Title: Effects of scent enrichment on behavioural and physiological indicators of stress in zoo primates – a preliminary study on red-ruffed lemurs and Lar gibbons

Short title: Effect of scent enrichment on the welfare of red-ruffed lemur and Lar gibbon

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Abstract

Almost half of the primate species recognized today worldwide are classified as endangered or critically endangered in the wild. Captive breeding is vital for primate conservation, with modern zoos serving a crucial role as breeders of populations acting as buffers against extinction, ambassadors of endangered species, and educators of the general public. However, captive populations may experience welfare issues that may also undermine their breeding success. In order to design and test a new scent enrichment programme to enhance the breeding success and well-being of critically endangered zoo primates, we carried out a preliminary study to assess the effects of lavender on captive red-ruffed lemurs (*Varecia rubra*) and Lar gibbons (*Hylobates lar*) as these species presently show a low success rate in captive breeding and are critically endangered in the wild. We combined behavioural observations and faecal endocrinology analyses to assess the effects of lavender on two captive troops (N = 8) housed at Dudley Zoo and Castle (UK). We recorded observations of natural species-specific and abnormal stress-related behaviours for 144 hr using instantaneous scan sampling. We collected 78 faecal samples and measured the faecal cortisol concentrations using radioimmunoassay. We found a significant effect by the scent enrichment on social interactions and stress-related behaviours (i.e., pacing and self-scratching), with both species reducing their rates of stress-related behaviours after they were exposed to lavender. We also found that both species displayed a significant increase in faecal glucocorticoids following the exposure to lavender. Our findings suggest that lavender may work as scent enrichment to decrease the stress levels of zoo primates across the major lineages, but its effects might change depending on the species-specific social lives and olfactory repertoires of primates.

Keywords: lavender, stereotypic behaviours, faecal cortisol, *Varecia rubra*, *Hylobates lar*

Introduction

Almost half of the total primate species recognized today worldwide are classified as endangered or critically endangered in the wild – primarily due to human activities [1]. Therefore, raising global scientific and public awareness of the plight of the world's primates is now vital [1]. The most important actions needed for ensuring the survival of these irreplaceable species are conservation, research, public education and outreach. Across all these contexts, zoos play a major role [2], as zoo animal populations are usually managed to educate the public regarding wildlife and their habitats, and to preserve endangered species through captive breeding and reintroduction programmes [3]. In this context, the maintenance of the genetic variation of such captive populations is imperative [4]. However, captive populations, potentially serving as buffers against extinction, experience problems that impair them from being viable for reintroduction into the wild. More specifically, zoo animal populations face reproductive challenges which have so far inhibited them from serving as viable 'reserve populations' [5]. Additionally, managing zoo populations is challenging because of the mismatch between natural and captive environments [6]. Primates have evolved distinct behavioural patterns, and difficulty in engaging in these behaviours can cause frustration or boredom, which, in turn, can lead to stress and development of abnormal behaviours [7] that may undermine their individual welfare and their breeding success.

To maintain captive healthy populations, with good genetic variability and thus high survival rates in case of reintroduction, modern zoos take part in conservation breeding programmes. As reproductive success is linked to how closely captive environmental conditions mirror those that primates would be experiencing in the wild [5], zoos also use environmental enrichments to manage captive populations. Environmental enrichments and conservation breeding programmes are strictly related, as enrichment is a dynamic process that changes an animal's environment, increasing its behavioural choices and prompting a wider range of natural and species-specific behaviours and abilities [8]. Furthermore, enrichment can contribute to promoting resiliency to stress, which helps animals recovering from adverse stimuli [9], improving both the exhibit from the visitor perspective and the reproductive performance of the hosted animals [10]. Enrichment can also foster the essential skills that animals need for their survival if reintroduced into their habitat [11].

Scent-based enrichments can be effective at increasing active behaviours in zoo animals and potentially improve their welfare [9,12,13]. However, some authors reported findings that are less clear or indicate that scent enrichment has little effect [14,15]. The delivery mechanism of the scent and the type of scents used are crucial for the implementation of novel olfactory enrichment programmes [16]. The majority of studies have used spices or essential oils rather than focusing on natural or biological scents, but this may not necessarily be appropriate for all species [14]. The main goal of olfactory enrichment is to improve welfare of animals in captive environments, but there is also the possibility that the use of scents can have further positive impacts in addition to this. For instance, scents may elicit behavioural and physiological responses and thus it is important to consider the use of olfactory enrichment to promote potential beneficial effects on reproductive success [17].

Primates are traditionally considered "microsmatic" (i.e., with a poor sense of smell) [18] and only a small proportion of studies on olfactory enrichment have been undertaken on primate species [19] with the majority of scents currently used in these studies remaining largely focused on essential oils, spices or herbs [14]. However, various lines of evidence suggest that chemical communication may be important in primates [20]. In particular, it has become increasingly clear that the sense of smell plays a crucial role in primate socio-sexual communication, with semiochemicals being important for kin recognition, mate choice and the regulation of socio-sexual behaviours [21]. However, little is known about the overall effects of olfactory enrichment on primate species.

The overarching aim of our work is to design and test a new scent enrichment programme to enhance the breeding success and well-being of critically endangered zoo primates. In this context, we carried out a preliminary study which aimed to assess the effects of lavender (which was chosen based on its effectiveness in humans and domestic animals; reviewed in [22]) on resting and social behaviour as well as on both behavioural and physiological indicators of stress in two captive primate species: red-ruffed lemurs (*Varecia rubra*) and Lar gibbons (*Hylobates lar*). These species are currently classified as either critically endangered (red-ruffed lemurs [23]) or endangered (Lar gibbons [24]), largely due to the deforestation, logging and hunting activities that threaten the habitat and survival of these species [23,24]. Therefore, designing and implementing strategies that improve welfare and breeding success of these species in captivity is particularly crucial. In this study, we predicted that lavender would reduce the stress levels of zoo red-ruffed lemurs and Lar gibbons, which would be reflected by significant changes in behavioural (i.e., decrease of the frequency of stress-related behaviours, and increase of the frequency of social behaviours) and physiological indicators of well-being when comparing before (i.e., baseline period) and after (i.e., post enrichment period) the scent enrichment.

Material and methods

Study subjects and housing

We studied two captive troops of red-ruffed lemurs (n = 3) and Lar gibbons (n = 5) housed at Dudley Zoological Gardens (UK). The troop of red-ruffed lemurs consisted of two related (brothers) adult males (15 y/o) and one unrelated adult female (14 y/o). The troop of Lar gibbons consisted of one adult male (16 y/o), one adult females (17 y/o) and their offspring – one adult female (6 y/o), two young males (2 y/o and 8 y/o, respectively).

We carried out behavioural observations and faecal sampling from September to December 2019 (red-ruffed lemurs) and from September to December 2018 (Lar gibbons). Both troops lived in an indoor enclosure (heated to 28 °C) with access to an outdoor enclosure (i.e., “visitor walkthrough” enclosure in the case of red-ruffed lemurs).

Study protocol

We divided the overall study period in three periods: baseline, scent enrichment, post enrichment. We collected behavioural data and faecal samples (see below “Assessment of welfare”) for four days per study period (12 days in total) over three months.

Scent enrichment

We cut white cotton sheets into 75 cm long and 5 cm wide strips, which we then soaked with 20 drops naissance 100% pure lavender essential oil diluted with 12 ml of cold boiled water. We prepared the scent cotton strips at early morning of each sampling day over the scent enrichment period. We positioned these strips around both indoor and outdoor enclosures; particularly, and we tied them approximately 1 m from the ground around the climbing frames as these were the most used areas of the enclosures.

Assessment of welfare

We have used both behavioural (e.g., naturalistic species-specific behaviours, stereotypic behaviours) and physiological (e.g., corticosteroid levels, regulated by hypothalamic-pituitary-adrenal-HPA axis) methods to assess the effects of scent enrichment.

Behavioural data collection

We collected behavioural data by instantaneous scan sampling [25] of some behaviours (Table 1), with behaviours recorded at 5-min intervals over the duration of six hours from 9am to 3pm, four days per study period (baseline, scent enrichment, post enrichment) over three months. We recorded a total of 144 hr of observations over the study period (excluding the pilot study), with 50 scan samples each sampling day on each group (see supporting information).

Table 1 - Ethogram

Behaviour	**Description**
Resting	Lying or sitting while awake, with eyes open and arms down by side of body.
Sleeping	Lying on back, front or side, eyes closed and whole body is relaxed
Grooming	Using fingers or mouth to pick through the coat, removing any foreign bodies from a conspecific
Play	Animal is engaging in activities such as chasing others, leaping around the enclosure etc. in a playful context
Self-scratching	An animal rubs their own body at a fast pace
Pacing	Animal walks back and forth in a distinct, unchanging pattern within the enclosure.

Hormone sampling and measurements

We collected faecal samples of all subjects every morning before behavioural observations, whenever defecation was observed and the identity of the animal was known. In total, we collected 78 samples (red-ruffed lemurs = 25; Lar gibbons = 53). The samples were stored in a freezer at 20°C right after collection. At the end of the study period, the collated samples were fully prepared by adding biological hazard labels onto each pot before being delivered in a refrigerated (dry ice) to the Department of Veterinary Medical Sciences and Animal Production Science of Bologna University for radioimmunoassay (RIA).

Cortisol concentrations were determined by RIA. All concentrations were expressed in pg/mg of faecal matter. The extraction methodology followed the methods of Fontani and colleagues [26]. In brief, five millilitres of a methanol:water (4:1 v/v) solution were added to 60 mg (wet weight) of faeces in capped-glass tube vials. The vials were then vortexed for 30 min using a multitube pulsing vortexer. After centrifugation at 1,500 g for 15 min, 5 ml ethyl ether (BDH Italia, MI, Italy) and 0.2 ml NaHCO3 (5%; Sigma Chemical Co., St. Louis, MO) were added to 1 ml of supernatant. This preparation was vortexed for 1 min and centrifuged for 5 min at 1,500 g. The ether portion was aspirated with a pipette, and evaporated under an airstream suction hood at 37°C. The dry residue was redissolved into 0.5 ml of 0.05 M phosphate-buffered saline (PBS; pH 7.5).

Cortisol was assayed in the faecal samples according to the method of [27]. The validation parameters of the analyses were as follows:

Cortisol. Sensitivity 3.10 pg/100 l; intra-assay variability 6.8%; interassay variability 9.3%; specificity (%), cortisol 100, corticosterone 9.5, 11¸-hydroxyprogesterone 8.3, cortisone 5.3, 11¸-desoxycortisol 5.0, progesterone 0.6, desoxycorticosterone 0.5, 20¸-dihydrocortisone 0.4, testosterone 0.3, aldosterone 0.1, dehydroepiandrosterone < 0.0001, 5¸-pregnenolone <0.0001, 17ˇ-estradiol < 0.0001, and cholesterol < 0.0001.

Statistical analyses

In order to assess the effect of scent enrichment on red-ruffed lemurs' and Lar gibbons' behaviour and FGCs, we first generated three behavioural categories from the individual behavioural measures we collected. More specifically, we generated: 1) a resting category by adding up our data on resting and sleeping behaviours; 2) a social category by combining our data on grooming and play; and, finally, 3) a stress category by combining our data on pacing and self-scratching behaviours. We included scratching in this category since this is commonly considered an indicator of anxiety [28]. We then used the *aov* function in R (v 4.0.2) [28] to run a 2x2 Analysis of Variance (ANOVA), in which the rates of resting, social and stress-related behaviours as well as FGC concentrations were set as dependent variables in separate models, while species (Lar gibbon vs red-ruffed lemur) and enrichment condition (before vs. after exposure to scent enrichment), along with their interaction, were entered as independent factors in all models. We excluded from the analysis an outlier from the faecal glucocorticoid data set. All models met the assumptions of homogeneity of variance and normality of residuals.

Results

The 2x2 ANOVA model that included resting rates as the outcome variable revealed only a significant main effect of Species, with red-ruffed lemurs resting more frequently than Lar gibbons, but there was no significant main effect of Enrichment condition or the interaction between Enrichment and Species on resting rates (Table 2).

Table 2. Results of the 2x2 ANOVA testing the effect of Enrichment condition, Species and their interaction on resting rates. Significant result is shown in bold.

Dependent variable	Independent variables	df	Sum square	F-value	P-value	Partial η^2
Resting	Enrichment	1	0.003	0.48	0.499	0.17
	Species	**1**	**0.266**	**51.23**	**< 0.001**	**0.8**
	Enrichment * Species	1	0.00	0.00	0.999	< 0.01
	Residuals	13	0.07			

By contrast, we found a significant effect of the interaction between enrichment condition and species on rates of social interaction (Table 3). Follow-up post-hoc t-tests revealed that, while Lar gibbons increased their rates of social interactions following their exposure to lavender (t(3.2) = 7, p = 0.005, d = 5.7, Figure 1), red-ruffed lemurs showed a reduction in rates of social interactions after being exposed to lavender (t(8.3) = 2.8, p = 0.02, d = 1.6, Fig. 1).

Table 3. Results of the 2x2 ANOVA testing the effect of Enrichment condition, Species and their interaction on social rates. Significant result is shown in bold.

Dependent variable	Independent variables	df	Sum square	F-value	P-value	Partial η2
Social	Enrichment	1	0.002	3.04	0.10	0.22
	Species	1	0.001	1.65	0.22	0.11
	Enrichment * Species	**1**	**0.005**	**6.46**	**0.02**	**0.33**
	Residuals	13	0.01			

Fig. 1. Effect of enrichment condition on rates of social interactions in red-ruffed lemur and Lar gibbon. Height of the boxes represents mean while whiskers represent standard error.

Rates of stress-related behaviours were significantly predicted by enrichment condition but not by species or the interaction between species and enrichment condition (Table 4). More specifically, both species reduced their rates of stress-related behaviours after they were exposed to lavender (Fig. 2).

Table 4. Results of the 2x2 ANOVA testing the effect of Enrichment condition, Species and their interaction on rates of stress-related behaviours. Significant result is shown in bold.

Dependent variable	Independent variables	df	Sum square	F-value	P-value	Partial η2
Stress-related behaviours	**Enrichment**	**1**	**0.0013**	**5.42**	**0.037**	**0.30**
	Species	1	0.0001	0.23	0.641	0.02
	Enrichment * Species	1	0.0001	0.41	0.533	0.03
	Residuals	13	0.0032			

Fig. 2. Effect of enrichment condition on rates of stress-related behaviours in both red-ruffed lemur and Lar gibbon. Height of the boxes represents mean while whiskers represent standard error.

Finally, we found that enrichment condition significantly predicted FGCs (Table 5): following the exposure to lavender, both species displayed a significant increase in FGC concentration, compared to before being exposed to the scent enrichment (Fig. 3).

Table 5. Results of the 2x2 ANOVA testing the effect of Enrichment condition, Species and their interaction on FGC concentration. Significant result is shown in bold.

Dependent variable	Independent variables	df	Sum square	F-value	P-value	Partial η2
FGC	**Enrichment**	**1**	**9.89**	**11.23**	**0.002**	**0.26**
	Species	1	2.06	2.34	0.135	0.06
	Enrichment * Species	1	1.13	1.28	0.265	0.03
	Residuals	36	31.72			

Fig. 3. Effect of enrichment condition on FGC concentration in both red-ruffed lemur and Lar gibbon. Height of the boxes represents mean while whiskers represent standard error.

Discussion

The effects of olfactory enrichment have been tested on several domestic, farm, laboratory and zoo-housed animals [29,30]. However, olfactory stimulation is still one of the least studied forms of enrichment (reviewed in [31]). In addition, there are mixed and conflicting conclusions regarding the benefits of olfactory enrichment on animal welfare, and this is particularly the case of primate species among which the overall effects of olfactory enrichment is currently unclear and understudied (reviewed in [22]).

In this study, we found a significant effect of the scent enrichment on social interactions, with red-ruffed lemurs showing a reduction and Lar gibbons exhibiting an increase in rates of social interactions after being exposed to lavender. Generally, several studies have found that scent enrichments may be effective at increasing active behaviours in individuals in zoo-housed animals, such as cheetahs (*Acinonyx jubatus*) [9], Californian sea lions (*Zalophus californianus*) [13] and Rothschild giraffes (*Giraffa camelopardalis rothschildi*) [12] but not in meerkats (*Suricata suricatta*) [15]. Regarding primates, Gronqvist and colleagues [32] showed that, among captive Javan gibbons (*Hylobates moloch*), olfactory enrichment significantly increased the frequency of natural species-specific behaviours, although individuals' interest in the olfactory enrichment decreased rapidly after the first day. By contrast, Baker et al. [16] tested the effects of olfactory enrichment on ring-tailed lemurs (*Lemur catta*) and found no significant effects on individuals' behaviours in the presence of the odours, a result which was also shown in a study with essential oils on gorillas (*Gorilla gorilla gorilla*) [14].

The opposite effect that scent enrichment exerted on rates of social interactions in the two study species, with increased rates of social behaviour in Lar gibbons and decreased rates of social behaviour in red-ruffed lemurs following exposure to the scent enrichment, might be related to the fact that these two species spent most of their time in different types of social behaviour. More specifically, Lar gibbons spent 72% of their social time playing, while red-ruffed lemurs spent 78% of their social time grooming. A wide range of studies has shown that frequency of play behaviour is strongly affected by the levels of exposure to stress the animal is experiencing. In rats (*Rattus rattus*), for example, prenatal exposure to a stressor significantly increases the latency to play [33]. Similarly, studies on both squirrel monkeys (*Saimiri sp.*), rhesus macaque (*Macaca mulatta*) and common marmoset (*Callithrix jacchus*) showed that play rates were higher in individuals who experienced low stress levels [34–36]. In this context, our findings that Lar gibbon spent more time playing after the introduction of the scent enrichment compared to before the exposure to lavender might reflect the fact that this species might have experienced lower stress levels after the exposure to the scent, which is consistent with our prediction. On the other hand, social grooming, a common affiliative behaviour in primates, has long been shown to have a stress-reducing effect. Work conducted on macaques has shown, for example, that either receiving or giving grooming lowers stress levels in the individuals involved in the interaction [36–42]. We, therefore, suggest that red-ruffed lemurs reduced their rates of social grooming following the exposure to the scent enrichment because the presence of lavender might have decreased their overall stress levels.

As we predicted, we found a significant effect on the scent enrichment on stress-related behaviours, with both red-ruffed lemurs and Lar gibbons reducing their rates of stress-related behaviours after they were exposed to lavender. This is consistent with our prediction that scent enrichment reduces behavioural indicators of stress (i.e., pacing and self-scratching). Similar results were reported in other species. Uccheddu and colleagues [43], for example, exposed domestic dogs to a variety of essentials oils, and found that some scents increased frequencies of behavioural indicators of relaxation while others decreased behavioural indicators of stress such as pacing and over-grooming. Similarly, a study on cheetahs and Sumatran tigers (*Panthera tigris sumatrae*) found that stereotypic pacing behaviour was significantly decreased in the presence of a hay ball with cinnamon [44].

Finally, our finding that both red-ruffed lemurs and Lar gibbons displayed a significant increase in FGCs following the exposure to lavender is at first puzzling and in direct opposite to our findings related to the behavioural indicator of stress (see above). This result, therefore, suggests that changes in behavioural indicators of stress such as pacing and self-scratching do not necessarily reflect physiological indicators of stress, such as FGCs. This is consistent with the work conducted by Higham and colleagues [45] showing that, among olive baboons (*Papio anubis*), both short-term (i.e., day-to-day) and long-term variation in rates of self-directed variation does not reflect changes in FGCs. This mismatch might be due to the fact that self-directed behaviours and FGCs may reflect two different types of stress. More specifically, self-directed behaviours likely reflect low-level acute stress, or anxiety [46], while FGCs likely reflect high-level chronic stress. Accordingly, self-directed behaviours have been found to increase in anxiety-inducing context, such as when animals are given anxiogenic drugs [47], or after aggression [48]. On the other hand, FGC levels have been shown to increase when animals are exposed to high levels of stress, such as in the presence of tourists [49] or when exposed to the odour of a predator [50]. Therefore, this suggests that researchers should, ideally, use *both* behavioural *and* physiological indicators of stress and anxiety in order to have a complete picture on the levels of stress an animal experiences and that behavioural indicators of anxiety should not necessarily be interpreted as indicator of glucocorticoid production.

The question remains, however, on why both red-ruffed lemurs and Lar gibbons exhibited higher FGC levels after exposure to scent enrichment compared to before scent enrichment was introduced. The main function of glucocorticoids is to mobilize the energy into the bloodstream from storage sites inside the body. This energy can then be used by tissues and organs, such as muscle, and brain. Consequently, glucocorticoids are generally produced every time the body needs energy, such as during stress response, when the animal might need energy, for example, to escape from a predator. However, there are other non-stress-related contexts in which animal body might need energy, such as early in the morning when an animal needs to start their physical activity. Accordingly, glucocorticoid levels tend to peak early in the morning. Here we argue that FGCs increased after the exposure to lavender because the introduction of the scent enrichment might have increased the animals' general activity pattern (e.g., locomotion, foraging), which might have required an increase in glucocorticoid production by the animals' body.

In general, it is crucial to consider the ecological/biological relevance of the scent enrichment to the species as this is likely to affect the results. Many scents, including lavender, are chosen based on their effectiveness in humans or domestic animals, but this may not necessarily be appropriate for all animal species [22]. In particular, as previous authors have suggested, important factors to consider for the implementation of novel olfactory enrichment programmes are the delivery of the scent and the type of scents used [16]. The majority of studies have used spices or essential oils rather than focusing on natural or biological scents; however, as with all types of enrichment, the biology of the species should be taken into account and its effectiveness should be continually monitored to inform best practices. For example, several studies have suggested that the use of either natural prey/predator odours or odours from conspecifics should be researched further [32]. Other studies have suggested the use of diffusers as a delivery method may be more effective [16].

Additionally, further studies have indicated that scents could be used in a number of combinations and introduced randomly to continue to add novelty to the enrichment programme and avoid the problem of habituation [9].

Interestingly, we found that scent enrichment exerted both behavioural and physiological effects on both red-ruffed lemurs and Lar gibbon. While the use of chemical communication and olfactory signals in lemurs has long been established [51–53], apes are commonly considered microsmatic. However, accumulating evidence seems to suggest that apes may also rely on olfactory signals and chemical communication. Henkel and Setchell [54], for example, recently showed that chimpanzees (*Pan troglodytes*) spend more time sniffing the urine of outgroup individuals than that of group members. Similarly, accounts of border patrolling among wild chimpanzees report how individuals are often seen sniffing the ground when near the territory of a neighbouring group [55]. Our findings showing that scent enrichment exerts both behavioural and physiological changes in Lar gibbon confirms that olfactory communication plays a key role in social interaction and physiological regulation not only in chimpanzees but also in other apes.

In conclusion, our preliminary study provides strong evidence of a beneficial effect of scent enrichment on animal welfare. Following exposure to the scent enrichment, animals spend more time play, and less time grooming and exhibit lower rates of stress-related behaviours. Our finding that both red-ruffed lemurs and Lar gibbon increased their levels of FGCs after exposure to lavender might be associated to an increase in activity after animals were exposed to the scent enrichment. Future work will need to expand the investigation of the effect of scent enrichment on animal welfare by focusing on a wider range of primate species and on a bigger sample size.

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Author contribution

Conceived and designed the experiment: SV. Performed the experiment: SS, BT, RP, GM, PAA. Analysed the data: SSKK. Wrote the paper: SV, SSKK, CY, EE, SF.

Supporting information caption

S1 Text. Dataset

The table includes both the raw data and the rates of resting, social and stress-related behaviours as well as the faecal glucocorticoid concentration (pg/mg) exhibited by red-ruffed lemur and Lar gibbon before and after exposure to the scent enrichment (lavender).

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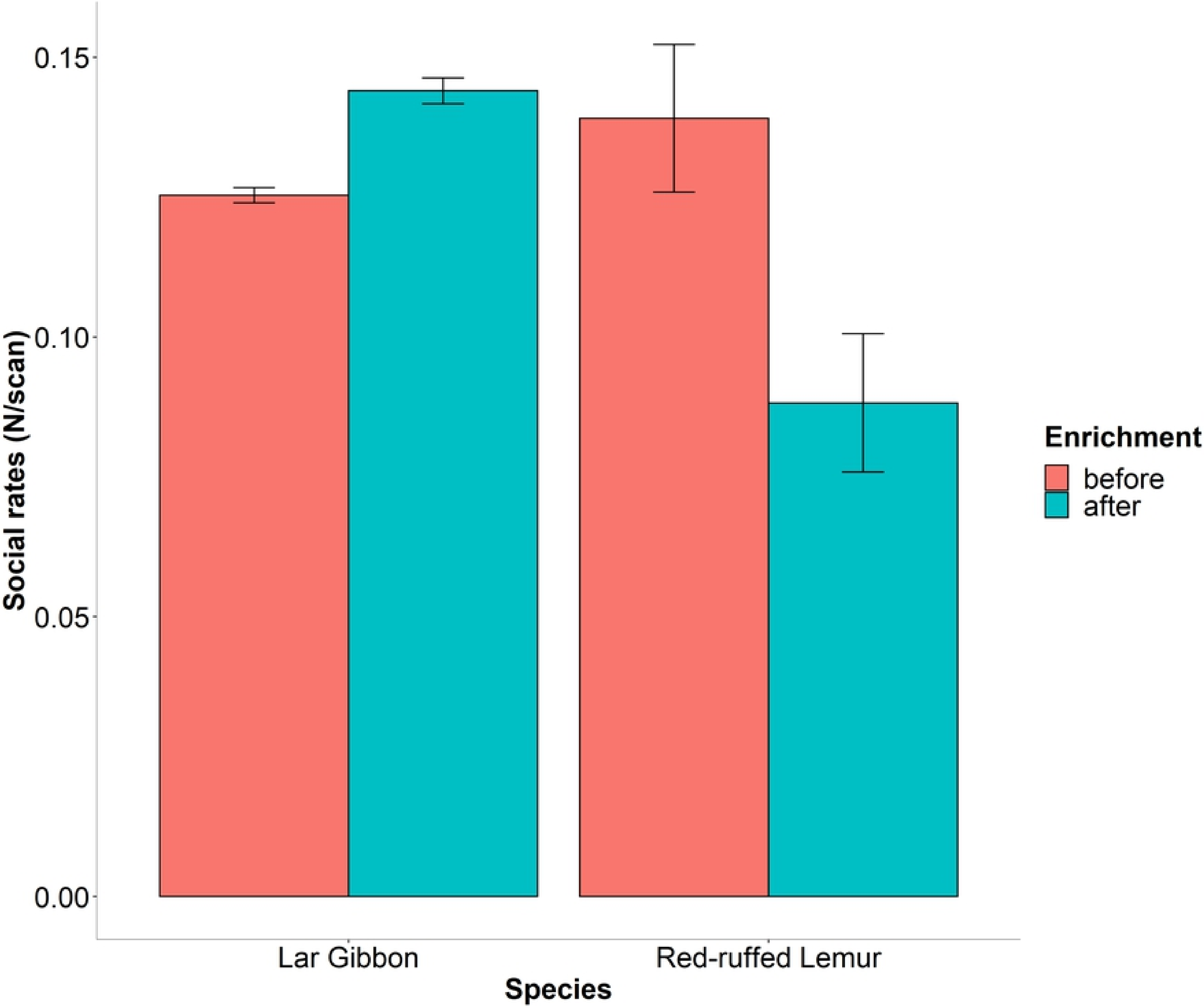
Social rates (N/scan)
0.15
0.10
0.05
0.00
Lar Gibbon
Red-ruffed Lemur
Species
Enrichment
before
after

Figure 1

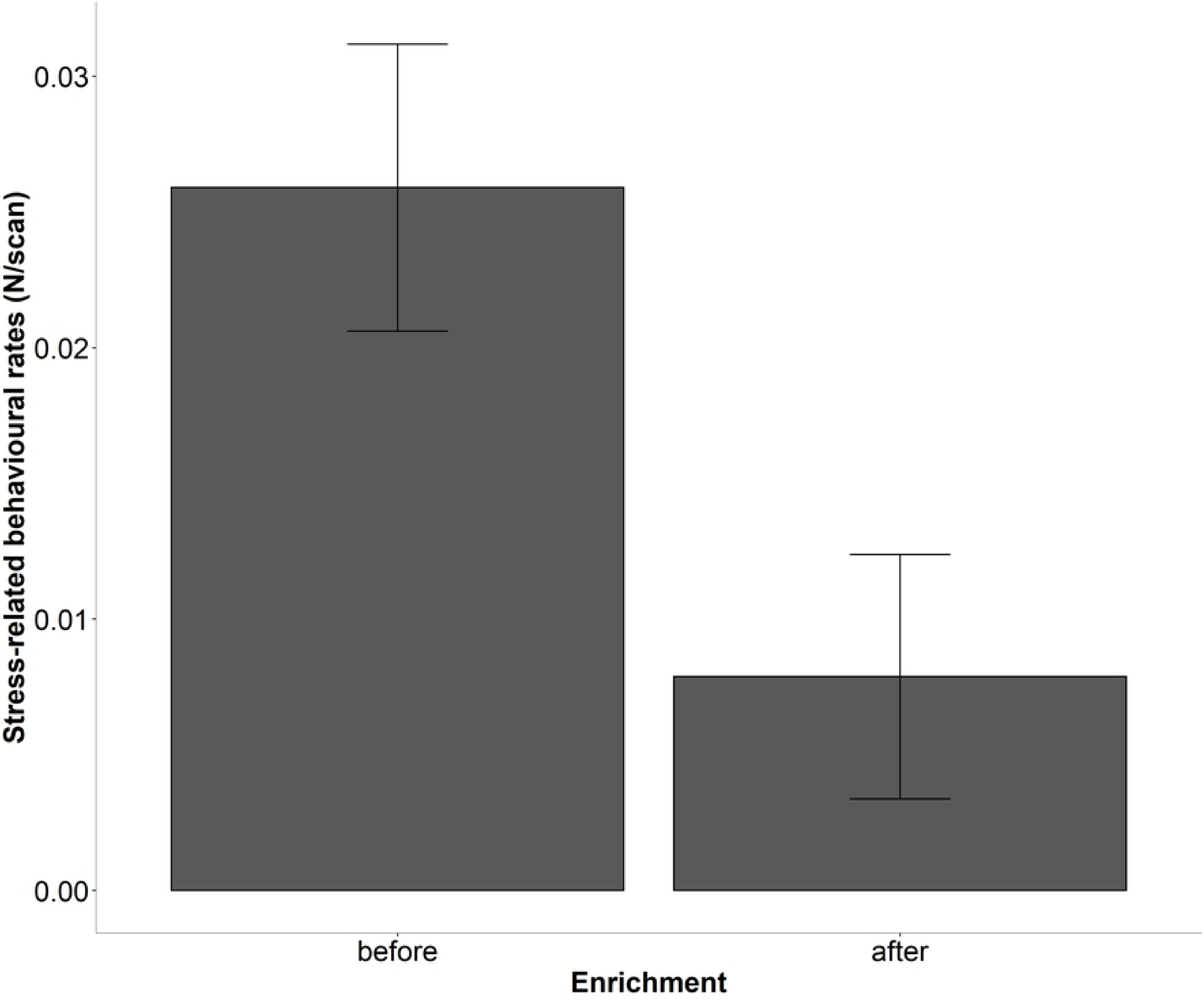
0.03
0.02
0.01
0.00
Stress-related behavioural rates (N/scan)
before
after
Enrichment

Figure 2

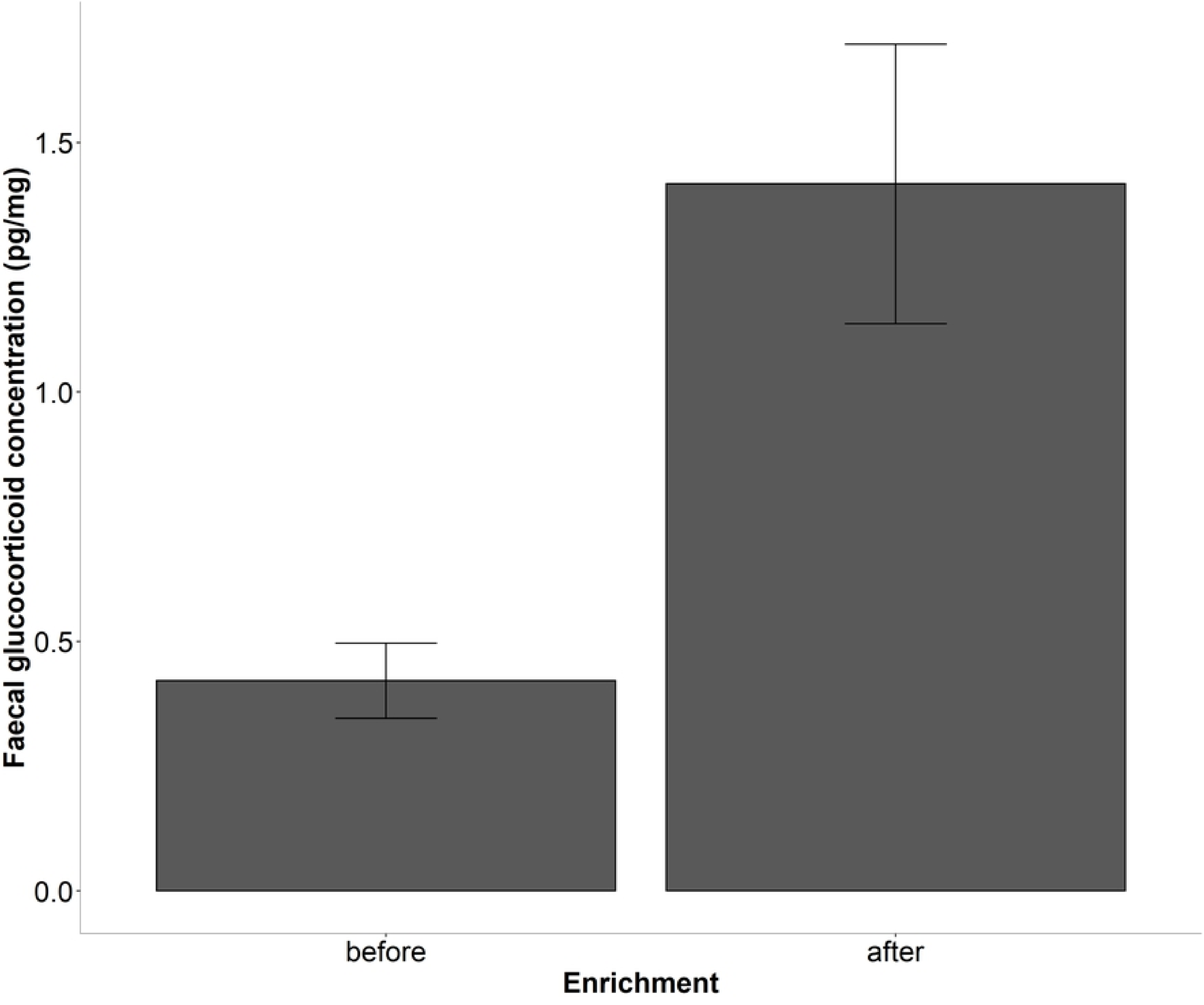
Faecal glucocorticoid concentration (pg/mg)
1.5
1.0
0.5
0.0
before
after
Enrichment

Figure 3