrig & Rytwinski, 2009; Witte et al., 2001; Tennessen et al., 2014). In addition, species may be able to tolerate high levels of interference (Herrera-Montes & Aide, 2011) and have their occurrence associated with locations close to sources of anthropic noise (Herrera-Montes & Aide, 2011; Alvarez-Berríos et al., 2016; Deichmann et al., 2017).

Considering that the human transportation network continues to rapidly expand throughout the world (Laurance et al., 2014), our results bring to light a problem that needs an urgent protocol for control (Kunc & Schmidt, 2019). It becomes even more urgent considering the global decline of amphibians (Ford et al., 2020). The highway network in Brazil is among the largest in the world with a total length of 1,720.9 km, of which 12.4 % is paved and 78.5 % is unpaved roads (National Confederation of Transport, 2021). Even with an understanding of the importance of acoustic communication for frogs and the effects of road noise on many species, it is not always politically, economically, and logistically feasible for governments to eliminate or reduce noise. There is no doubt that many policy approaches set noise standards in order to limit noise levels. However, it is important to establish norms that balance economic and environmental factors, since in the development of standards the priority is aimed at human benefit (Blickley & Patricelli, 2010; Kunc & Schmidt, 2021). In Brazil, there are no laws or regulations that require noise control and monitoring in areas uninhabited by humans. Thus, there is no noise control in nature reserves such as national and state parks bordered by roads, even though in some parts of the world this is already happening (e.g. Dumyahn & Pijanowski, 2011; Pijanowski et al., 2011). The results of our study showed that road noise is present in areas of high biodiversity and can force frog species to decrease or increase vocalisation activity. Road noise has the potential to mask and disturb frog calls as it overlaps the frequencies of their vocalisations. Our findings show the importance of establishing norms for noise control and monitoring in natural areas.

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Activity budgets, responses to disturbance and novel behaviours in captive mountain chicken frogs *Leptodactylus fallax*

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Mountain chicken frogs *Leptodactylus fallax* are assessed as Critically Endangered on the IUCN Red List due to threats including chytridiomycosis and habitat loss. Ex-situ populations underpin species survival, but captive management is hampered by incomplete species knowledge, including its behavioural biology. In sixteen adult frogs, we investigated enclosure usage, nocturnal activity budgets, and behavioural responses to varying levels of husbandry-related disturbance through instantaneous scan sampling of camera trap footage over forty-two consecutive nights. Enclosure usage was quantified through the application of modified Spread of Participation Indices (mSPI). We present the first detailed activity budgets published for this species and found that broad activity patterns of captive animals corresponded well with their wild conspecifics; taking refuge during the day and emerging at dusk into exposed areas of the enclosure. Some behaviours, especially hunting and bathing, were partially phased throughout the nocturnal period. Enclosure use was not even, with disproportionately large amounts of time spent using nest boxes and deep leaf litter, even at night, and the converse in thin leaf litter and elevated perches. Principal Components Analysis (PCA) identified a bold-shy behavioural grouping (movement and resting opposed to refuge behaviours) that was consistent across husbandry-related disturbance conditions. Randomisation analyses showed that disturbance significantly affected multiple measured behaviour, especially feeding, social interactions and vocalisation; the impact was associated with the degree of disturbance. Novel behaviours were also documented, which are thought to be related to courtship. Our data provide insights into the behaviour of mountain chicken frogs and highlight potential trade-offs between enclosure maintenance, veterinary intervention, and animal welfare.

Keywords: Amphibians, behaviour, mSPI, disturbance, welfare, Zoo

INTRODUCTION

Amphibians are undergoing a global mass extinction; 42 % of amphibian species are at risk of extinction, mediated principally by habitat destruction and emerging infectious diseases (Scheele et al., 2019). The mountain chicken frog *Leptodactylus fallax* was once widely distributed among the Eastern Caribbean islands. The introduction of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), alien predatory species, habitat loss and over-harvesting caused significant declines and the species is now restricted to the islands of Dominica and Montserrat (Adams et al., 2014). The species is assessed as Critically Endangered with fewer than 200 individuals remaining in the wild on Dominica and being likely extinct on Montserrat (IUCN SSC ASG, 2017). Mountain chickens are nocturnal and terrestrial, and one of the largest extant frog species (Jameson et al., 2019) and exhibit a unique mode of reproduction (Gibson & Buley, 2004). Males form territories around terrestrial burrows which they guard against rival males and to which they attract females through advertisement calling. Within the burrow, the frogs create a foam nest in which larvae develop into metamorphosis, fed with infertile eggs by the mother (see Gibson & Buley, 2004). They have cultural and economic value to local communities (Nicholson et al., 2020). These factors have fuelled an international collaborative effort to save this species in the wild (Adams et al., 2014; Jameson et al., 2019).

Creating a sustainable captive population is essential for the long-term survival of this species (Adams et al., 2014; IUCN SSC ASG, 2017). Multiple captive populations were established, between 1998 and 2011, at Metro Toronto, Jersey and ZSL London Zoos and in a breeding centre in Dominica, as safety net populations and for conservation education. These populations did not ultimately contribute to conservation translocations (Gibson & Buley, 2004; Cunningham et al., 2008; Tapley et al., 2014; Jameson et al., 2019). In 2009, in response to disease mediated population collapses, 50 wild mountain chickens were collected from Montserrat and were distributed to multiple European institutions where breeding has produced frogs for translocations, and founded a separately managed non biosecure population for staff training and public education (Hudson et al., 2016).

Although successful captive breeding has led to an increase in this captive population size (Jameson et al.,

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Figure 1. Mountain chicken enclosure at ZSL London Zoo, illustrating enclosure layout and overlapping camera angles providing coverage of the entire enclosure.

2019), managing mountain chickens in captivity has proven problematic, mainly due to the highly specific conditions required by this species in captivity (Tapley et al., 2015; Donaldson, 2019; Jameson et al., 2019). The physiological requirements of captive mountain chickens are becoming better understood (Fitzgerald et al., 2007; Dierenfeld et al., 2008; King et al., 2011; Jaffe et al., 2015; Tapley et al., 2015; Jayson et al., 2018; 2018b; Croci et al., 2019; Donaldson, 2019; Jameson et al., 2019; Ashpole et al., 2021; Michaels et al., 2021; White et al., 2021). Although very basic information on habitat use and activity patterns is known from wild populations (Schwartz & Henderson, 1991; Daltry, 2002; see Jameson et al., 2019), detailed information on activity budgets and patterns is lacking for both wild and captive mountain chickens. Behavioural data are important to inform the management of wild and captive animals (Estevez & Christman, 2006; Ross et al., 2009). We investigated baseline enclosure usage and nocturnal activity patterns in captive mountain chicken frogs and assessed whether these were affected by husbandry-related disturbance.

MATERIALS & METHODS

Ethics statement

This study was reviewed by the Zoological Society of London (ZSL Projects Database ref. number WHB7) and, as all data were collected from observations of normal husbandry practice, it was not deemed necessary to undertake full ethical review, nor was the study subject to the Animals (Scientific Procedures) Act 1986. The work is in line with the BHS Ethics Policy.

Study subjects

We observed sixteen adult mountain chicken frogs housed at ZSL London Zoo; eight captive-bred males, seven captive-bred females (ages ranging from 1 year 6 months – 1 year 8 months at time of study) and one wild-collected female (17 years 1 month in captivity, estimated age c. 23 years, at time of study). Frogs were housed in four groups of two females and two males, which were the experimental units in this study.

Enclosure Design and husbandry

Frogs were housed in 2x2 m dens with bark chip substrate, aligned back-to-back with a brick wall and wire mesh separating them (Fig. 1). Furnishings consisted of leaf litter, 2 x large artificial palm leaves, 2 x clay-lined nest boxes with tubes for entrance. Detailed husbandry information is given by Michaels et al. (2021). Animals were fed three times a week, but food insects were almost always present in dens between feedings, as frogs do not capture all items immediately. In order to collect accurate temperature data, four data loggers (EasyLog EL-USB-1-LCD, Lascar Electronics, Wiltshire, UK) were evenly distributed across each enclosure (refer to Fig. 2) and recorded temperature every five minutes throughout the study. Mean temperature across enclosures was 24.26 °C with a range from 22.61–25.39 °C.

Observations and disturbance events

Frogs were observed between 11 April (night 0) until and including 23 May 2021 (night 42), during which period the frogs were in their breeding season. Observations occurred between the hours of 1700 and 0830 the next day. Lights (full spectrum specialist lighting arrays - see Michaels et al., 2021 for details) within the dens were on until 1800 after which they remained switched off until 0745 the following morning.

Observations were recorded using two time-lapse infra-red based night vision video cameras (Crenova Trail Camera 186 PH760) per den, which captured the entire den in combined field of view. Therefore, frogs were only out of sight when in refuges (nest boxes and other refugia) and so were coded as showing refuge behaviour (see below; Table 1).

Review of preliminary diurnal footage showed almost no observations of visible frogs and so data collection was limited to the nocturnal period, including a brief period of light at the start and end of each recording. As there was no internet or other communications access in the frog facility, all data was stored locally and omission of diurnal recording substantially reduced required incursions into the facility to change storage cards, as well as reducing personnel interactions during the COVID-19 pandemic.

Table 1. Ethogram used for coding mountain chicken frog behaviour

Behaviour	Definition
Refuge use	Stable position - partially visible or not visible at all. Using furnishing/substrate to conceal itself. Note: in this study if the animal is out of sight, we are determining it as a 'refuge use' behaviour.
Resting	50 % or more of the body is visible and stationary.
Movement	Can be observed either crawling (whole body movement across the enclosure in a slow manner with the body near to the ground), hopping (fast process whole body movement jumping across the enclosure using their hind limbs) or climbing (whole body movement with the intention of ascending). Climbing usually occurs on top of the window ledge of each enclosure, ascending up the wired mesh. If a frog is stationary on the mesh, this too is classified as movement.
Hunting	Actively stalking prey (for an example slow body movement towards prey item with eyes orientated towards it) or lunging on prey and consuming.
Intra-specific social interaction	Any social interactions observed such as following a conspecific to a different area of the enclosure or moving towards a stationary conspecific in close proximity (within three body lengths), submissive or dismissive interaction, an act of dominance such as chasing or fighting.
Vocalisation	Single, or multiple continuous calls.
Bathing	Bathing in water bath either alone or with a conspecific
Other	Any other notable behaviours that have not been categorised as focal behaviours.

Cameras were programmed to record continuous four-minute video clips with an interval time of fifteen minutes; these video clips were later analysed by the observer.

An ethogram (Table 1) was developed based on a subset of the footage. Individual frogs were not reliably identifiable on camera trap footage, so nocturnal observations were conducted using instantaneous scan sampling for each den. The number of frogs performing each behaviour was counted in each den every five seconds from each four-minute video clip and data were recorded in Microsoft Excel for Windows 10. Ad-hoc descriptive observations of behaviours of note were also made contemporaneously. Total counts of each behaviour per den per night (i.e. for the entire nocturnal period) were calculated and used for analysis.

Frogs were exposed to different intensities of disturbance due to routine husbandry procedures during the observation period. Data for all dens collected prior to night 29 were designated as baseline disturbance intensity (disturbance intensity A), characterised by standard husbandry with no recent or current disturbance.

Dens 2 and 4 were simultaneously subject to a full substrate change. This involved briefly capturing and containing frogs in their nest boxes, while all furnishings were removed from the den and cleaned, and the bark mulch substrate was removed and replaced with fresh material. This took approximately 60 minutes per den between containment of frogs and release from the nest box, and took place between 0900 and 1100.

In the morning prior to night 29, three frogs in den 2 (disturbance intensity C) and one frog in den 4 (disturbance intensity D) underwent invasive health checks. An invasive health check is part of recommended health screening for the captive population (Jameson et al., 2019) and consists of catching frogs by hand, transport to veterinary facilities, isoflurane anaesthesia, blood sampling, radiography and ultrasonography,

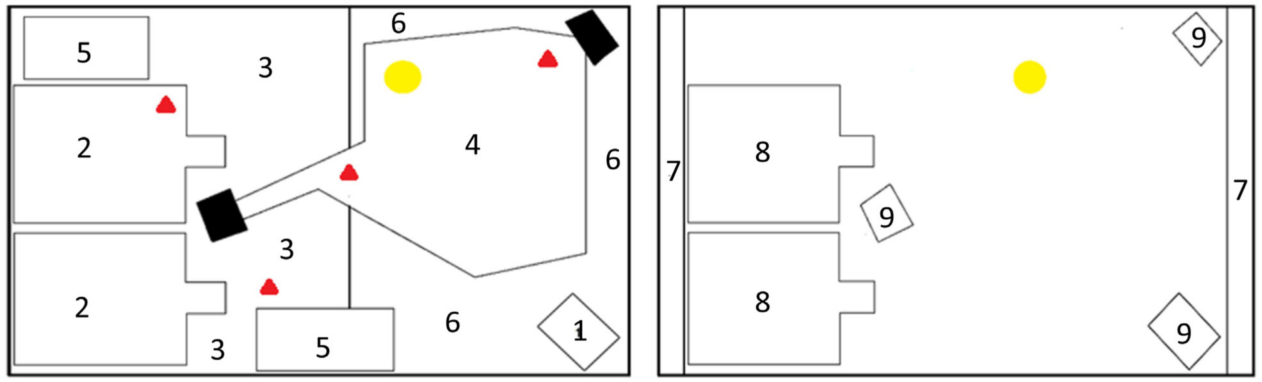


Figure 2. Diagrams illustrating resource zones within mountain chicken enclosures. Zone numbers correspond to Table 2 and are defined as 1: Large 'tipped over' plant pot lined with leaf litter; 2: Enclosed, nest box lined with modelling clay; 3: Thin layer of leaf litter; 4: Large palm leaves with a thick layer of leaf litter underneath; 5: Water dish; 6: Thick layer of leaf litter; 7: Window ledge; 8: Thin layer of leaf litter on top of the nest box; 9: The top surface of the 'tipped over' plant pot. The left-hand panel shows floor-level resources, the right represents the higher tiers, red triangles show the location of the 4 data loggers, yellow circles indicate the location of basking lamps and black polygons show positions of plant pots filled with substrate that do not provide a floor-level refuge but do for elevated resource zones (zone 9, right-hand panel).

Table 2. Resource zones into which enclosures were divided for mSPI analysis

Zone	Description of each zone	Biological relevance	Percentage cover for each enclosure (%)			
			1	2	3	4
1	Large 'tipped over' plant pot lined with leaf litter	Empty plant pot, filled with leaf litter acts as a retreat within the warm zone	3.7	3.4	3.9	3.4
2	Enclosed, nest box lined with modelling clay	Nest building, refuge, within the cool zone	9.1	9.0	8.8	9.1
3	Thin layer of leaf Litter	Leaf litter unsuitable for hiding as too shallow. Includes a temperature gradient from warm to cool zones across the enclosure	30.4	29.4	29.9	30.7
4	Large palm leaves with a thick layer of leaf litter underneath	Artificial plants and leaf litter provide effective refuges; incorporates a temperature gradient across enclosure from warm to ambient zones	16.1	15.3	15.9	15.9
5	Water dish	2 x large plastic tray, filled with fresh water daily. Ambient and cool temperature zone.	5.8	5.6	5.2	6.0
6	Thick layer of leaf litter	Deep leaf litter layer suitable for hiding with temperature gradient from warm to ambient zones.	12.2	11.7	11.7	12.6
7	Window ledge	An 80 mm ledge positioned 50 cm off the ground. When on top of the window ledge, frogs can see into other enclosures housing mountain chicken frogs. Fully exposed so cannot use zone as refuge.	5.8	9.3	9.8	5.4
8	Thin layer of leaf litter on top of the nest box	Fully exposed, cannot use this zone for refuge. Elevated so frogs can see rest of enclosure, but not high enough to see into neighbouring enclosures. Cool zone of enclosure.	11.1	11.0	10.8	11.1
9	The top surface of the 'tipped over' plant pot	Fully exposed, cannot use this zone as refuge. Elevated so frogs can see rest of enclosure, but not high enough to see into neighbouring enclosures. Warm zone of enclosure.	5.8	5.3	4.9	5.8

followed by recovery and return to the den. The process took approximately 90 minutes from capture to release for all involved frogs together.

Therefore, dens 2 and 4 experienced direct disturbance; they were subject to a full substrate change as well as some individuals undergoing capture and health checks. Dens 1 and 3 experienced no direct disturbance, but both were subject to indirect disturbance from dens 2 and 4 (see above) due to the prolonged presence of humans and vibrations from moving refugia and substrates (disturbance intensity B).

These arrangements resulted in four disturbance intensities: A - pre-disturbance baseline, B - indirect disturbance - substrate change, C - high intensity direct disturbance - 3 frogs removed from den 2 for health checks, and D - low intensity direct disturbance - 1 frog removed from den 4 for health checks.

Enclosure usage

To evaluate enclosure usage, we used Plowman's (2003) modified Spread of Participation Index (mSPI). This equation produces values ranging from 0–1; a value of 0 indicates that frogs use all zones equally whereas a value of 1 suggests that frogs favour only one zone of the enclosure.

$$\mathrm{mSPI} = \frac{\sum |f_o - f_e|}{2(N - f_{e\,\mathrm{min}})}$$

f_o= observed frequency of observations.

f_e= expected frequency of observations in a zone, calculated as the total number of observations multiplied by the proportion of the enclosure represented by the zone.

$|f_o - f_e|$= absolute value of the difference between f_o and f_e

$\sum$= sum of all zones

N = total number of observations in all zones

$f_{e\,\mathrm{min}}$= expected frequency of observations in the smallest zone

Dens were zoned based on biological relevance, i.e. based on qualitatively different microhabitats and relevance to different behaviours (Fig. 2.; Table 2). At the same time as behavioural monitoring, the number of frogs in each zone was recorded; the total number of observations in each zone was recorded for each den for each night. For refuge zones (see Table 2; zones 1, 2, 3, 4 & 6), video footage was reviewed to confirm frogs entering and moving between refuges to ensure that frogs were counted in the correct refuge type. If location could not be confirmed, data were omitted. If a frog was partly in two zones at the same time, the zone containing the majority of the frog was recorded; if a frog inhabited two zones equally, the zone containing the head was recorded; if the head was positioned where the boundary was down the midline between each zone, the zone that the frogs' eyes were oriented towards was recorded. mSPI was calculated per night per den.

Statistical analyses

Statistical analysis was conducted using R version 4.1.1 in RStudio Version 1.4.17 for Windows (R Core Team, 2021). An Alpha of 0.05 was used throughout the study unless otherwise indicated.

Activity budget phasing

Total counts of each behaviour exhibited by all frogs during the undisturbed phase of the study were calculated. These data were partitioned between three segments of the nocturnal period (1700–2159, 2200–0259, 0300–0830) to assess phasing inactivity budgets across the night. Behavioural counts split across time period were assessed visually as interdependence between observations (as they were from the same individuals) prevented formal analysis without pseudoreplication.

Behavioural changes in response to disturbance

In order to minimise the number of behavioural variables analysed, following Martin & Réale (2008), we used a Principal Components Analysis using FactoMineR (Lê et al., 2008) and Factoextra (Kassambar & Mundt, 2020) to identify behavioural groupings that were consistent across both before and after disturbance conditions following. Data were standardised with mean 0 and standard deviation 1 prior to analysis so that the covariances were robustly comparable. Eigenvalues >1 were used to select dimensions retained, and relative weightings for each behaviour in each dimension were used to identify the dimensions primarily representing those behaviours. Hunting was not included in this process as it was identified as a variable of specific interest given keeper anecdote that food intake appeared to reduce after disturbance (see Discussion). A dimension comprising movement, refuge and resting was identified as present before and after disturbance, and the co-ordinates associated with this dimension were used for analysis, along with raw data for remaining behaviours.

We compared baseline (A) to disturbed (B, C or D) data for each frog enclosure. We used the shuffle function within the Mosaic package (Pruim et al., 2017) to run randomisation analyses with 10,000 iterations in order to test for an effect of disturbance on behavioural counts and mSPI. Randomisation is a valid strategy for analysing small- and single-n samples and is useful when working with small sample sizes in zoo contexts (Dugard et al., 2012). The residual (i.e. the difference) between the means of each before-after disturbance pair was used as a test statistic. The data were then shuffled randomly 10,000 times and a new test statistic calculated; a two-tailed p-value was derived for each comparison from the overlap of simulated test statistics with the observed test statistic. Bonferroni corrections were applied to adjust for multiple comparisons.

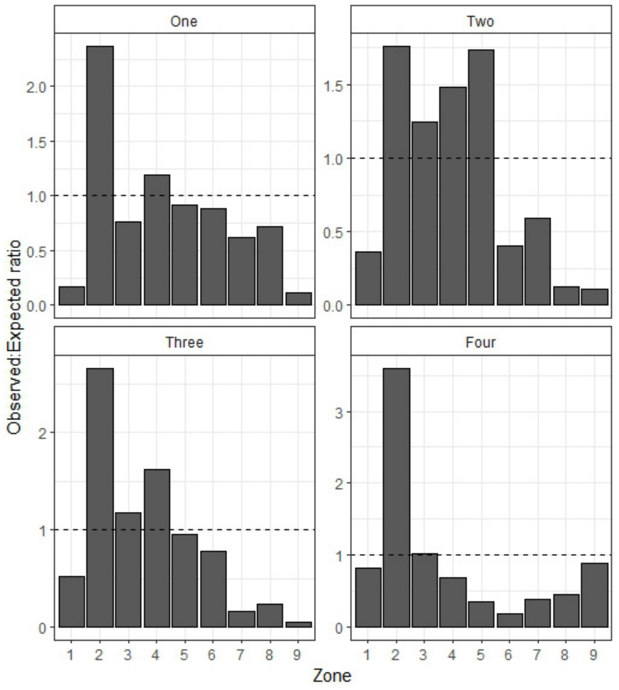


Figure 3. Ratios of observed:expected (as calculated via modified spread of participation indices) counts of frogs in each den (one–four) occupying each enclosure zone under pre-disturbance baseline conditions. The dashed line indicates where observed and expected counts did not differ.

RESULTS

Baseline enclosure usage, behavioural phasing and activity budget

Mean (SD) mSPI, pre-disturbance, for dens 1–4 were 0.35 (0.084), 0.38 (0.080), 0.38 (0.09), 0.48 (0.148), respectively. Figure 3 shows total observed zone usage compared with expected values under perfectly even zone usage; patterns between dens were similar with over-use of zone 2 and under- or quasi-expected use of other zones.

Figure 4 shows mountain chicken frog nocturnal activity prior to disturbance; all frogs spent the majority of their time either resting (51.7 %) or taking refuge (38.6 %). Frogs spent 5.4 % of their time using the water baths. Less than 10 % of time was spent in social interactions, movement and hunting, combined. Frogs remained active throughout the night, but behaviours do not appear to have been equally distributed across the three time periods for some behaviours, with animals tending to engage in active behaviours (movement, hunting, vocalisation) earlier in the night, and bathing behaviour later in the night, while the other behaviours are more evenly distributed (Fig. 5). Note that the final time period is slightly longer (30 minutes) than the first and second periods.

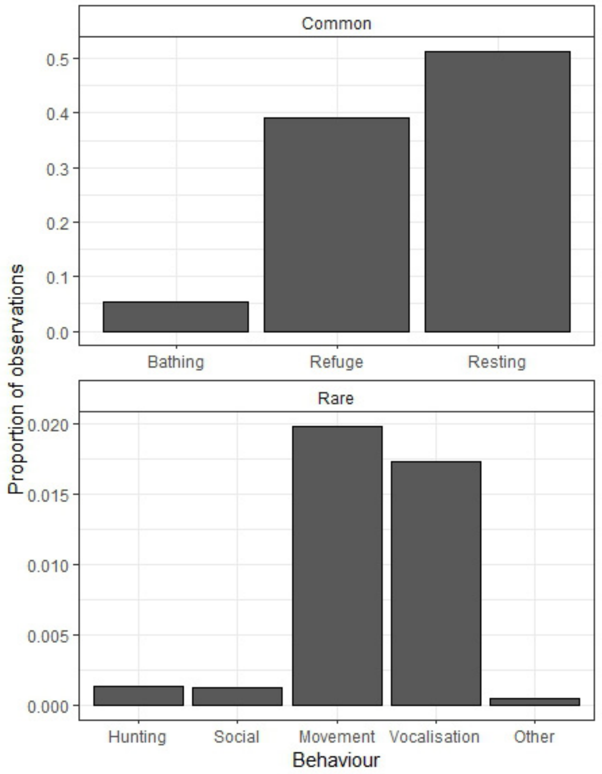


Figure 4. Proportion of observations of each behaviour type under pre-disturbance conditions aggregated across all dens and all nights; the top pane contains common behaviours, the lower pane rare behaviours.

Effects of disturbance from substrate change and invasive health checks

We retained only the dimension relating to movement, resting and refuge. The PCA identified a grouping between movement, resting and refuge, with movement and resting both negatively correlated with refuge (average co-ordinates 0.62, 0.799 and -0.89, respectively; see Table 4). Randomisation analysis showed that disturbance had a significant effect on several behaviours. Most strikingly affected was hunting behaviour, which reduced significantly under all disturbance intensities. The behavioural grouping comprising movement, resting and refuge showed significant reduction (i.e. reduction in movement and resting and increase in refuge) after disturbance intensity C (catch-up of three out of four frogs) in den 2, but not in other dens (i.e. not in dens 1 or 3 under intensity B, or in den 4 under intensity D). Social interaction significantly decreased in both dens 2 and 4 (following catch-up of three and one of four frogs, respectively); vocalisation also decreased in den 2. (Fig. 6; see Table 3 for statistical outcomes).

Other behaviour

Two novel behaviours for mountain chicken frogs were observed on camera trap footage. These are described in Table 4. Behaviour 1 was coded as a social interaction, and Behaviour 2 as an other behaviour.

DISCUSSION

Review of preliminary footage showed that mountain chickens were almost entirely out of sight when lights were on and emerged from refugia when lights were switched off. These findings correspond well with previous research on their wild conspecifics; wild frogs were observed to retreat during the daytime and move into exposed, open areas at dusk (Jameson et al., 2019; Daltry, 2002). Temperatures in enclosures reflected natural temperatures of mountain chicken frog microhabitat in Dominica (20–25 °C) and Montserrat (21–24 °C), as did the photoperiods (Jameson et al., 2019), suggesting that frogs in this study were exposed to environmental cues broadly similar to those experienced by free living frogs.

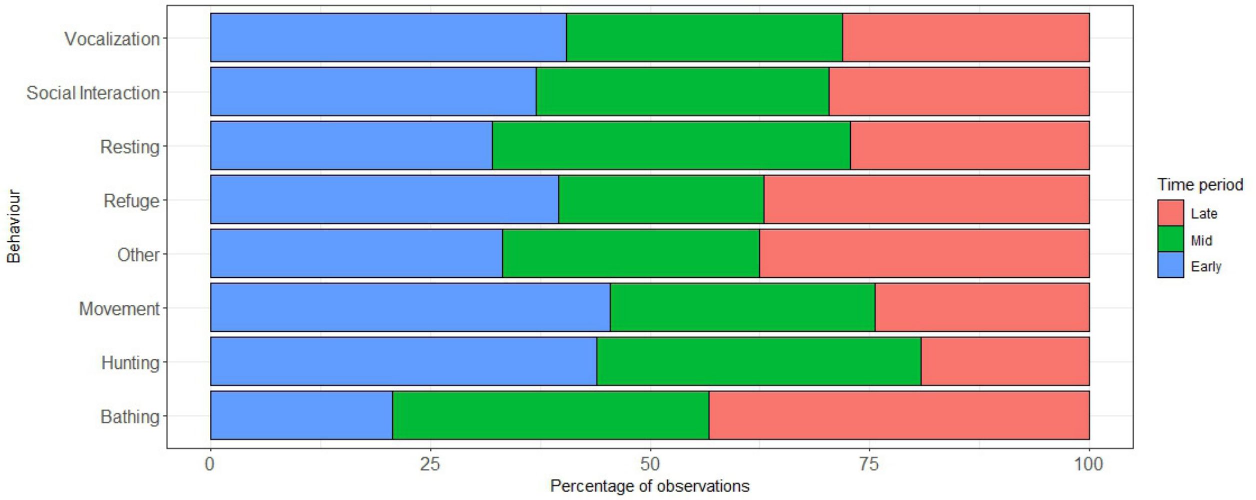


Figure 5. Total proportion of observations of each behaviour, expressed as a percentage of all observations of that behaviour, split between the early (blue), mid (green) and late (pink) thirds of the nocturnal period.

Table 3. Statistical outcomes (p-values) of randomisation tests, to three decimal places, comparing behaviours before and after disturbance in each of four dens. Bonferroni corrections were applied; significant values after corrections are in bold

		P value (2 tailed)						
Den	**Treatment comparison**	**Bathing**	**Hunting**	**Social interaction**	**Vocalisation**	**Other**	**mSPI**	**Bold/shy grouping**
1	A vs B	0.500	**<0.001**	0.018	0.417	0.180	0.732	0.075
2	A vs C	0.597	**<0.001**	**0.010**	**0.009**	0.34	0.440	**<0.0001**
3	A vs B	0.583	**<0.001**	0.364	0.067	0.4	0.391	0.020
4	A vs D	**<0.001**	**<0.001**	**0.004**	0.444	0.060	**<0.001**	0.052

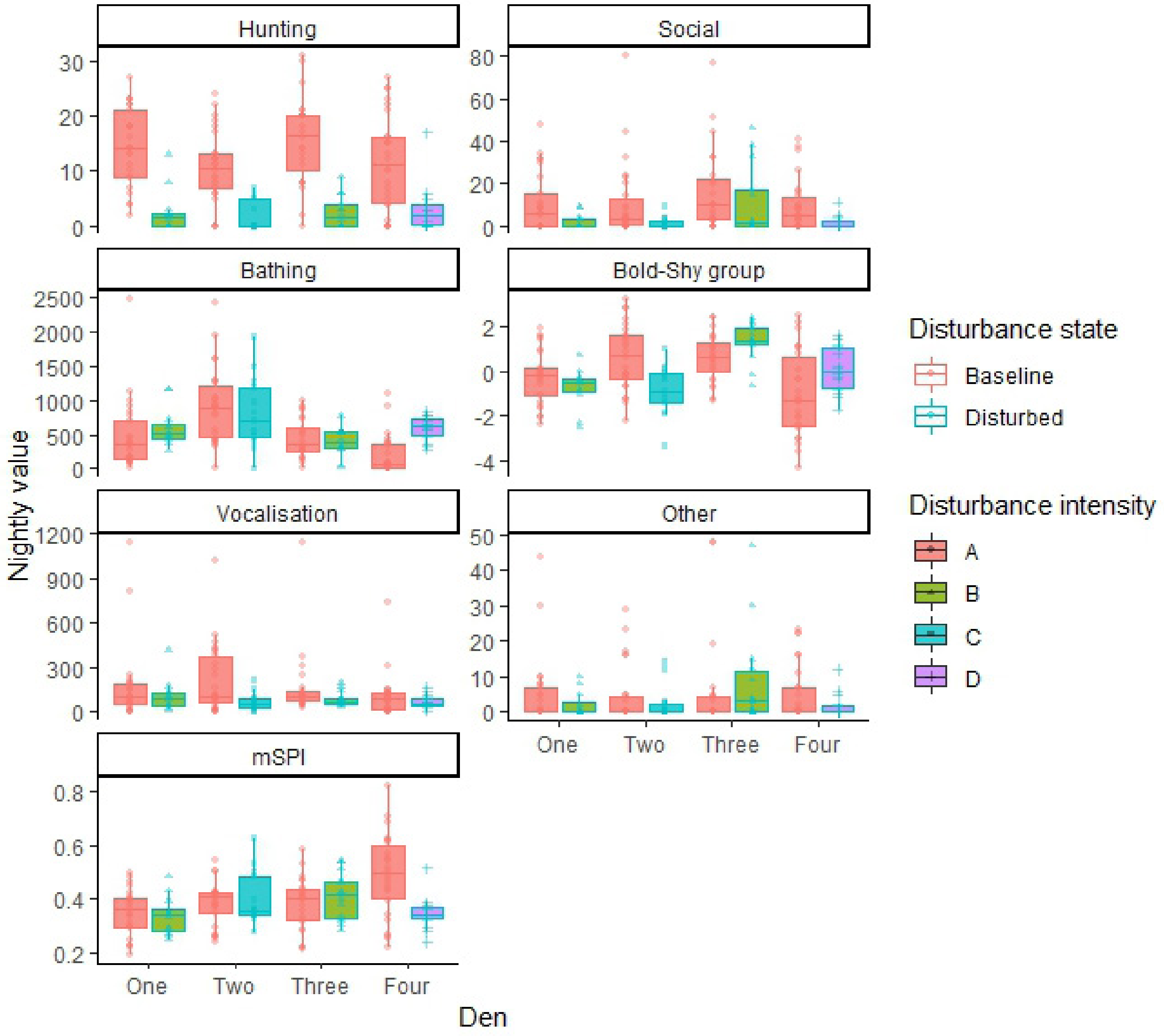


Figure 6. Nightly counts of each mountain chicken frog behaviour and mean mSPI, split by disturbance state and intensity, and by den. Boxplots show median, interquartile range and outliers (to which randomisation analysis is robust) identified as > 1.5* IQR above Q3 or below Q1. Refer to Table 3 for relevant statistical comparisons, which can only be made within dens.

Our data show that some behaviours appear to be partially phased throughout the night (Fig. 5). Although all behaviours were observed in all parts of the nocturnal period, frogs tend to emerge soon after dark and engage in hunting and movement behaviours, but bathe primarily in the final part of the night. Other behaviours are more evenly distributed. Frogs absorb water to assist with digestion of food, with the mass of water absorbed roughly equalling food intake (Hillman et al., 2009), so frogs may bathe more later in the night to absorb water after feeding earlier in the night. The inability to see inside nest boxes and refugia, where frogs spent around 40 % of their time, is a limiting factor to fully elucidating activity budgets, as we do not know whether these spaces conceal additional social interactions or other behaviours.

Enclosure usage under undisturbed conditions measured through mSPI was slightly uneven; adjustments to the design of dens could expand the proportions of dens covered by favoured resource zones. Zone 2 (nest box) was particularly over-used, whereas zones 6 and 8 (thin leaf litter zones), and 7 and 9 (elevated zones) were particularly

Table 4. Co-ordinates and contributions associated with the PCA dimension grouping movement, refuge and resting behaviours, with a comparison of outcomes from all data, and data from before and after disturbance. As consistency across conditions was established for this grouping, co-ordinates from the dimension across all data could be used. Other behavioural groupings did not demonstrate consistency across conditions and so raw counts of individual behaviours were used for randomisation analysis.

	All data			Before disturbance			After disturbance		
Behaviour	Average co-ordinates	Average contributions	Eigenvalue	Average co-ordinates	Average contributions	Eigenvalue	Average co-ordinates	Average contributions	Eigenvalue
Movement	0.62	17.01		0.67	18.424		0.60063	14.68	
Refuge	-0.89	35.47	2.46	-0.92	34.80	2.43	-0.84662	29.16	2.46
Resting	0.79	27.98		0.79	25.55		0.79	25.55	

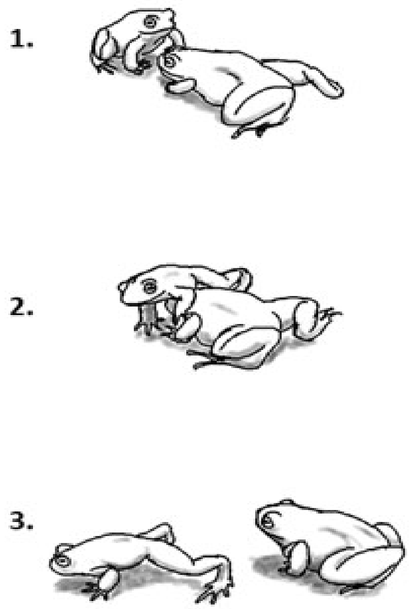
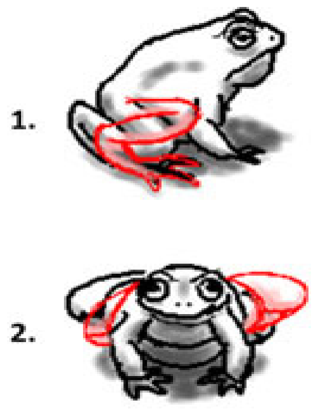
under-used (Fig. 3). The over-usage of the nest box during the nocturnal period highlights the importance of this resource. Through anecdotal observation of this species in captivity during the day, frogs typically use the nest box as a diurnal retreat (Tapley, Servini & Michaels, pers. obs.) and our data show that this pattern is maintained at night. This is particularly important for territoriality as nesting-sites form the core resource in, especially male, frog territories (see Jameson et al., 2019). As such this over-use is unlikely to be a welfare concern, as it reflects the tendency of mountain chicken frogs to live in burrows and other small subterranean spaces (Jameson et al., 2019; Daltry, 2002). However, it does indicate that adequate nest box space should be provided for the number of frogs present in an enclosure and that other refugia may not be equivalent as a hide. The under-use of thin leaf litter indicates the importance of deeper leaf litter zones (zone 4, which was not under-used) for mountain chickens. Our data therefore support the use of deeper leaf litter, under which frogs can be completely covered, across the enclosure to cater to frog behaviour; however, this must be balanced with the need for frogs to find food easily and before it has lost nutritional quality and to be exposed to UVB lighting (Jameson et al., 2019). The under-use of elevated positions is likely a result of these exposed zones being used primarily for behaviours associated with territory defence and mate attraction (Jameson et al., 2019). Although not used as frequently as expected by chance, frogs did regularly use these zones, despite being difficult to access. This highlights that mountain chickens are agile animals and that captive environments should provide opportunities for climbing, elevated resting and vocalisation. Frogs regularly climbed to more than 1.5 m above ground level on mesh walls and used perches at approximately 1 m height for vocalising from a resting position.

In alignment with other authors (see Jameson et al., 2019), we observed substantial bouts of combat between male frogs sometimes lasting more than four minutes, whereby animals attempted to flip one another using their nuptial spurs to grasp opponents while pushing against the floor with their hind legs. These fights appeared to be energetically expensive and support observations of scratches and other injuries in male frogs thought to derive from nocturnal combat (Jameson et al., 2019).

We also made several novel observations of behaviour in mountain chicken frogs. These behaviours were included in broader behavioural categories, but specific description may be useful in managing this species in captivity and understanding its natural history in the field. In line with Daltry & Gray (1998) and Daltry (2002), we observed reproductive advertisement behaviour (primarily vocalisation) from males outside of the nest box entrance and in elevated, exposed positions. However, in a novel observation, on multiple occasions amplexus was observed outside the nest box in exposed, flat areas. Couples either separated or moved into nest boxes while still amplectant.

We observed previously undescribed social behaviours between males and females. In Behaviour 1 (Table 5), which was conserved in sequence between several observations, a female frog approaches a vocalising male near to a nest box entrance. The female's body posture is low and flat to the ground, usually calling softly, in which position she crawls very slowly forwards, attempting to move underneath the male. Once moving, female vocalisation stops. Meanwhile the male vocalises continuously, often in alternating synchrony with other males, reflecting wild observations of males alternating rather than overlapping their respective calls (Davis et al., 2000). When the female makes physical contact, with her snout touching the underside of the male's throat, the male stops vocalising and crawls away. The function of this behaviour is not clear, but given that it only occurred between females and males, and incorporated vocalisation from both sexes, the slow movement and exaggerated posture is likely linked to courtship. In Behaviour 2 (Table 5), male frogs moved their folded hind legs side to side with increasing frequency and then came to rest. Again, the function for this behaviour is not clear, but it is again likely linked to communication between individual frogs; sometimes frogs were not clearly associated with another frog when performing this behaviour. The exaggerated and repeated movements are almost certainly visible to more distant frogs within an enclosure since mountain chickens possess good night vision (see Jameson et al., 2019).

Table 5. Novel behaviours in mountain chicken frogs described from video footage from this study

Image	Description and/or timeline of behaviour
	Behaviour 1: These images demonstrate a timeline of Behaviour 1, which appears to be part of courtship. 1. The male (left, image 1) was positioned near the entrance of the nest box in an exposed position and vocalised continuously throughout the night; alternately synchronised with a male in another enclosure; it appeared that the female (right, image 1) was attracted to his calling. 2. The female (right, image 2) positions her body flat to the ground then slowly attempted to crawl under the male (left) from the anterior end. 3. The male immediately stopped vocalising and crawled away from the female (image 3). 4. A few seconds later, he continued vocalising throughout the night. The timescale of this behaviour can vary; some sequences can last for 20 seconds whereas others can last up to 3 minutes. To view an example of this behaviour sequence, watch our video (BHS video A, 2023)
	Behaviour 2: This behaviour was observed repeatedly throughout the study period. Here, frogs were seen to move their hind limbs side to side while shifting their abdomen side to side at the same time; at the beginning of the behaviour, the movement is slow and then continuously accelerates to the end. Frogs were observed carrying out this behaviour either alone or close to a conspecific. The timescale of this behaviour lasts for approximately 10 seconds. The behaviour is shown from lateral (image 1) and frontal (image 2) aspects; red lines indicate the moving parts. To view an example of this behaviour sequence, watch our video (BHS video B, 2023; BHS video C, 2023)

PCA identified a consistent behavioural grouping of movement, resting and refuge, with the latter being opposed to the former two. This combination of behaviours follows a typical bold-shy axis (e.g. Martin & Réale, 2008), with behaviours associated with sitting out (movement and resting in open areas) negatively correlating with hiding. Disturbance significantly and substantially affected multiple measured behaviours. The greatest number of significantly affected behaviours were seen in the most disturbed frog dens (dens 2 and 4; disturbance intensities C and D), and the fewest changes in the least disturbed groups (dens 1 and 3; disturbance intensity B; Fig. 6). Most strikingly was the enormous and significant reduction in hunting behaviour under all disturbance intensities, and reduction in social interactions (disturbance intensities C and D) and vocalisations (disturbance intensity C) under the highest disturbance intensities. Frogs in the den with the highest disturbance intensity (den 2, intensity C) also showed a significant shift of behaviour away from bold-orientated behaviours (movement and resting) towards shy-orientated behaviours (refuge). These results correspond to anecdotal reports of mountain chickens from keepers suggesting that similar disturbance causes feeding behaviour to decrease and potential association between interference with frogs and reproductive failure (Jameson et al., 2019). However, our results show that the impact of disturbance goes beyond feeding and impacts other aspects of behaviour, especially where disturbance was direct rather than indirect, and at higher levels of disturbance intensity.

As behaviours were counted at an enclosure level, differences between disturbance intensities C (3/4 of frogs captured) and D (1/4 frogs captured) are likely the result of the differential dilution of behavioural changes based on the number of anaesthetised frogs per group.

These results demonstrate that disturbance may have key impacts on behaviours involved in feeding and reproduction. Given that consistent captive reproduction, unnaturally small adult size and nutritional disease are frequently a challenge for the ex-situ conservation of this species (King et al., 2011; Tapley et al., 2015; Jameson et al., 2019), our data strongly suggest that disturbance for husbandry and especially health checks may be a contributing cause. In alignment with this, Bell (2002) found that a factor responsible for unsuccessful captive breeding in Archey's Frogs *Leiopelma archeyi* was regular handling.

mSPI was not significantly affected by disturbance in frogs exposed to indirect disturbance (dens 1 and 3; disturbance intensity B) compared with the baseline, or in one of the dens with higher disturbance intensity (den 2; intensity C) but was significantly reduced in the other directly disturbed group (den 4, intensity D; Fig. 6). Given that a lower mSPI is typically associated with better welfare (see above), this result is unexpected. It is possible that the increase in resting behaviour and reduction in refuge behaviour in den 4 caused frogs to spend more time out of the refuges and in other resource zones. It is unclear why direct disturbance did not have this effect (nor even an effect approaching significance) and interpretation of this result is therefore tentative. However, this result suggests that behavioural data combined with mSPI can provide a better understanding of the impact of husbandry than mSPI alone, and that reducing mSPI does not necessarily indicate positive change if it is caused by negative behavioural shifts.

Health checks are imperative, particularly as mountain chickens are known to develop potentially fatal diseases within captivity (Ashpole et al., 2021), but our results show a clear tension between the need for health checks and the need for minimising disturbance. Importantly, the effects of disturbance were not limited to those dens including frogs that were captured, but were identified for some behaviours in dens that were indirectly disturbed by keepers catching frogs in neighbouring dens. This result highlights the sensitivity of mountain chicken frogs to disturbance and suggests that captive colonies might be best maintained where disturbance can be limited. Keeper anecdotes suggest that feeding may be suppressed for up to one month in disturbed mountain chickens (Jameson et al., 2019; Michaels & Servini, pers. obs.). Our data are not able to assess latency to return to normal behaviour, but taking these anecdotes as a guide, behavioural impacts may be both substantial and long lived, particularly as some collections may disturb frogs on a more frequent than monthly basis (Michaels, pers. obs.), which may prevent return to normal behaviour from ever happening. Further research is needed to assess rates of recovery from handling to determine impacts of behaviour, and the time taken for frogs to recover to their normal activity patterns. The use of in-nest box cameras to elucidate behaviour exhibited within these chambers is also recommended to better understand not only what the frogs do inside the boxes, but also to identify further effects of disturbance and other husbandry practices on behaviour.

In other anurans, disturbance from zoo visitors, handling and veterinary procedures are known to cause stress responses that may be detected through corticosteroid stress hormones and sex hormones (Narayan, 2013; Narayan et al., 2012a; 2013), behaviour (Boultwood et al., 2021) and dermal bacterial communities (Antwis et al., 2014) and that these effects may be dependent on the degree of disturbance (Narayan et al., 2011; 2012b). However, inter-specific differences appear, based on very limited data, to exist in that some taxa do not show stress hormone responses to relatively invasive procedures (e.g. Antwis et al., 2014b). Impacts of stressors on behavioural repertoires and resource use have not, to the authors' knowledge, been investigated in other anurans. However, our data suggest that mountain chicken frogs mount a stress response to environmental disturbance, restraint and veterinary procedures, although stress hormone analysis, which was outside the scope of this study, would be useful to link behavioural to physiological changes. The impact of disturbance on reproductive behaviours seen in mountain chicken frogs in the present study may be mediated by the strong and fast-acting impacts of restraint on sex hormones in other anurans (Narayan et al., 2012).

Our methods are limited by the necessity to collect data at the enclosure level. Although we controlled frog numbers and sex ratios, the ability to follow individual animals would provide higher resolution data on behaviour and response to putative stressors compared with our data, as well as increasing sample size and enabling more detailed statistical analysis. We did see imperfect alignment in results between the two enclosures exposed to indirect disturbance (disturbance intensity B), which indicates some variation in response between dens. Individual-level data, or collection of data from more groups, would also allow better assessment of behavioural effects for this reason.

Overall, our data provide initial insights into nocturnal behaviours, activity patterns and budgets for mountain chicken frogs and demonstrate sensitivity to disturbance and restraint in this species. These findings may be incorporated into best practice captive husbandry protocols for the species. Despite the utility of our data, important blind spots include the behaviour exhibited by frogs while within nest boxes, and the behavioural recovery period after disturbance. Further research into these areas, as well as work to link behavioural outcomes to physiological and endocrine mediators, is strongly recommended.

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SHORT NOTE

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First report of chytridiomycosis in the Southern Yungas Andean forest: a threat to the endangered La Banderita marsupial frog *Gastrotheca gracilis*

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This work reports the first record of *Batrachochytrium dendrobatidis* (*Bd*) infection in the endangered La Banderita marsupial frog *Gastrotheca gracilis* from the Southern Yungas Andean forest. We analysed swab samples from the oral discs of 20 tadpoles and histological sections from two post-metamorphic individuals. We found 60 % of the tadpoles to be infected, and the histological sections revealed the presence of zoosporangia of *Bd* in different maturation stages. The signs of infection confirm the presence of *Bd*, which may pose a threat to the endangered La Banderita marsupial frog populations.

Keywords: conservation, *Batrachochytrium dendrobatidis*, chytridfungus infection, Hemiphractidae, tadpoles

Globally, amphibians are experiencing a major decline, rendering 60 % of the amphibian species being Endangered (IUCN, 2022). There is a consensus among scientists and conservationists that emerging infectious diseases, such as chytridiomycosis caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans*, are among the main factors that contribute to this global decline (Martel et al., 2013; O'Hanlon et al., 2018; Scheele et al., 2019; Fisher & Garner, 2020).

La Banderita marsupial frog *Gastrotheca gracilis* Laurent 1969, is the southernmost species of the family Hemiphractidae (Duellman, 2015). *Gastrotheca gracilis* is a threatened species listed as Endangered on both National and International Red Lists (Vaira et al., 2012; IUCN, 2019). The main threats to this marsupial frog are habitat loss by clear-cutting of primary forests, wildfires and reproductive habitat alteration by domestic animals and human activities (IUCN, 2019). After the rediscovery of this species in 2011 (Akmentins et al., 2012), the three known populations of *G. gracilis* have been monitored and seem stable despite the active threats (pers. obs.). In 2018, a population supplementation program of *G. gracilis* was established with the support of the Amphibian Ark in the ex-situ facilities of the HorcoMolle Experimental Reserve of the National University of Tucumán (REHM-UNT), with the target of recovering the population of this threatened species in Los Sosa Provincial Reserve in Tucumán province, Argentina (Akmentins, 2019).

Chytridiomycosis is a potential threat to the amphibians of the Southern Yungas Andean forest (Akmentins et al., 2012; 2014). In Bolivia, *Bd* prevalence values of up to 30 % were reported for the Hemiphractidae family (Burrowes & De la Riva, 2017). In this work, we combine molecular and histological analysis to evaluate the presence of *Bd* in a wild population of *G. gracilis*.

On 12 January 2019, we captured 67 tadpoles of *G. gracilis* in Los Sosa Provincial Reserve (27° 00' S; 65° 40' W, 1455 m.a.s.l.), Tucumán Province, Argentina. Following the swabbing protocol proposed by Fisher et al. (2018), we collected samples with swabs (DELTA LAB-300201) from the oral disc of the largest 20 tadpoles and released them immediately after. Swabs were stored in labelled refrigerated cryogenic vials until the laboratory processing. We extracted DNA using the DNeasy Blood & Tissue commercial kit (Qiagen ®), and we quantified *Bd* DNA following the protocol proposed by Boyle et al. (2004). We conducted a real-time amplification (qPCR) using a StepOnePlus thermal cycler (Applied Biosystems ®). Thermal profiling of the reaction was performed at 95 °C for 20 seconds, then 50 cycles (90 °C for 1 second, followed by 60 °C for 20 seconds). Each plate included a negative control (UltraPure™ DNase/RNase-free distilled water) and standard curve from 0.01–1000 zoospore genome equivalents as positive control for the qPCR.

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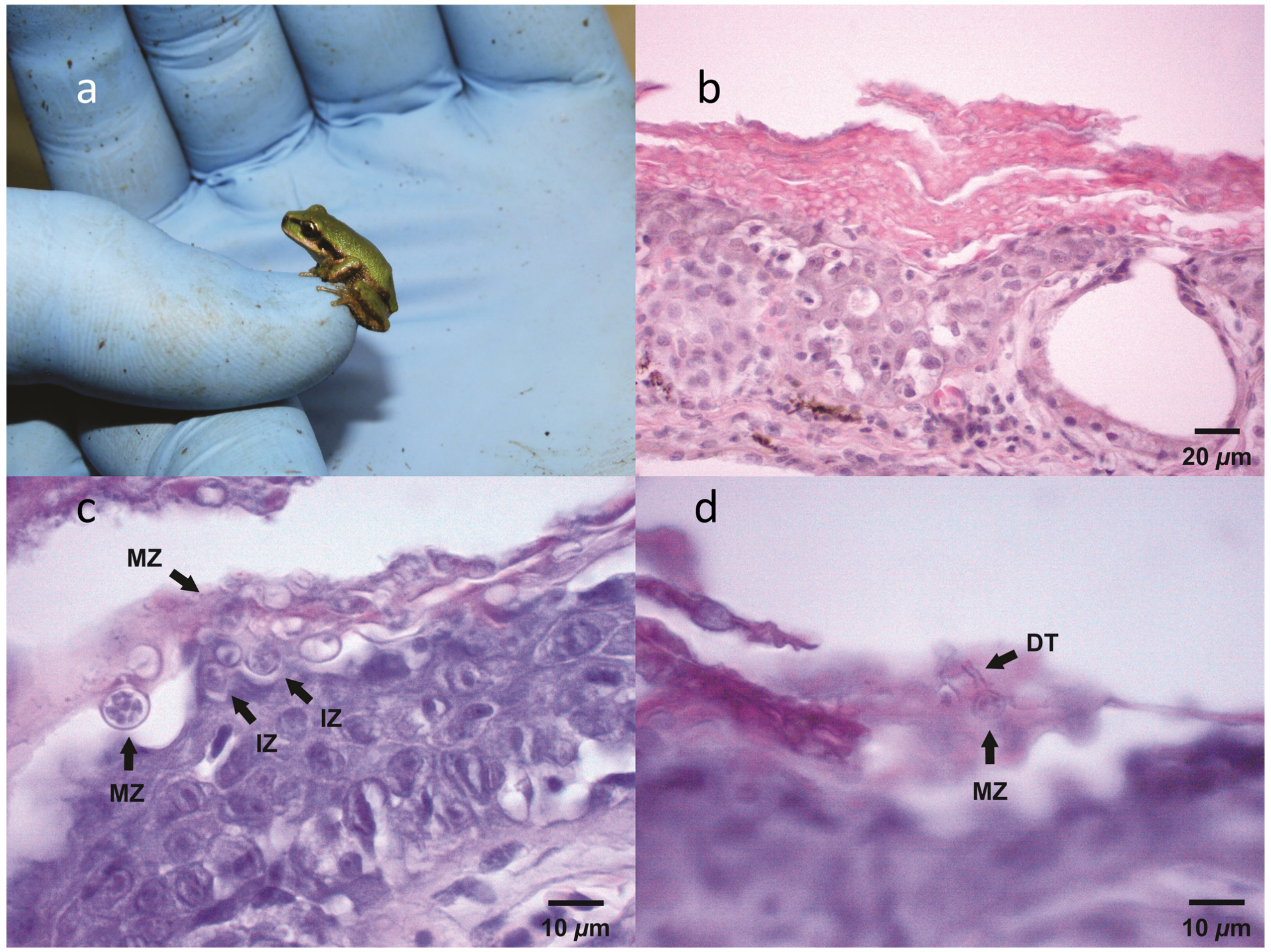

Figure 1. Post-metamorphic individual **(a)** and photomicrographs of the epidermis of La Banderita marsupial frog *Gastrotheca gracilis* maintained in the ex-situ facilities of the HorcoMolle Experimental Reserve, Tucumán, Argentina. Epidermis sections showing **(b)** signs of hyperplasia and hyperkeratosis of the stratum corneum; **(c)** zoosporangia of *Batrachochytrium dendrobatidis* in mature (MZ) and immature (IZ) stages; and (d) detail of a mature zoosporangium (MZ) containing discrete basophilic zoospores and discharge tube (DT).

All samples were run duplicate, and we used StepOne v2.3 software (Applied Biosystems ®) to estimate *Bd* DNA loads in zoospores equivalents from the amplification curves. We considered a *Bd* positive sample if *Bd* DNA was amplified.

The remaining 47 tadpoles were kept in captivity in the ex-situ facilities of the HorcoMolle Experimental Reserve of the National University of Tucumán (REHM-UNT) until they completed metamorphosis. The tadpoles were maintained in a fish tank (dimensions: 100 x 50 x 40 cm), with a density of one tadpole per two litres of dechlorinated tap water. Food was provided every other day, alternated between dry and fresh food. Food rations per tadpole were 0.05 g of flake fish food (i.e. dry food) and 1 g of pumpkin (i.e. fresh food). Room temperature was maintained at 24 °C, with a 12 h light cycle. Swab samples from the oral disc in these 47 tadpoles were not collected. We performed a post-mortem histopathological analysis from the skin of the pelvic patch in two 12-day-old post-metamorphic individuals. The skin samples were formalin-fixed and paraffin-embedded. A5 μm histological sections of the skin was obtained, used hematoxylin and eosin stain, and determined the life cycle stages of *Bd* (Berger et al., 1999; 2005).

The percentage of infected individuals with *Bd* in the 20 analysed tadpoles of *G. gracilis* was 60 %. Loads of *Bd* were between 0.05 and 42.17 zoospore equivalents. In total, 12 (25.5 %) of the 47 post-metamorphic individuals of *G. gracilis* died after showing a combination of signs of infection compatible with chytridiomycosis infection (i.e. poor body condition, lethargy and inappetence). We observed hyperplasia and hyperkeratosis of the stratum corneum (a sign of *Bd* infection) and the presence of zoosporangia of *Bd*. The zoosporangia were detected in at least three different maturation stages: immature, mature containing numerous basophilic zoospores and with formed discharge tubes, and empty mature (Fig. 1).

Our observations represent the first record of *Bd* in the endangered *G. gracilis* and the first record of *Bd* in the Southern Yungas Andean forest. *Bd* has been reported in other Andean ecoregions of the Neotropics, such as the Northern Yungas forest, Puna, and Inter-Andean Mesothermy Valley. In many locations, the amphibian population decline and local extinction has been associated with the emergence of *Bd* in the Southern Andes (Barrionuevo & Mangione, 2006; Burrowes & De la Riva, 2017). The presence of *Bd* in the Southern Yungas Andean forest, the ecoregion with the highest percentage of endemic and threatened species of Argentina, is concerning (Lavilla & Heatwole, 2010; Vaira et al., 2017). The three southernmost marsupial frog species inhabit Argentina, and all of them have suffered substantial population declines since the 1990s (Akmentins et al., 2012). Our results suggest that *G. gracilis* is susceptible to infection by *Bd*. Monitoring local amphibian populations in combination with *Bd* surveillance is desirable to assess the impact of this pathogen on the persistence of populations of this and other frog species.

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