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Effect of weather conditions and presence of visitors on adrenocortical activity in captive African penguins (*Spheniscus demersus*)

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ABSTRACT

A number of potential stressors are present in captive environments and it is critically important to identify them in order to improve health and welfare in *ex situ* animal populations. In this study, we investigated the adrenocortical activity of a colony of African penguins hosted in an immersive zoo in Italy, with respect to the presence of visitors and local microclimatic conditions, using the non-invasive method of assessing faecal glucocorticoid metabolites (FGMs). The penguins' exhibit is a large naturalistic outdoor enclosure, which closely reproduces the natural habitat of this species. Data collection took place from the beginning of June to the end of August 2014, during the period of maximum flow of visitors. We carried out 12 sampling periods, each involving 2 consecutive days; during the first day we counted the visitors and we registered the meteorological data, and on the second day, we collected the faecal samples, which amounted to a total of 285 faecal samples. Our results showed that the number of visitors did not influence the adrenocortical activity of the African penguins. Conversely, the local microclimatic conditions did influence the physiological stress on these birds. We found that an increase of the daily mean temperature induced a significant increase in FGM concentrations, although humidity and wind speed had a moderating effect on temperature and reduced the heat-induced stress. Moreover, we calculated two climatic indices, commonly used to assess the thermal discomfort in animals, namely the THI (Temperature-Humidity Index) and WCI (Wind Chill Index), and we detected a positive relationship between their values and the FGM levels, demonstrating that these indices could be useful indicators of weather discomfort in African penguins. Our study shows that the simulating naturalistic conditions could have significant benefits for zoo animals, such as reducing the negative effect of visitors. Nevertheless, it should be taken into account where the zoological facility is located and if the local microclimatic conditions are compatible with the hosted species, to ensure that they do not differ greatly from their natural habitat.

1. Introduction

A wide range of environmental challenges threatens the welfare of animals housed in artificial habitats. For zoo animals, the number of potential environmental stressors can be numerous, and the effects are often species-specific (Scarlata et al., 2013). Improving health and welfare of *ex situ* populations is one of the main goals of zoological facilities worldwide (Melfi, 2009; Howell-Stephens et al., 2012). Therefore, identifying these conditions, which are associated with high levels of stress in captive animals, is critically important to achieve these aims. There are a number of potential stressors in captive environments, such as inadequate housing conditions and inappropriate social interactions, which could restrict the natural behaviour of zoo animals (Scarlata et al., 2013; Khonmee et al., 2014). In addition, captive animals are exposed to a continuous human presence. The presence of people at zoo exhibits could be perceived by animals as a negative influence, a form of enrichment, or simply a changing variable with no evident effect (Hosey, 2000; Davey, 2007; Hosey, 2008). Whether the zoo visitors have a stressful effect on the animals seems to depend on many factors, such as the temperament of the species or individuals (Fernandez et al., 2009), the animals' body size (Davey, 2007), and the enclosure design (Davey, 2007; Hosey, 2000). Particularly, it is known that the zoo exhibit design influences the well-being of captive animals (Morgan and Tromborg, 2007; Young et al., 2013). Due to the logistics of space and enclosure size, the variety of environmental conditions available for zoo animals is

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normally reduced compared to the wild (Young et al., 2013). Moreover, animals in zoos are often subjected to microclimatic conditions that can differ greatly from their natural habitat, and to which their species is not usually adapted (Morgan and Tromborg, 2007). While the effect of visitors on the welfare of captive animals has been widely studied for more than four decades (Hosey, 2000; Davey, 2007; Hosey, 2013), few studies have investigated the microclimatic conditions that result from outdoor zoo enclosures (Young et al., 2013) and their effect on captive species.

Assessing hormone levels in animals is a widely accepted method to determine the sources of stress, which could influence the welfare of captive animals (Wielebnowski, 2003; Howell-Stephens et al., 2012). The physiological stress response in vertebrates results in an increased secretion of glucocorticoid hormones (GCs) subsequent to the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Möstl and Palme, 2002; Touma and Palme, 2005; Sheriff et al., 2011). The main GCs produced by the adrenal gland are cortisol and corticosterone, with the latter being predominant in birds (Möstl et al., 2005; Palme et al., 2005; Cockrem, 2007). If exposure to a stressor persists, chronic GC secretion can lead to deleterious consequences on animal behaviour, individual fitness and reproductive success, and can induce immunosuppression (Munck et al., 1984; Liptrap, 1993; Munck and Naráy-Fejes-Tóth, 1994). One way to evaluate physiological responses to environmental stressors is by analysing the excretion of GC metabolites in faeces (Schwarzenberger, 2007; Scarlata et al., 2013). This non-invasive method permits the monitoring of the animal's endocrine status without the necessity to capture or handle the animals, which may compromise the accurate assessment of stress (Millspaugh and Washburn, 2004; Goymann, 2012). In addition, faecal glucocorticoid metabolites (FGMs) represent an average, pooled value of excreted steroids, and are therefore less affected by diurnal fluctuations and pulsatile hormone secretion (Palme, 2005). Monitoring adrenal activity through non-invasive faecal hormone sampling is rapidly gaining popularity as a tool to assess zoo animal welfare (Clark et al., 2012) in order to develop the best management practices, also with respect to endangered species.

The African Penguin (*Spheniscus demersus*) is an endemic seabird of the coastal region of South Africa and Namibia, and is the only member of the Order *Sphenisciformes* which breeds in Africa (Shelton et al., 1984). African penguins are found from central Namibia (Hollam's Bird Island) to South Africa's Eastern Cape province (Bird Island) (Crawford et al., 2011). African penguins at this latitude have developed adaptations for cold marine environments, due to the presence of cold up-welling currents, while the air temperatures, modified by cool sea breezes, are generally moderate (Frost et al., 1976). The current conservation status of this species is "Endangered", and is included in the Red List of Threatened Species of the IUCN (International Union for Conservation of Nature). The population in the wild of this species has dramatically decreased in recent years to less than 75,000–80,000 mature individuals (BirdLife International, 2013). This decline is mainly due to loss of habitat, reduction of fish stocks, environmental pollution (including oil spills), and egg collection (Barham et al., 2006; Crawford et al., 2011). African penguins are exhibited in zoos and aquaria all over the world, and as they face an elevated risk of extinction, *ex situ* conservation programs are becoming increasingly crucial.

Here, we investigated the physiological stress related to environmental conditions of a colony of African penguins hosted in an immersive zoo (Zoom Torino, Cumiana, Italy), using the non-invasive method of assessing faecal glucocorticoid metabolites (FGMs). Previous studies have demonstrated that this method is useful for detecting and highlighting potentially stressful situations, which may influence the welfare of penguins, and it allows the detection of individual differences of FGM concentrations (Anfossi et al., 2014; Ozella et al., 2015). At the time of study, there were 54 penguins (26 females and 28 males) in the colony. We carried out 12 sampling days of faecal collections; during each sampling day the collection of samples took place from 9:00 AM to 8:00 PM. The faeces collected during the different sampling days did not belong to the same penguins, and we considered the FGM average that related to the penguin colony as a whole. Moreover, there was variability concerning the number of samples, collected on each sampling day, between males and females, penguins' age, and time of day (morning, afternoon and evening). During the 12 days before the faecal collections, we registered the number of visitors and the microclimatic conditions. The data collection took place during summer 2014 (from the beginning of June until the end of August), when the weather conditions in Cumiana are usually warm and muggy. This period included the maximum flow of visitors for the zoological facility, mainly due to the presence of a swimming pool for the use of visitors, adjacent to the penguins' pond.

The main objectives of this study were: (1) to evaluate any differences in FGM concentrations between penguins of different gender and ages, and between time of day and sampling days; (2) to assess if the presence of visitors and the microclimatic conditions could influence the adrenocortical activity in these birds; (3) to evaluate if two climatic indices, namely the THI (Temperature-Humidity Index) and WCI (Wind Chill Index), are related to FGM levels.

2. Materials and methods

2.1. Study site and animals

The study was conducted at an immersive zoo called Zoom Torino (44°56′N, 7°25′E) located in Cumiana (Italy), and accredited by the EAZA (European Association of Zoos and Aquaria). The biopark covers an area of about 16 ha with naturalistic enclosures that closely reproduce the natural habitat of the different species. The exhibits provide an opportunity for visitors to view the various animal species through the use of open-faced exhibits and glass panels, without evident barriers or cages. All the exhibits have a large outdoor area, with flooring and plant elements designed to simulate natural habitat conditions, and an indoor area for the night shelter.

The penguins are housed in an exhibit, which reproduces the habitat of "Boulder Beach", a natural nesting site in South Africa, near Cape Town (Fig. 1). The exhibit covers an area of 1500 m^2, including a pond of 120 m^2 (water depth maximum 3 m; temperature constantly maintained at 15 °C). The exhibit substrate is made from sand and pebbles; trees and bushes are present to serve as hiding places or cover for penguins, and artificial nests are present in sufficient numbers to accommodate each pair. An indoor area is used only as a quarantine zone, and these animals are therefore exposed both day and night to the weather. Visitors can view the penguins from three points of the exhibit: a wooden footbridge that crosses the enclosure, where they are separated from the animals by a low wooden fence; from two glass panels, which allow the underwater viewing of the animals; and from two other glass panels, which separate the penguins' pond from the swimming pool for visitors, which is adjacent to the penguin exhibit. This point also allows the underwater viewing of the animals.

At the time of the study there were 54 African penguins (26 females and 28 males) in the colony, all of which were born and reared in captivity; 28 penguins were transferred from another European zoo in 2009, while 26 penguins were born at Zoom Torino. Penguins of age 3–12 months were classified as "juveniles";

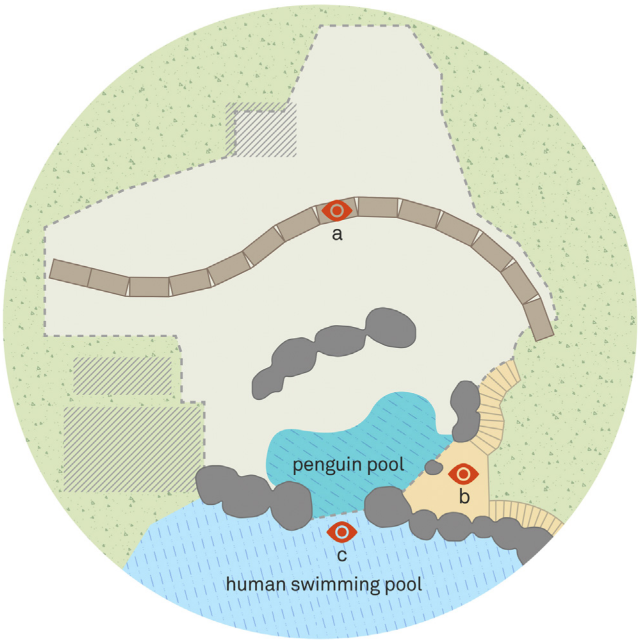

Fig. 1. Diagram of the penguin exhibit at Zoom Torino. The visitors can view the penguins from three observation points of the exhibit: (a) from a wooden footbridge that crosses the enclosure (b) from two glass panels, which allow underwater viewing of the animals; and (c) from two other glass panels, which allow underwater viewing of the animals, and which separate the penguins' pond from the swimming pool for visitors.

penguins younger than 2 years old, with adult plumage, who were looking for a mate and/or a nest were classified as "prospectors" (Warham, 1990); penguins aged 2–20 years were classified as "adults", and penguins older than 20 years were "old", according to the average lifespan of African penguins (10–27 years; Pearce, 2011). Nesting chicks (younger than 3 months old) were not involved in this study as they always remained within the nest.

2.2. Data collection

Data collection took place in summer 2014, during the period of maximum flow of visitors, from 2nd June (corresponding to the opening of the swimming pool) to 25th August. Each sampling period involved 2 consecutive days: during the first day we carried out (1) the visitors' count and (2) the meteorological data registration. On the second day of sampling, the (3) collection of faecal samples took place. We carried out 12 sampling periods, for a total of 24 days of observation. In a previous study, we demonstrated that in this species the FGM levels begin to increase after 5 h from a punctual stress (*i.e.* capture and immobilization of animals for 5 min) and decrease within 30 h (Ozella et al., 2015). Therefore, we assume that FGM concentrations assessed during the day of faecal collection reflected the relative chronic stress accumulated during the previous day.

2.2.1. Number of visitors

In order to count the number of visitors at the penguin exhibit, instantaneous scan sampling method (Altmann, 1974) was used. The visitors were photographed at the three points of permitted penguin observation: (a) the wooden footbridge, (b) the glass panels, and (c) the glass panels that separated the penguins' pool from the visitors' pool (Fig. 1). Three sampling sessions were carried out during each sampling day; the first sampling took place just after the opening of the biopark (10:00 AM), the second sampling took place after the first feeding time of the penguins (12:00 PM), and the third sampling took place after the second feeding time of the penguins (4:00 PM). During each sampling session, we performed a total of 60 scan samples: 20 scan samples for each observation point, every 5 min. A grand total of 2160 scan samples were taken. Moreover, in order to evaluate the daily number of visitors, Zoom Torino kindly provided the number of people admitted into the biopark, and the people admitted into the swimming pool adjacent to the penguins' pond. The visitors admitted to the swimming pool can also visit the biopark, but the visitors admitted to the biopark do not have access to the swimming pool.

2.2.2. Microclimatic parameters and climatic indices

The meteorological data were obtained from the meteorological station of "ARPA Piemonte" (Agenzia Regionale Protezione Ambiente del Piemonte) located in Cumiana (Italy), about 1 km from the penguins' exhibit. The daily meteorological data registered by the station are: 24 h mean temperature (°C), 24 h mean relative humidity (%), 24 h mean wind speed (m/s), and 24 h accumulated rainfall (mm). Moreover, two climatic indices, the THI (Temperature-Humidity Index) (Thom, 1959) and the WCI (Wind Chill Index) (Tucker et al., 2007) were calculated. Climate indices are commonly used to assess the degree of thermal discomfort in humans and animals, by combining different microclimatic parameters into a single measure (Van laer et al., 2014). The THI was originally developed by Thom (1959) as a "discomfort index" to estimate the levels of discomfort for human beings during summer months. Subsequently, the THI was used to predict the effects of environmental warmth in farm animals (Segnalini et al., 2011).

The THI combines air temperature and relative humidity in a single parameter:

$$\mathrm{THI} = 0.8 \times T + [\mathrm{RH} \times (T - 14.4)] + 46.4$$

where T is air temperature in °C and RH is the relative humidity in decimal form.

The WCI was initially used to evaluate thermal comfort for humans (Siple and Passel, 1945), and it has also been used to assess thermal conditions in a variety of animal species (Mader et al., 2010; Angrecka and Herbut, 2015). The WCI relates ambient temperature and wind speed, and one of the most commonly used algorithms for calculating WCI is the formula according to Tucker et al. (2007):

$$\mathrm{WCI} = 13.12 + 0.62 \times T - 13.17 \times [\mathrm{WS}]^{0.16} + 0.40 \times T \times [\mathrm{WS}]^{0.16}$$

where T is air temperature in °C and WS is wind speed in km/h.

2.2.3. Collection of faecal samples

A total of 235 samples were collected. Faecal collection was carried out on the days after counting the visitors and registering the meteorological data. During each sampling day, sample collection took place from 9:00 AM to 8:00 PM. "Morning" was considered as the time from 9:00 AM to 12:00 PM, "afternoon" from 12:00 PM to 6:00 PM, and "evening" from 6:00 PM to 8:00 PM. The birds were observed from a distance of higher than 5 m, through binoculars to avoid disturbing the animals, by a researcher standing motionless outside the exhibit. Each penguin involved in the study was identified by means of a coloured flipper band on one of its wings. After a defecation event, the researcher entered the exhibit and gathered the expelled faeces. Sample collection took place immediately after defecation to avoid bacterial and microbial degradation (Millspaugh and Washburn, 2004). As urinal and faecal excretion are combined in birds, we only collected the faecal portion from droppings, which was distinguishable by colour (Ninnes et al., 2010). Samples were gathered in cryovials and frozen immediately after collection at −20 °C (Millspaugh and Washburn, 2004; Palme et al., 2005; Sheriff et al., 2011).

Throughout the study period, the penguins' diet was not modified in order to avoid alterations in gut flora and, as a consequence, steroid metabolism (Sheriff et al., 2011).

2.3. Faecal glucocorticoid metabolite extraction

FGM extraction was carried out following the procedure described by Anfossi et al. (2014). Briefly, penguin faeces were transferred to a 15 ml tube and extracted with 5 ml of methanol: water (70: 30, v/v). After centrifugation (to remove sand and particulate matter of the exhibit), supernatants were transferred to a weighted tube, and the amount of the extracted sample was calculated by the difference between the total weight of the extract and the weight of the extraction solvent. Sample extracts were immediately stored at −20 °C until required for analysis.

2.4. Enzyme immunoassay (EIA)

Before FGM analysis, the immunoassay was validated for suitability in African penguins by testing specificity, precision, limit of detection, and accuracy (Anfossi et al., 2014). The EIA is based on a polyclonal antibody raised against cortisol-3-(O-carboxyme thyl)oxime-BSA (Bovine Serum Albumin). The experimental protocol has been previously reported (Anfossi et al., 2014). Briefly, calibration curves were obtained by dispensing 150 µl of the conjugate (cortisol-3-(O-carboxymethyl)oxime-horse radish peroxidase diluted at 1.5 mg L^{-1} in 20 mM TRIS buffer pH 8.0, supplemented with 0.3 M NaCl, 1% BSA, and 0.1% Tween 20) and 50 µl of corticosterone (diluted in 35% methanol at concentrations ranging from 0 to 50 µg l^{-1}) into immunoreactive wells obtained by coating with the polyclonal antibody. After a 1-h incubation, wells were washed, and colour development was obtained by 30 min incubation with TMB (200 µl per well). A volume of 50 µl of sulphuric acid (2 M) was used as a stop solution, and absorbance was recorded at 450 nm. Unknown sample concentrations were measured by replacing the corticosterone standard solution with sample extracts diluted at a ratio of 1 + 1 with water and then 1 + 9 with 35% methanol. All standards and samples were measured in duplicate.

2.5. Data analysis

The average FGM concentration (expressed as mean ± SEM) was calculated for each sampling day, gender, age class, and time of day. Data were tested for normality and equality of variance. To assess differences in FGM levels between sexes, the *Welch two sample t-test* was used. To assess differences in FGM levels between age classes and times of day (morning, afternoon, and evening), the *Welch one-way ANOVA* was used. The average FGM concentrations in each sampling day were compared using the *Welch one-way ANOVA*, followed by the *post hoc Waller-Duncan test*.

The average number of visitors (a) at the wooden footbridge, (b) at the glass panels, and (c) at the glass panels that separated the penguins' pool from the visitors' pool, were calculated. Before evaluating the effects of environmental variables on FGMs, a preliminary analysis was conducted to assess a possible relation between number of visitors number (both instantaneous and daily number of visitors) and microclimatic parameters, by means of the *correlation Spearman test*. To evaluate a possible relation between average number of instantaneous visitors at each observation point (a, b, and c), the *correlation Spearman test* was used. Likewise, to assess a possible relation between the average number of instantaneous visitors and the daily evaluation of the number of visitors, the *correlation Spearman test* was used.

We performed *Principal Component Analysis* (PCA) on microclimatic parameters in order to assess their variation and for data visualization. Afterwards, the *General Linear Model* was used to investigate the effects of the number of visitors and microclimatic parameters on FGM levels. Moreover, to assess the relationship between the THI and the WCI, with FGM concentrations, a simple linear regression was performed.

Statistical analyses were carried out using the R software, Version 3.1.2 (R Core Team, 2014), $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Differences of FGMs between genders, age classes, times of day and sampling days

The average FGM concentrations for each sampling day, gender, age class, and time of day are indicated in Table 1. The FGM concentrations did not differ between gender (*Welch two samples t-test*: $t = -0.446$, $p = 0.655$), age class (*Welch one-way ANOVA*: $F = 2.196$, $df = 3$, $p = 0.097$), or times of day (*Welch one-way ANOVA*: $F = 0.638$, $df = 2$, $p = 0.530$). Mean FGM concentrations differed significantly between sampling days (*Welch one-way ANOVA*: $F = 2.065$, $df = 11$, $p = 0.032$), and the *Waller-Duncan post hoc test* showed that FGM levels were significantly higher on 9th June compared to: 3rd June ($p = 0.026$), 14th July ($p < 0.001$), 24th July ($p = 0.004$), 28th July ($p = 0.046$), 30th July ($p = 0.023$), 1st August ($p = 0.026$), and 18th August ($p = 0.003$); conversely, FGM levels were significantly lower on 14th July compared to 23rd June ($p = 0.033$) and 25th August ($p = 0.044$) (Fig. 2).

3.2. Correlations between number of visitor and microclimatic parameters

There were no significant correlations between the number of visitors and the microclimatic variables.

3.3. Correlations between the number of instantaneous visitors and daily visitors

The average number of instantaneous visitor counted at each observation point and the number of daily visitors provided by Zoom Torino are indicated in Table 2. The average number of instantaneous visitor counted at the wooden footbridge (a) was positively and significantly correlated with the average number of visitors counted at the glass panels (b) ($\rho = 0.78$, $p = 0.004$), and with the daily evaluation of people admitted to the biopark ($\rho = 0.79$, $p = 0.003$). The average number of instantaneous visitors counted at the glass panels that separated the penguins' pool from the visitors' pool (c) was positively and significantly correlated with the daily evaluation of people admitted to the swimming pool adjacent to penguins' pond ($\rho = 0.9$, $p < 0.001$). Therefore, the mean numbers of instantaneous visitors counted at observation points (a) and (c) were used for the subsequent statistical analyses to assess the visitor effect on FGM levels.

3.4. Weather features of sampling days

The daily meteorological data provided by the meteorological station of "ARPA Piemonte" (Agenzia Regionale Protezione Ambiente del Piemonte) located in Cumiana (Italy), are indicated in Table 2. The accumulated rainfall showed very low variability during the observation period (the rainfall was 0 mm for 10 days out of 12) (Table 2), therefore this parameter was not included in the subsequent statistical analyses. The first two components of the PCA explained 99.9% of the whole variance (PC1 = 94.1%, PC2 = 5.8%), and we represented these two PCA components in

Table 1
FGM values (ng/g) expressed as the mean ± SEM for each sampling day and for the total sampling period. Daily FGM mean values were also calculated for each gender, age class and time of day. Sample sizes are indicated in square brackets.

Sampling day (faecal collection)	FGM daily values	Gender		Age classes				Time of day		
		Female	Male	Juvenile	Prospector	Adult	Old	Morning	Afternoon	Evening
3rd June	1985 ± 1308 [18]	1957 ± 1258 [9]	2013 ± 1431 [9]	2072 ± 699 [1]	2116 ± 1524 [4]	1503 ± 1051 [8]	2635 ± 1606 [5]	1444 ± 577 [6]	2216 ± 1567 [11]	2697 ± 217 [1]
9th June	5357 ± 5470 [17]	5815 ± 5678 [6]	5108 ± 5617 [11]	1661 ± 197 [1]	5062 ± 5076 [5]	4957 ± 5759 [7]	7351 ± 6964 [4]	6609 ± 6463 [9]	3949 ± 4046 [8]	–
23rd June	4342 ± 4850 [9]	4810 ± 6316 [5]	3757 ± 2950 [4]	6754 ± 342 [1]	8073 ± 10,710 [2]	2233 ± 2033 [4]	3622 ± 2920 [2]	5336 ± 5111 [7]	860 ± 510 [2]	–
4th July	3677 ± 3582 [14]	3369 ± 4303 [6]	3907 ± 3235 [8]	–	5346 ± 4652 [4]	1849 ± 1356 [6]	4749 ± 4356 [4]	4625 ± 4324 [8]	2413 ± 1959 [6]	–
11th July	3497 ± 3434 [21]	3864 ± 4002 [9]	3221 ± 3099 [12]	1412 ± 254 [1]	3974 ± 3428 [2]	3071 ± 3256 [15]	6003 ± 4966 [3]	3514 ± 4011 [8]	3486 ± 3205 [13]	–
14th July	1644 ± 1360 [22]	1277 ± 809 [8]	1854 ± 1583 [14]	3425 ± 918 [2]	1475 ± 824 [2]	1619 ± 1471 [14]	926 ± 493 [4]	1373 ± 1000 [6]	1735 ± 1480 [12]	1778 ± 1746 [4]
24rd July	2405 ± 1730 [23]	2206 ± 1299 [10]	2559 ± 2040 [13]	2163 ± 1442 [3]	1053 ± 686 [3]	2356 ± 1849 [12]	3480 ± 1707 [5]	2018 ± 1704 [8]	2828 ± 1983 [11]	2018 ± 902 [4]
28th July	3135 ± 2400 [16]	3026 ± 2074 [7]	3220 ± 2750 [9]	3932 ± 893 [2]	2242 ± 1065 [3]	3307 ± 3033 [7]	3103 ± 2846 [4]	4014 ± 2495 [8]	2419 ± 2197 [7]	1106 ± 437 [1]
30th July	3041 ± 3119 [23]	3521 ± 3870 [11]	2601 ± 2324 [12]	7297 ± 6201 [2]	3439 ± 4727 [4]	1862 ± 1173 [14]	5175 ± 3217 [3]	1140 ± 620 [8]	4215 ± 3695 [12]	3415 ± 2772 [3]
1st August	3105 ± 2857 [24]	2473 ± 1938 [11]	3639 ± 3442 [13]	4099 ± 5065 [2]	2477 ± 1697 [6]	3131 ± 3323 [12]	3468 ± 2672 [4]	2264 ± 2624 [6]	3024 ± 3056 [14]	4647 ± 2454 [4]
18th August	2261 ± 2020 [21]	2157 ± 2189 [8]	2325 ± 1998 [13]	2766 ± 3572 [3]	1826 ± 1408 [3]	2317 ± 2204 [10]	2106 ± 1315 [5]	2414 ± 723 [3]	2205 ± 2463 [13]	2312 ± 1380 [5]
25th August	3490 ± 4310 [27]	2767 ± 3601 [14]	4268 ± 4995 [13]	–	6368 ± 6795 [8]	2693 ± 2087 [13]	1381 ± 1138 [6]	3385 ± 1529 [5]	3689 ± 5123 [18]	2725 ± 2859 [4]
Total	3058 ± 3245 [235]	2951 ± 3287 [104]	3142 ± 3221 [131]	3566 ± 2996 [18]	3851 ± 4461 [46]	2531 ± 2600 [122]	3440 ± 3301 [49]	3341 ± 3686 [82]	2948 ± 3149 [127]	2703 ± 2024 [26]

order to understand the weather features of sampling days (Fig. 3). The two components separated sampling days with high temperature and low humidity and wind (8th June, 22nd June, 27th July and 3rd July), and one day with high temperature and humidity (23rd July). Moreover, there were sampling days with low temperature, low humidity and high wind (*e.g.* 16th August and 10th July), and one day with high humidity, low temperature and low wind (29th July).

3.5. Effects of visitor numbers and weather conditions on FGMs

A General Linear Model was used to investigate the effects of mean temperature, mean wind speed, mean relative humidity, and mean number of visitors at observation points (a) and (c), on mean FGM concentrations in faeces collected the day after registering the microclimatic parameters and visitor counts. Mean temperature, mean wind speed, and mean relative humidity were considered both separately and also as interactions terms: the mean temperature and mean relative humidity (named "Temp*Humidity"), the mean temperature and mean wind speed (named "Temp*Wind"), the mean relative humidity and the mean wind speed (named "Humidity*Wind"). The results showed that the factors that affected adrenal activity were the mean temperature and the three factors considered as interaction terms, *i.e.* Temp*Humidity, Temp*Wind, and Humidity*Wind. Conversely, mean wind speed, mean relative humidity, and mean numbers of instantaneous visitors counted at observation points (a) and (c) were not significant ($F = 11.16$, $R^2 = 0.88$, $p = 0.036$). Mean FGM concentrations were higher when the mean temperature ($p = 0.008$), and Humidity*Wind increased ($p = 0.038$); FGM concentrations were lower when Temp*Humidity ($p = 0.018$), and Temp*Wind increased ($p = 0.02$) (Table 3).

3.6. Relationships between climatic indices (THI and WCI) and FGMs

The relationship between the THI and FGMs is described by the equation: $y = 181.96 \times -9173.73$ ($F = 6.388$, $R^2 = 0.32$, $p = 0.03$). The mean FGM concentrations increased as the THI values increased, and for each 1 unit increment of THI, we observed an increase of 182 ng/g of FGMs (Fig. 4). The relationship between Wind Chill Index (WCI) and FGMs is described from the equation: $y = 248.22 \times -1788.56$ ($F = 7.886$, $R^2 = 0.38$, $p = 0.018$). The mean FGM concentrations increased as the WCI values increased, and for each 1 unit increment of WCI, there was an increase of 248 ng/g of FGMs (Fig. 5).

4. Discussion

In this study, we evaluated the effects of environmental factors, weather conditions and presence of visitors, on the adrenal stress status of a colony of captive African penguins hosted in a zoological facility in northwest Italy. Overall, the results suggest that the local microclimatic conditions may affect the adrenocortical activity in the African penguins. While, the visitors' presence had a neutral effect on the physiological stress of the penguins. Furthermore, we found a positive relationship between daily FGM concentrations and two climatic indices, commonly used in human beings and domestic animals: Temperature-Humidity Index (THI) and Wind Chill Index (WCI).

Before investigating the adrenocortical response in African penguin in relation to environmental factors, we analysed gender-, age- and diurnal-differences in FGM levels within the penguin colony. Gender differences in adrenal activity have been previously reported in several species and may be due to different physiological and behavioural aspects (Touma and Palme, 2005). Our results

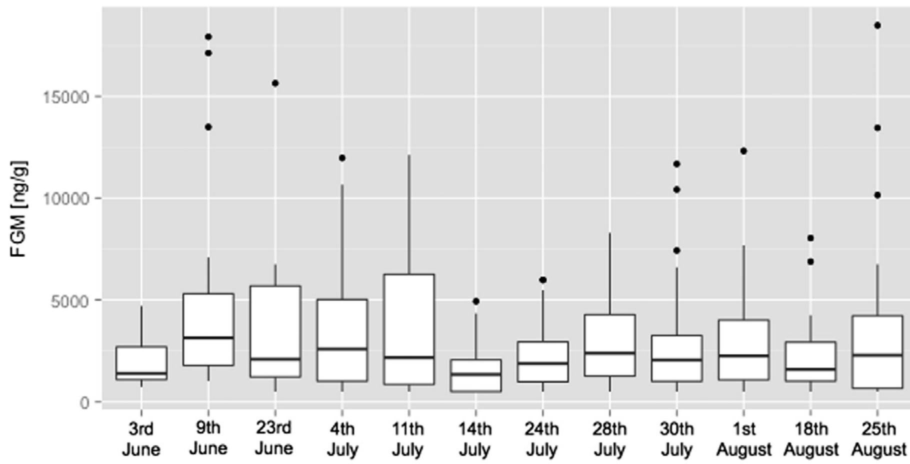

Fig. 2. Mean values of FGMs (ng/g faeces) in each sampling day (Summer 2014). The box and whisker plots illustrate the interquartile range, and the black lines indicate the median. The error bars extend from the box to the highest and lowest values. The circles indicate the outlier data.

Table 2
Environmental variables. Microclimate variables: 24 h mean temperature (°C), 24 h mean relative humidity (%), 24 h mean wind speed (m/s), and 24 h accumulated rainfall (mm). Instantaneous number of visitors: mean number of visitors photographed at observation points (a) at the wooden footbridge, (b) at the glass panels, (c) at the glass panels that separated the penguins' pool from the visitors' pool. The daily number of visitors provided by Zoom Torino: number of people admitted to the biopark and number of people admitted to the swimming pool adjacent to the penguins' pond.

Sampling day (Meteorological data registration and visitors' count)	Microclimate variables				Instantaneous visitors' number			Daily visitors' number	
	Mean temperature (°C)	Mean relative humidity (%)	Mean wind speed (m/s)	Rainfall (mm)	Mean visitors' number at observation point (a)	Mean visitors' number at observation point (b)	Mean visitors' number at observation point (c)	Visitors' number admitted at biopark	Visitors' number admitted at swimming pool
2nd June	17.9	73	1.2	0	5	10.4	0	2643	20
8th June	25.2	63	1.2	0	3.9	8.2	4.4	2938	881
22nd June	23.5	74	0.9	0	6.3	10.6	3.7	2397	873
3rd July	22.6	72	1.1	0	4.6	2.64	3.6	1109	1220
10th July	19.5	65	1.3	0	1.6	1.5	1.24	655	630
13th July	20	76	1.1	0	3.6	4.7	0.8	2080	806
23rd July	22	84	1.1	0	0.1	0.2	1.3	341	519
27th July	23	74	1.3	0	5.1	4.8	8.6	2999	2811
29th July	17	94	1.2	9.6	0.06	0.06	0	108	36
31st July	20.4	70	1.3	4.6	3.2	4.3	6.3	1081	1260
16th August	19.5	62	1.6	0	7.3	6.5	5	2677	2683
24th August	19.9	76	1.5	0	4.8	10.9	4.3	2969	1764

did not show any differences in FGM concentrations between the sexes. This result agreed with those obtained in other avian species, such as the Mourning dove (*Zenzaida macrura*) (Washburn et al., 2003) and the Golden eagle (*Aquila chrysaetos*) (Staley et al., 2007). Conversely, a sex-dependent excretion of FGMs does exist in certain bird species, such as the European stonechat (*Saxicola torquata rubicola*) (Goymann, 2005), the Peregrine falcon (*Falco peregrinus*) (Staley et al., 2007), and the Adelie penguins (*Pygoscelis adeliae*) (Ninnes et al., 2010). As suggested by Touma and Palme (2005), different factors may be responsible for gender-specific differences and should be considered when measuring FGMs of a given species. As a relationship exists between the hypothala mic–pituitary–adrenal (HPA) and the hypothalamic–pituitary–go nadal (HPG) axes, and both testosterone and oestrogen modulate the response of the HPA axis (Toufexis et al., 2014), gender differences in FGMs could vary depending on the biological stage of birds, such as breeding period.

In addition, it is essential to consider the life stage at which an individual is observed, in order to link physiological measures with environmental parameters (Wilcoxen et al., 2011). The decline of corticosterone secretion is age-related in birds, due to a decrease in adrenal capacity, which contributes to a reduction in the stress response (Heidinger et al., 2008; Wilcoxen et al., 2011). Our results did not show any age-related differences, and there was no decrease in FGM levels with age. A previous study conducted on this penguin colony (Ozella et al., 2015) showed a possible age-related difference in FGM levels, which was, however, connected to different past-experience with the applied stressor (*i.e.* handling of animals) and also with the reproductive status. In the current study, all the animals had already experienced the relevant stressors and the data collection period did not coincide with the breeding period.

Furthermore, we did not find any diurnal variation in FGM concentrations during the data collection period. While well-defined circadian rhythms of plasma GCs have been described in several avian species (*e.g.* Rich and Romero, 2001; Romero and Remage-Healey, 2000), only a few studies have focused on diurnal variation in FGMs in birds (*e.g.* Carere et al., 2003). A diurnal variation in FGM concentrations was found in small birds, such as the Great tit (*Parus major*) (Carere et al., 2003), which usually defecate more frequently. However, our results are in agreement with those obtained by Baltic et al. (2005) in Black grouse (*Tetrao tetrix*), and Thiel et al. (2005) in Western capercaillie (*Tetrao urogallus*). These birds have similar heights and weights to the African penguin, and we hypothesize that the longer gut passage time, and consequently the more infrequent defecation, may preclude detecting diurnal

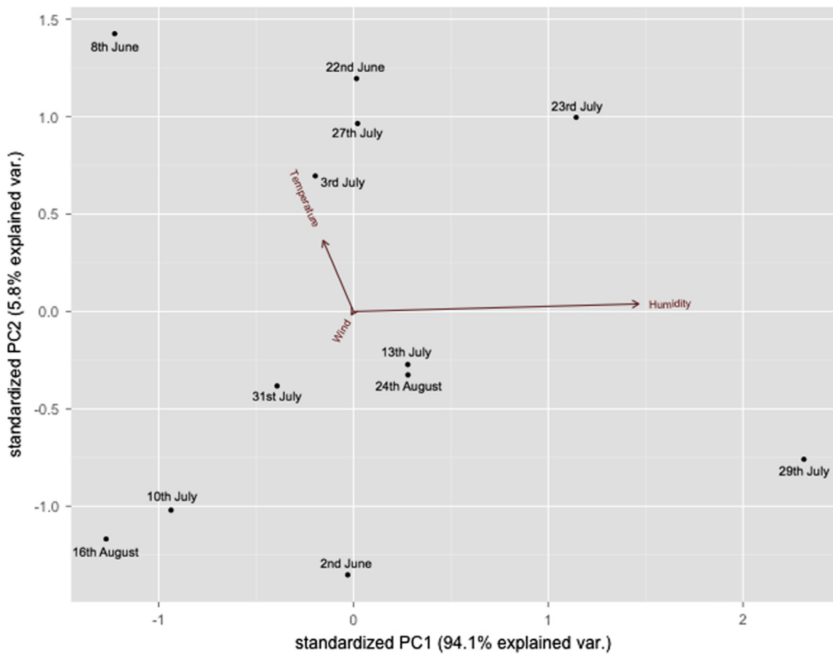

Fig. 3. Plot of the variation of sampling days in relation to daily mean temperature, daily mean wind speed and daily mean relative humidity.

Table 3
General Linear Model results predicting the effects of mean temperature, mean wind speed, mean relative humidity, mean number of visitors at observation points (a) and (c), and the interaction terms Temp*Humidity (mean temperature and mean relative humidity), Temp*Wind (mean temperature and mean wind speed), Humidity*Wind (mean relative humidity and mean wind speed) on mean FGM concentrations in African penguins in Summer 2014.

Variable	Estimate	Standard error	*t*-Value	*p*-Value
Intercept	−54253.452	15460.593	−3.509	0.039
Mean temperature	4599.934	731.537	6.288	0.008
Mean relative humidity	−70.527	176.669	−0.399	0.716
Mean wind speed	20887.205	10348.692	2.018	0.136
Visitors' number (a)	−202.749	78.030	−2.598	0.08
Visitors' number (c)	79.117	78.026	1.014	0.385
Temp*Humidity	−22.216	4.774	−4.653	0.018
Temp*Wind	−2326.402	519.916	−4.475	0.02
Humidity*Wind	386.153	108.961	3.544	0.038

changes in FGM levels. However, in line with our previous study about this species (Ozella et al., 2015), we did not find any diurnal variation in FGM levels during the breeding season. Consequently, we consider the FGM levels obtained as a pooled measure over a certain time period, representing a cumulative secretion, as suggested by several other authors (*e.g.* Goymann et al., 1999; Palme, 2005; Keay et al., 2006; Cockrem, 2007; Palme, 2012).

Daily mean FGM concentrations varied across the data collection period and they significantly differed between sampling days. Two environmental conditions were considered as possible causes for this variation: the number of visitors and the weather conditions. In the context of captive animal research, 'visitor effects' describes the influence of visitors on the welfare of zoo animals (Choo et al., 2011). Hosey (2000) categorized three classes of different effects that zoo visitors may have on exhibited animals, namely a source of stress, a source of enrichment, or relatively neutral. Commonly, visitor effects have been investigated using the behavioural assessment of captive animals (Davey, 2007), and more recently, several studies have utilised physiological parameters, such as the measurement of FGM concentrations (*e.g.* Pirovino et al., 2011; Clark et al., 2012; Sherwen et al., 2014, 2015). Our results showed that the number of visitors did not influence the adrenocortical activity of African penguins measured through assessing FGM daily mean concentrations. These findings suggested that visitors to the zoological facility had a neutral impact on the physiological stress of the penguins. Other studies conducted in immersive zoos with naturalistic exhibits (Choo et al., 2011; Sherwen et al., 2014) showed similar results, demonstrating that the number of visitors had no impact on the welfare of captive animals. The exhibit design of naturalistic zoos simulates the natural habitats of animals, thus providing direct benefit to captive animals by promoting naturalistic behaviour (Sha et al., 2013). At the same time, the absence of a visible physical barrier fosters a more positive attitude by the public towards the animals (Tofield et al., 2003). Despite the limited number of studies that have investigated animal responses to visitors in immersive zoos, they all report minor effects of the presence of visitors on animal behaviour (Sherwen et al., 2014). It is possible that naturalistic exhibits allow animals greater freedom of movement, enhancing choice and control over their interactions with visitors (Davey, 2007), which may reduce potentially negative impacts from the public. Nevertheless, potential undesirable implications of naturalistic exhibits must be considered. For example, the removal or reduction of the physical barriers and the safety mechanisms leave animals unprotected from direct trauma and injuries due to irresponsible visitor behaviour or wild animal predators (Sha et al., 2013). Furthermore, as the naturalistic exhibits are commonly large outdoor areas, animals are greatly exposed to surrounding environments, as well as to the local microclimate. However, few studies have investigated the influence of microclimatic conditions on the welfare of captive animals hosted in outdoor enclosures (Young et al., 2013). A few recent studies on captive animals, such as African lions (*Panthera leo*), Siberian tigers (*Pantera tigris*) (Young et al., 2013) and gorals (*Naemorhedus griseus*) (Khonmee et al., 2014) show that high temperature could affect animal welfare. In particular, authors observed an increase of glucocorticoids in gorals during the summer months and this result could reflect a form of heat stress in this species (Khonmee et al., 2014). Instead, the effect of weather parameters on glucocorticoid production has been widely studied in various livestock species (Silanikove, 2000), in order to assess the consequences of stress on productivity (Hill and Wall,

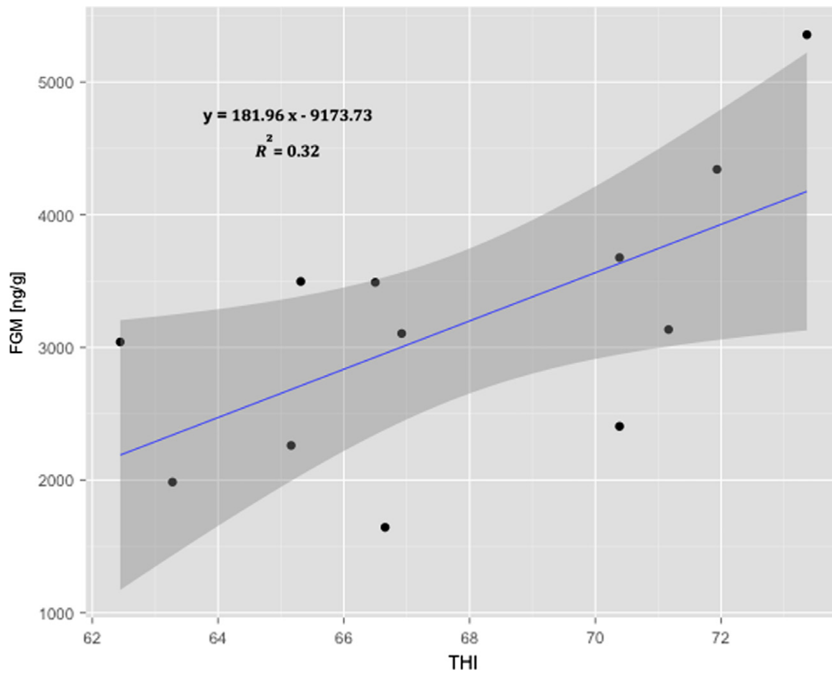

Fig. 4. Relationship between Temperature-Humidity Index (THI) and FGMs (ng/g). Shaded area indicates the 95% confidence intervals.

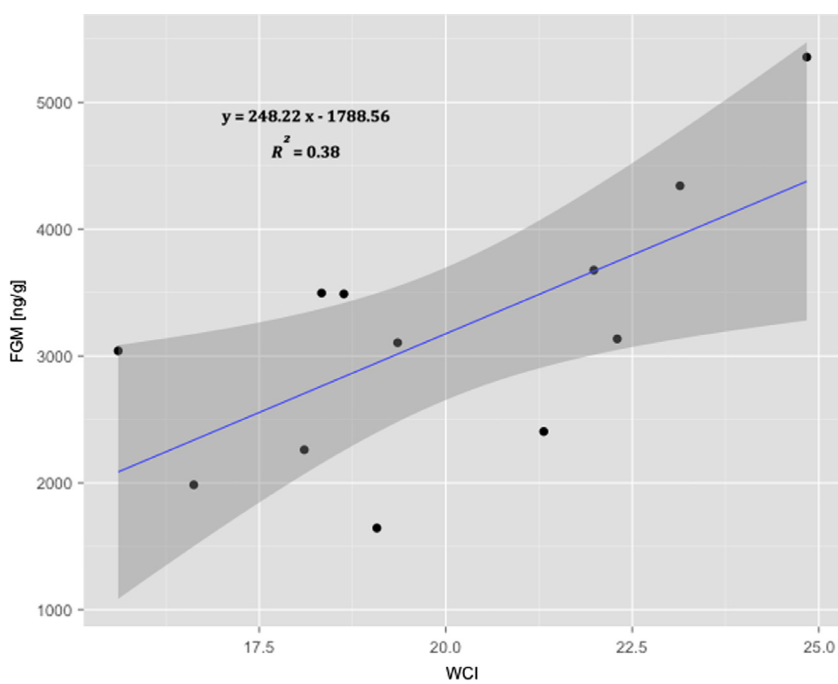

Fig. 5. Relationship between Wind Chill Index (WCI) and FGMs (ng/g). Shaded area indicates the 95% confidence intervals.

2015). In particular, high ambient temperature, wind speed and humidity are recognized as environmental stressing factors for farm animals (Silanikove, 2000).

In this study, we have shown, for the first time in this species, that adrenocortical activity in captive African penguins is influenced by local microclimatic conditions. Specifically, the increase of the mean temperature induces an increase of FGM concentration, when the daily temperature is considered as a single predictor variable. The mean relative humidity and the mean wind speed, considered as single parameters, did not influence daily FGMs.

However, when we considered the interaction between the mean temperature and the mean relative humidity, as well as the mean temperature and the mean wind speed, we observed a negative relationship with FGM concentrations. Humidity and wind speed could have a moderating effect on temperature, which subsequently reduces the heat-induced stress and consequently the adrenal activity in African penguins. Conversely, the interaction between humidity and wind speed was related to the increase in FGM concentrations. We hypothesize that these results can be explained by the different microclimate conditions at the latitude of the zoological facility and the natural habitat of this species.

"Boulder beach" is one of main natural nesting site of African penguins and is located several kilometres to the south of Simon's Town, South Africa. According to Köppen and Geiger (1930), the climate of Simon's Town is temperate-humid, and is classified as Csb: mesothermal with temperatures in the warmest month being less than 22 °C. Although the data collection period – summer 2014 – was not a particularly hot summer for the standards of north Italy, and the microclimatic conditions were variable, we found that the warmest day, 8th June, with a mean daily temperature >24 °C, was related to the highest daily level of FGM. Conversely, when the daily mean temperature was ⩽20 °C in the 2 days – 3rd June and 14th July – there was the lowest daily level of FGM. Indeed, African penguins start to become heat-stressed at air temperatures of 24 °C, when they have been observed panting (Frost et al., 1976), a behavioural adaptation to increase heat release with respiratory evaporation. Moreover, in order to adapt to hot climates, these penguins breed in burrows dug in guano, which maintains a constant microclimate, with high relative humidity, buffered temperatures and little exposure to the wind (Frost et al., 1976).

As also demonstrated by our results, the weather factors did not act separately, and their interactions influenced animal welfare in an extremely complex way (Van laer et al., 2014). To account for this factor, different climatic parameters have been combined into singles indices, such as the THI and the WCI, to quantify the degree of discomfort and potential production loss in domestic livestock (Van laer et al., 2014). The THI or heat index was developed for describing the degree of heat stress at various combinations of temperature and humidity (Hill et al., 2004), while the combined effects of temperature and wind are often expressed as WCI. This index is commonly used to estimate effective temperature perceived by animals rather than mere air temperature (Piao and Baik, 2015). We calculated the THI and the WCI, and we detected a positive relationship between the increase of the THI and the WCI with the FGM concentrations, demonstrating that these indices could be useful indicators of weather discomfort in African penguins. Nevertheless, further research may be necessary to establish threshold values of climatic indices for African penguins under different management conditions and during different seasons.

In conclusion, our study has shown that monitoring of FGMs is a valuable tool for advancing our understanding of adrenal function and stress responses in captive animals, and can enhance *ex situ* management of threatened species. Our results indicate that the naturalistic exhibits could have significant benefits for captive animals, such as a reduction of the visitor effect. Nevertheless, the location of the zoological facility should be taken into account and if the local microclimatic conditions are compatible with the hosted species and do not differ greatly from their natural habitat. Above all, the identification of conditions that influence the welfare of captive animals is critically important in order to implement *ex situ* conservation programs in zoo facilities.

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