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The long-term impact of infant rearing background on the behavioural and physiological stress response of adult common marmosets *(Callithrix jacchus)*

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Abstract

Although triplet litters are increasing in captive colonies of common marmosets, parents can rarely rear more than two infants without human intervention. There is however much evidence that early life experience, including separation from the family, can influence both vulnerability and resilience to stress. The current study investigated the behavioural and hypothalamic pituitary adrenal (HPA) axis response to the routine stressor of capture and weighing in adult common marmosets (*Callithrix jacchus*), reared as infants under 3 different conditions: family-reared twins (n=6 individuals), family-reared animals from triplet litters where only 2 remain (2stays: n=8) and triplets receiving supplementary feeding from humans (n=7). In the supplementary feeding condition, infants remained in contact with each other when they were removed from the family. There were no significant differences (P>0.5) in cortisol level or behaviour between the rearing conditions. In all conditions, salivary cortisol decreased from baseline to post-capture, which was accompanied by increases in agitated locomotion. Family reared 2stays demonstrated significant cortisol decreases from baseline to post capture (post 5 mins: P=0.005; post 30 mins: P=0.018), compared to the other conditions. Family reared twins displayed significantly more behavioural changes following the stressor than the other conditions, including significant increases in scent marking (post 5 mins and post 30 mins: P=0.028) and significant decreases in inactive alert (post 5 mins: P=0005; post 30 mins: P=0.018), calm locomotion (post 5 mins: P=0.028; post 30 mins: P=0.046) and proximity to partner (post 5 mins: P=0.046). There were increases in behaviour suggesting reduced anxiety, including significantly more exploration post-capture in supplementary fed triplets (post 5 mins: P=0.041), and significantly more foraging post capture in family reared 2stays (post 5 mins and post 30 mins: P=0.039). However, as differences between rearing conditions were minimal, supplementary feeding of large litters of marmosets at this facility did not have a major effect on stress vulnerability, suggesting that this rearing practice may be the preferred option if human intervention is necessary to improve survival of large litters.

Key words: marmosets, rearing, cortisol, behaviour, stress response, animal welfare

1. Introduction

Although the most frequently used New World Primate in laboratory research and testing, there are problems associated with the breeding and rearing of common marmosets (*Callithrix jacchus*). While twins are most often seen in the wild (Sousa et al., 1999; Windle et al., 1999), births of triplets are often just as common in captivity, which is associated with greater infant mortality (Ash and Buchanan-Smith, 2014) and perinatal stress (Riesche et al., 2018). As parents can rarely successfully rear more than two offspring, infant marmosets can be hand-reared to help improve the survival of larger litters (3+ infants) (Hearn and Burden, 1979). Such practices include complete hand-rearing, involving permanent family absence, and partial hand-rearing, in which one or all infants are removed from the family for certain periods of the day for supplemental feeding (Ash, 2014).

However, as marmosets are adapted to be immersed in a rich social environment from birth, family life is extremely important for their development (Dettling et al., 2007), with much research demonstrating that the stress of early parental loss can increase fear, as well as alter baseline activity and stress responsivity of the HPA axis in adulthood (primates- reviewed in Parker and Maestripieri, 2011; Pryce et al., 2002). Several studies have used the parental separation paradigm to look at later responses to separation and novelty. Maternally deprived primates showed significantly greater behavioural disturbance and less exploration of objects and food in a novel environment (Spencer-Booth and Hinde, 1971: rhesus macaque, *M. mulatta*; Capitanio et al., 1986: pigtail macaque, *M. nemestrina),* and were rated as less sociable than controls (Caine et al., 1983: *M. nemestrina*). Peer-reared primates have also been found to display high levels of fear and hyperemotional behaviour in a novel environment, compared to mother-reared animals (Capitanio, 1986: *M. nemestrina;* Higley et al., 1992a: *M. mulatta*).

There also appears to be some dysregulation of the HPA axis, with animal literature mostly suggesting heightened stress responses following early life stress (review: Fogelman and Canli, 2019). For example, in a longitudinal study, Higley et al (1992b) found that young rhesus macaques exposed to repeated social separations had increased plasma cortisol concentrations both prior to and during the stressor. However, other studies have failed to find differences in cortisol response (Winslow et al., 2003: *M. mulatta*) or have found lowered plasma cortisol levels (Champoux et al., 1989: *M. mulatta*), as well as reduced responsiveness following social separation, dexamethasone suppression and ACTH challenge, compared to mother-reared infants (Capitanio et al., 2005: *M. mulatta*), which may be due to an altered set point of the HPA axis. These inconsistent findings could be due to a number of factors, including type, number and severity of stress events, general housing conditions, timing of study, as well as species and developmental stage (Pryce et al., 2002). The link between cortisol activity and stress can therefore often be unclear (e.g. Mormede et al., 2011).

Early deprived (ED) common marmoset infants, removed from their natal group each day and placed alone in a cage for variable durations and times from post-natal day 2-8, have been found to spend more time in contact with parents and less time in social play, as well as exhibit reduced mobility and contact calls when isolated in a novel cage as juveniles (Dettling et al., 2007), suggesting they were more anxious and behaviourally inhibited than controls. Early deprivation was also associated with altered physiological parameters, including diminished basal cortisol levels (Dettling et al., 2002), as well as elevated norepinephrine levels and systolic blood pressure (Dettling et al., 2007; Pryce et al., 2004). Early parental separation can therefore have detrimental consequences in marmosets, increasing subsequent fear and vulnerability to stressors.

However, while separation from the family early in life can lead to deficits in behavioural development, as well as alterations in physiology (Parker and Maestripieri, 2011), there is accumulating evidence that exposure to early life stress could promote resilience to stress in adulthood. Parker et al (2004; 2019) exposed juvenile squirrel monkeys *(Saimiri sciureus*) to varying ‘doses’ of early life stress. In response to subsequent stressors, the animals given 1 or 2 stressors, including weekly one hour separations at 17 weeks old, a time when they naturally develop independence, had fewer indicators of anxiety, displaying less maternal clinging, more exploration and food consumption in a novel environment, as well as diminished HPA activation. Similarly, removal of the mother at weaning resulted in fewer distress calls, more time in proximity to peers and smaller increases in cortisol in ‘stress-inoculated’ squirrel monkeys (Lyons et al., 1999). However, those with either no interventions or 3 stressors did not differ in their heightened response (Parker et al., 2019). Results therefore suggested that stress-inoculated monkeys were less anxious than non-inoculated monkeys (Parker et al., 2004). However, it is unknown whether these effects persist across the lifespan.

Parker and Maestripieri (2011) suggest that while exposure to excessive early life stress or minimal stress may undermine coping ability and leave individuals vulnerable, overcoming moderate stress when young, which is not overwhelming, but challenging enough to evoke acute anxiety, may enhance emotional regulation and protect individuals against adverse effects. The resilience model of stress development therefore assumes a J shaped curve (Parker et al., 2019). As marmoset infants are in almost constant body contact with a family member for the first weeks of life, separations very early on are ‘non-biological’ events (Dettling et al., 2002). Rearing methods are therefore an important issue when caring for captive primates, with husbandry practices often advocated without sound knowledge of their effect on physiological and behavioural development. As few studies have followed animals beyond one year of age, more longitudinal studies in marmosets would yield important information on long-term consequences.

This study aims to investigate behavioural and cortisol responses to the mild routine stressor of capture and weighing in adult common marmosets, to assess the stress vulnerability of individuals born and reared under different backgrounds. Undisturbed family-reared twins were compared to both family-reared triplets (where only 2 remain) and supplementary fed triplets, exposed to early life family separations. Based on previous research (Dettling et al., 2002; 2007), it was hypothesised that supplementary fed marmosets would be more vulnerable to stress, displaying altered HPA axis function and heightened behavioural agitation to capture and weighing, compared to the other rearing conditions, if this practice mimics stressful primate parental separation paradigms. Alternatively, as marmosets are adapted to being transferred between carriers at a young age (Ingram, 1977), separation from the family with litter mates, as well as predictable timing of separation and early exposure to novel humans and situations, may lead to stress resilience.

2. Method

2.1 Study animals

Twenty-one marmosets were studied, housed at Dstl, Porton Down, UK (aged between 1 year 7 months and 2 years 7 months). All animals were purpose bred in captivity. Each marmoset was housed in vasectomised male-female pairs, as stock animals (generally from approximately 20 months old, following a period of same-sex housing after removal from the natal group at 18 months). Common marmosets reach sexual maturity at 18-24 months (Hearn, 1982), and so recommended age to remove young from the captive family group is 18 months and above. In the wild, common marmosets remain in the groups until adulthood, before leaving to find breeding partners (Ferrari and Digby, 1996).

In certain pairs, only one member was sampled (n=7), as their partner did not fit one of the conditions (i.e. was born as a singleton or quadruplet), while on all other occasions (n=14) both animals in the pair were studied. Partners were allocated randomly from available animals at the time of pairing, and so were not often of the same rearing background (see Table 1 for rearing conditions, including whether both in the pair or only one individual was sampled). Members of a pair are not independent of each other, leading to pseudoreplication (Lazic, 2010), but recording them simultaneously was necessary in order to increase the sample size. Not all marmosets born into the same litter were sampled in adulthood, as they had been allocated for different studies at the lab.

Table 1: Study animals in each rearing condition, including whether both or only one individual in a pair was sampled.

	Rearing condition		
	Family reared twin	Family reared 2stay	Supplementary fed triplet
Pair			
1	P1 (m)	P1 (f)	
2		P2 (f)	P2 (m)*
3		P3 (f)*/ P3 (m)	
4	P4 (f)		P4 (m)
5	P5 (f)*		P5 (m)*
6		P6 (f)/ P6 (m)	
7	P7 (m)	P7 (f)	

Table 1 continued: Study animals in each rearing condition, including whether both or only one individual in a pair was sampled.

	Rearing condition		
	Family reared twin	Family reared 2stay	Supplementary fed triplet
Individual			
1			I1(m)
2			I2 (f)*
3	I3 (f)		
4			I4 (m)
5		I5 (m)*	
6	I6 (m)		
7			I7 (m)*

m=male; f= female

*Missing 1 cortisol sample

2.1.1 Study conditions

Marmosets were studied in three rearing backgrounds, based upon practices carried out at the breeding facility, and so no manipulations solely for the purpose of the study were necessary.

Condition 1:

As controls, condition 1 had six family-reared twins (3 male, 3 female). Infants were born as twins and left undisturbed in the family group, except for monthly weighing.

Condition 2:

To examine potential differences in born litter size (i.e. twins v. triplets), condition 2 had eight family-reared marmosets from triplet litters where only 2 remain, due to loss of the third (known as 2stays) (3 male, 5 female). These marmosets were born as triplets, but one sibling either died naturally or was euthanised due to low weight (<27g) at less than 5 days old, leaving the remaining two infants to be raised undisturbed in the family.

Condition 3:

To examine potential differences in rearing background (i.e. family reared v. human intervention), condition 3 had seven supplementary fed triplets (6 male, 1 female). From postnatal day 1, the family member carrying the infant/s was encouraged to the front of the homecage with a piece of marshmallow, and the infant/s were gently removed. All three infants were taken out of the family group together, wrapped in towelling and placed in an incubator every day for 2 x 2 hours (8:00-10:00, 16:00-18:00), during which time each infant was handled for approximately 5 mins while they were fed SMA milk by care staff. The infants received four feeds per day until they were 20 days old (0.5ml of milk/feed at 1 week, 1-1.5ml milk at 2 weeks and 1-2.5ml milk at 3-4 weeks). This was reduced to three feeds, with one 2-hour morning incubator session and one afternoon feed with no incubator session, until 25 days old. From 26-30 days old there were 2 feeds per day, with no incubator time, and then from 31-41 days old there was only one feed per day (up to 5ml milk after 4 weeks old). After this time, they were left with their family. This gradual reduction in feeding time aimed to mimic the young marmoset's natural weaning off the mother's milk. Although full weaning occurs after week 8, solid food is often eaten before this through sharing or stealing (Yamamoto, 1993).

2.1.2 Housing and husbandry of adults

The marmosets were housed in cages measuring 100cm wide x 60cm deep x 180cm high, lined with wood chippings and furnished with a nestbox, wooden platforms, perches, ropes, suspended toys and a wire veranda. All marmosets had *ad libitum* access to water, and food was delivered twice a day (primate pellets were fed in the morning, and a variety of fruit was provided in the afternoon). Malt loaf, egg, rusk, mealworms, dates, peanuts and bread were provided on alternate days, and gum Arabic and milkshake (with added vitamin D) were given twice a week. Additional environmental enrichment, such as cardboard boxes and mealworm feeders, were given once a week to introduce novelty. Temperature and humidity were at 23-24ºC and 55 +/- 10% respectively. Lighting was provided on a 12-hour light/dark cycle.

Marmosets from all rearing conditions were weighed at 10 days, and then each month of their life at the colony. Once a week, all animals received a human socialisation programme, in which technicians sat in the homecage and offered food to the family, as well as syringe training to accept banana milkshake, in preparation for receiving medication. Study methods were approved after review by the Stirling University Psychology Ethics Committee and the facility involved, and complies with legal and ethical requirements in the UK.

2.1.3 Weighing and cage change procedure

Weighing is a necessary routine event, that provides a good opportunity to assess how individuals cope with a mild stressor, without inflicting any stress for the sole purpose of the study. A standardised procedure was employed, based on current practice:

Weighing took place between 9:00 and 10:00. The marmoset was hand caught by grasping the base of the tail and then holding around the chest. They were given a brief health check and then placed into a small, plastic box on the weigh scales where they had no visual or olfactory contact with their pair member, before being released into a new clean cage. The old cage was then removed for washing. The whole process took approximately 5 minutes/marmoset. The marmosets were in view of other pairs in the room while in the homecage. Although a routine husbandry procedure, previous studies have found that capture and handling can be physiologically stressful for captive primates (e.g. *C. jacchus*: Bassett et al., 2003; *M. mulatta*: Reinhardt et al., 1995; *S. sciureus:* Hennessy et al., 1982). Stress-related behaviour, including reductions in inactivity, accompanied by increases in self-scratching and scent marking, have also been observed in marmosets following capture (Bassett et al., 2003; Cilia and Piper, 1997).

2.2 Cortisol response

2.2.1 Saliva collection and cortisol assay

Salimetrics Oral Swabs (SOS) coated with banana were used to collect the saliva. One end of the swab was presented through the wire cage front for 5 minutes and the marmoset allowed to lick and chew the end to deposit saliva. Previous studies have shown this to be an effective, non-invasive method for saliva collection in the marmoset (Ash et al., 2018). Banana is a favoured flavour, which reliably encourages chewing (Cross et al., 2004). After habituating the monkeys to the SOS (for 5 mins on 3 days), saliva was sampled on three baseline days in the week prior to weighing, between 9:00-10:00. Samples were taken at similar times for each individual animal, to ensure compatibility and avoid variation due to circadian rhythm (Cross and Rogers, 2004).

Two saliva samples were then collected after capture and weighing, at 0-5 minutes and 25-30 minutes, prior to behavioural observations (see section 2.3.1). Salivary cortisol is thought to reflect acute changes in the non-protein bound 'free', biologically active fraction of the hormone (Higham et al., 2010). Although we do not have a full understanding of the time course of salivary cortisol in non-human primate species, previous research has found significant changes in concentration from 0-45 mins post stressor (e.g. *C. jacchus:* Bowell, 2010; *P. troglodytes*: Heintz et al., 2011; Laudenslager et al., 2006). Therefore, 0-5 mins and 25-30 mins were chosen to detect changes. Figure 1 describes the timeline for each monkey, including their rearing background, housing changes from infancy to adulthood, and timings of behaviour and cortisol collection for the study.

The samples were first cut to fit in the storage tube, sealed and marked with subject ID, time and date. They were then spun in a centrifuge for 15 mins at 1500 RPM, to separate the saliva from the swab, and stored at -80 ^{o}C. They were analysed using Salimetrics® Salivary Cortisol Enzyme Immunoassay Research Kits, which we have validated previously for use in common marmosets. Plates were run as per the manufacturer's instructions (Salimetrics®, 2012a), using standards in the range 82.77, 27.59, 9.19, 3.06, 1.02, 0.33 nmol/L. All samples were run in duplicate, at a dilution of 1:5000.

2.3 Behavioural response

2.3.1 Behavioural observations

One individual conducted all behaviour observations and cortisol collection. Baseline and post-weighing data were recorded for each animal. Baseline behavioural data were collected over three days a week before weighing, to match the post-weigh time points. Behaviour was then observed 5 minutes and 30 minutes after weighing. Five-minute observations were conducted at each time point, using focal sampling. Behaviours were recorded using 30 second instantaneous sampling for longer duration behaviours and all-occurrence sampling for short duration behaviours, expressed as estimated percentage of sample time when in view and frequency per sample time respectively.

Coded behavioural data were collected using The Observer^{R} V8.0 (animal behaviour) recording software (Noldus Information Technology; Wageningen, Netherlands). Behaviours of particular interest included activity (locomotion, inactivity, exploration, foraging), social (proximity) and stress-related (self-scratch and scent mark) (definitions based on Stevenson and Poole, 1976; predictions of increases or decreases in response to stress based on Bassett et al., 2003; Badihi, 2006). Intra-observer reliability for each behaviour ranged from 80-100%. Table 1 provides a full description of each behaviour, including behaviour recording method.

Table 2: Behavioural categories recorded at baseline, 5 minutes and 30 minutes post capture and weighing for marmosets in each condition (family reared twins, family reared 2stays and supplementary fed triplets)

Behaviour	*Definition*
Stress-related *(increase when stressed)*	
Inactive alert [a]	The animal remains stationary, alert and aware of the surroundings, without engaging in any other activity.
Agitated locomotion [a]	The animal moves between locations rapidly. Its gait is not relaxed.
Proximity [a]	The animal is stationary, sitting, crouching or lying next to another individual, with some form of physical contact.
Scratch [b]	The animal rapidly moves its hand or foot, drawing its claws across the fur or skin.
Scent mark [b]	The animal sits and rubs its anogenital area on a branch or other area of the enclosure (anal scent mark), or rubs its sternal area along a substrate (sternal scent mark).
Positive *(decrease when stressed)*	
Inactive rest [a]	The animal is stationary, usually with the tail curled around the body or through the legs, its eyes open or closed.
Calm locomotion [a]	The animal travels between locations by walking, running, climbing or jumping, its gait relaxed.
Explore [a]	The animal investigates objects in the environment by handling, sniffing, gently biting, licking or attending to them whilst walking around them.
Eat/forage [a]	The animal is engaged in any activity directly related to acquiring or ingesting food.
Other [a]	Any other behaviour not noted above, or animal cannot be seen by the observer.

a. Instantaneous sampling (long duration behaviour)
b. All-occurrence sampling (short duration behaviour)

2.4 Statistical Analysis

Means were calculated from the three baseline cortisol and behavioural values for each individual, to obtain one baseline value for use in the analysis, in attempt to reduce any large variations. As data were approximately normally distributed (as determined by the Kolmogorov-Smirnov test) within the rearing conditions, parametric tests could be conducted to look at differences between baseline and post capture cortisol values in each of the three conditions. Due to some missing data points (where samples were not collected or analysed successfully: n= 7), each time point was examined using paired samples t tests, to prevent any data from being lost in repeated measures ANOVAs (which only include subjects with all data points). A one way ANOVA was conducted to look at any differences between the rearing conditions at each of the 3 time points (t tests were not needed for the between subject design, which did not lose any data points). All cortisol data presented were uncorrected for banana (see Ash et al., 2018), as variations in banana concentration are likely to have minimal effects on the assayed cortisol concentration (Cross et al., 2004). Results are presented as means (+/- 1SE).

No transformation was successful in making behavioural data normally distributed, and so non-parametric tests were conducted. ‘Other’ was not analysed, as it was only recorded for accurate estimations of relevant behaviours. Friedman tests were used to look at time point differences within each rearing condition for the nine behaviours. Significant results were followed up with Wilcoxon post hoc tests. Kruskal Wallis tests were used to look at each time point between the rearing conditions. Despite multiple analyses being carried out, Bonferroni adjustments were not made, to allow maximum information to be extracted from the data, and independent assessment of the validity of results (Caldwell et al., 2005). Results are presented as median and IQR (+ min and max value). All statistical analysis was conducted in SPSS. Level of significance was 0.05.

Due to the small sample size, effect sizes were also calculated to look at differences in cortisol concentration (calculated with Cohen’s d using z scores), as well as frequencies and durations of behaviour (calculated using eta-squared with Mann U z scores) between the 3 conditions.

3. Results

3.1 Cortisol Data

3.1.1 Comparison between baseline and post capture data within rearing condition

For family reared twins and supplementary fed triplets, while cortisol concentration was lower at post 5 mins and post 30 mins than at baseline, this was not significant. Family reared 2stay cortisol level was however significantly lower at post 5 mins (Paired samples t test: $t(6)=4.40, P=0.005$) and at post 30 mins ($t(6)=3.24, P=0.018$) than at baseline. Figure 2 displays the mean cortisol values (+/-1 SE) at each time point for each rearing condition, as well as the significant changes found in family reared 2stays.

3.1.2 Effect of rearing condition on cortisol response

There was no significant difference in baseline cortisol levels between rearing conditions, although variation was very large at this time point, particularly for supplementary fed triplets. Cortisol levels at post 5 mins and post 30 mins were also not significantly different between the rearing conditions.

High effect sizes were however found between family reared twins and family reared 2stays at 0-5min post stressor (0.890), as well as between family reared twins and supplementary fed triplets (0.727), and family reared 2stays and supplementary fed triplets at 25-30 min post stressor (0.951). Supplementary fed triplets had higher cortisol concentrations than family reared 2stays and family reared twins, and family reared 2stays had higher cortisol concentrations than family reared twins post stressor. Effect sizes between the conditions were low at baseline.

3.2 Behavioural Data

3.2.1 Comparisons between baseline and post capture data within rearing condition

Stress-related short duration behaviour

In family reared twins, scratching (Friedman test: $X^2(2)=6.70, P=0.035$) at baseline was significantly higher than at post 5 mins (Wilcoxon test: $Z=2.23, P=0.026$). Scent marking ($X^2(2)=9.33, P=0.009$) at baseline was significantly lower than at post 5 mins ($Z=-2.20, P=0.028$) and at post 30 mins ($Z=-2.20, P=0.028$). No short duration stress-related behaviour changed significantly in family reared 2stays or supplementary fed triplets. Figure 3 displays the median frequencies (with IQR, min and max) spent in each short duration stress-related behaviour in each condition.

Stress-related long duration behaviour

In family reared twins, inactive alert ($X^2(2)=6.82, P=0.033$) was significantly lower at post 5 mins than at baseline ($Z=-2.03, P=0.042$) and at post 30 mins ($Z=-2.04, P=0.041$). Agitated locomotion ($X^2(2)=8.67, P=0.013$) was significantly higher at post 5 mins than at baseline ($Z=-2.21, P=0.027$). Proximity to partner ($X^2(2)=5.16, P=0.076$) was significantly higher at baseline than post 5 mins ($Z=-2.00, P=0.046$).

In family reared 2stays, agitated locomotion ($X^2(2)=5.16, 0.076$) was higher at post 30 mins than at baseline ($Z=-2.04, P=0.041$). In supplementary fed triplets, there was an increase in scent marking ($X^2(2)=6.53, P=0.038$) from baseline to post 5 mins and post 30 mins, although this was not significant when further analyzed with Wilcoxon tests. Agitated locomotion ($X^2(2)=6.63, P=0.036$) was significantly lower at baseline than at post 5 mins ($Z=-2.03, P=0.042$) and post 30 mins ($Z=-2.02, P=0.043$). Figure 4 displays the median estimated percentage of sample time (with IQR, min and max) spent in each long duration stress-related behaviour for each condition.

Positive behaviour

In family reared twins, calm locomotion ($X^2(2)=6.52, P=0.038$) was significantly higher at baseline than post 5 mins ($Z=-2.20, P=0.028$) and post 30 mins ($Z=-2.00, P=0.046$). In family reared 2stays, foraging ($X^2(2)=10.00, P=0.007$) was significantly higher at post 5 mins than baseline ($Z=-2.06, P=0.039$) and post 30 mins ($Z=-2.06, P=0.039$). In supplementary fed twins, exploration ($X^2(2)=5.29, 0.071$) was significantly higher at post 5 mins than at baseline ($Z=-2.04, P=0.041$). Figure 5 displays the median estimated percentage of sample time (with IQR, min and max) spent in each long duration positive behaviour for each condition.

3.2.2 Effect of rearing condition on behavioural response

There were no significant differences in any behaviour recorded between family reared twins, family reared 2stays and supplementary fed triplets at baseline, post 5 mins or post 30 mins. Effect sizes were small (0.37 and below) between the 3 conditions for each of the 9 behaviours analysed.

3. Discussion

Early interactions with caregivers can have an important role in development, with the quality of early life experience enhancing stress vulnerability or resilience (Parker and Maestripieri, 2011). It was hypothesised, based on numerous primate models, that early family separation would lead to adverse developmental consequences, including altered HPA axis function and increases in anxiety-related behaviour following mildly stressful routine procedures (e.g. Dettling et al., 2002; 2007). However, the present study found minimal behavioural and physiological differences in response to capture and weighing amongst marmosets of different rearing backgrounds.

4.1 Effect of rearing condition on cortisol response

There was no significant effect of rearing condition on cortisol level, with all decreasing from baseline to post capture time points. We found similar reductions in cortisol concentration post stressor in a previous study (Ash et al., 2018), which researchers have suggested may be due to social buffering, the ability of a companion to ease the stress of challenging situations (Gilbert and Baker, 2010), or ‘blunting’ of the HPA axis following a prolonged period of stress (Loman and Gunnar, 2010). Although there was only a significant difference in cortisol over the timepoints for family reared 2stay marmosets, the lower variation at baseline between individuals in this condition may account for the significant result. The previously reported diminished basal cortisol levels in early separated common marmosets (Dettling et al., 2002) were therefore not seen in the current study, and instead support research finding similar cortisol levels in peer reared and mother-reared animals (e.g. Clarke, 1993). However, studies investigating the effect of rearing background on HPA axis activity have been inconsistent, and a comprehensive meta-analysis found no significant association between early life stress and cortisol (Fogelman and Canli, 2018).

We did however find large effect sizes in cortisol concentration post-stressor between the conditions, which suggested that, although there were reductions in cortisol from baseline, supplementary fed triplets and family reared 2stays may have higher cortisol levels than family reared twins following capture and weighing. Prenatal experience, including uterine crowding and competition for resources, can have profound effects on physiological development (review: Riesche et al., 2018). Results can therefore be considered potentially suggestive of differences in HPA activity between rearing conditions, albeit being somewhat preliminary. It is possible that a larger sample would help clarify results.

4.2 Effect of rearing condition on behavioural response

Despite little significant changes in cortisol, marmosets in all conditions exhibited striking increases in agitated locomotion following capture and weighing. Scent marking also increased in both family reared twins and supplementary fed triplets following capture and cage change, which has been observed in response to potential stressors in previous studies (Bassett et al., 2003). Although there were no differences between rearing conditions, some minor differences emerged when looking at each condition separately. Behaviour changed more significantly from baseline to post capture for family reared twins, compared to the other conditions. There was significantly less calm locomotion, inactive alert and proximity, as well as self-scratching, following capture, suggesting more unsettled movement. There were few significant behavioural changes in family reared 2stay marmosets, except for an increase in foraging directly after capture. Supplementary fed triplets also engaged in increased positive behaviour, with more exploration directly after capture.

Therefore, while there were some stress-related behavioural changes in family reared 2stays and supplementary fed triplets, there were also some increases in behaviour suggesting reduced anxiety. Results are therefore contrary to previous work reporting early deprived primates to be more anxious and behaviourally inhibited (Dettling et al., 2007: marmosets; Higley et al., 1992a: *M. mulatta*). Instead, results are more consistent with Parker et al's (2004; 2019) stress inoculation studies, which demonstrated that brief separations from the family lead to less negative arousal, and more exploration and food consumption in a novel environment. 2stays and supplementary fed triplets in the current study may therefore be better able to deal with challenges in the laboratory, as they have experienced moderate stress, either prenatally or in early life (Parker and Maestripieri, 2011), due to loss of a litter mate or family separation.

Variation in effects of early life stress

Features of early life stress, including type, duration, frequency and developmental stage can all play a role in the diverse range of developmental outcomes (Parker and Maestripieri, 2011). Rearing primates in isolation can have devastating effects on development and behaviour, with effects of family separation often greater when they deviate significantly from the norm, particularly when the individual is separated very early on, and the deprivation is longer lasting and more complete (Parker and Maestripieri, 2011). It is therefore likely that if one marmoset infant were removed and kept alone, effects would be much less subtle than seen in the current study**.** As supplementary fed marmosets were taken out with their siblings for brief periods, and integrated back into the family as soon as possible, any adverse effects may have been minimized. Supplementary fed marmosets were also fed at predictable times, building positive experiences with humans from an early age. As common marmosets are co-operative rearers (eg. Ingram, 1977), infants are naturally adapted to be passed between helpers, which may help to explain the differences found between this species and the very maternally bonded macaques used in many primate models of parental separation (Lindburg, 1971).

Individual factors such as genotype and personality can also play a role in response to stress (Parker and Maestripieri, 2011), and so certain experiences may not always lead to the same outcome in different individuals. Marmosets in each condition may therefore have differing levels of emotionality and sociality, as well as different coping styles, which could help explain the multivariate response patterns (Koolhaas and Reenen, 2016). While active responses, such as agitated locomotion, are associated with greater activation of the sympathetic system and the release of adrenaline, passive responses, including inactivity, are associated with activation of the parasympathetic system, leading to greater fluctuations in cortisol (Cross and Rogers, 2006; Korte et al., 2005).

Limitations of the study

Several factors may help to explain our limited significant findings. Relationships between early life stress and stress responsivity are complex, and often mediated by the presence of additional risk variables (review: Fogelman and Canli, 2019). For example, in humans, family tension and quality of care has been found to influence the impact of early parental loss (Bloch et al., 2007; Luecken, 2000; Flinn and England, 1995; Gunnar et al., 1996). Observations of behaviour in the family group, which is known to differ markedly between marmoset families (Yamamoto, 1993), would therefore have been beneficial. Adult experience and social support can also have an influence (Carere and Maestripieri, 2013). Therefore, behaviour of the current partner may have influenced results. A larger sample size would enable us to conduct more complex models, including the combination of partner conditions (i.e. a family reared twin with a supplementary fed triplet) and the relationship between them.

As well as these unmeasured factors, it is difficult to disentangle the outcomes of rearing and litter size, as only triplets received supplemental feeding. However, the conditions studied were based on management practices at the facility, and so reflect real-life situations for laboratory-housed marmosets (see Schutz-Darken et al., 2019 for details of commonly used rearing methods). The little differences between the conditions may also suggest that the marmosets had habituated to the routine capture and weighing. Although the behavioural agitation observed indicates it is unlikely that the marmosets studied were completely habituated, the monthly process may not have been sufficient to elicit any significant differences between the conditions. The procedure lasted only 5 minutes, compared to many experimental stressors, such as the social separation test, which can last at least 1 hour (e.g. Parker et al., 2019). The timing of the weighing procedure may also have led to high morning concentrations of cortisol, which may be at ceiling levels and so less sensitive to any increases (Pryce et al., 2002). However, for ethical reasons, we did not wish to impose any manipulations, such as additional stressors, for the sole purpose of the study. Other non-invasive measures of stress, such as Alpha amylase, may however be useful.

We also could not control for the possible buffering effect of animal care procedures, with the lack of any major differences between rearing conditions in the current study potentially due to the socialisation and training programmes that all animals at the colony received. Early exposure to positive human interactions has been shown to be beneficial for captive primate welfare, helping to improve their ability to cope with routine laboratory procedures (Bassett et al., 2003; Bowell, 2010). Brief periods of daily handling have also been found to reduce the physiological stress response in other species (rats: Meaney et al., 1985).

Despite these limitations, the current study provides some evidence that this particular supplementary feeding procedure, involving keeping the infants together, along with a regular human socialisation programme, appears to minimise the potential negative behavioural and physiological effects of early family separation. Therefore, while removal from the natal group is not recommended, if it is possible to keep infants with their family, it may be the preferred option if human intervention is necessary to improve marmoset infant survival.

5. Conclusion

The present study investigated whether rearing background had a long-term effect on response to a routine stressor in common marmosets. Behaviourally, capture elicited some stress in all animals, although this was not reflected physiologically. Although some very minor differences were seen in cortisol and behavioural responses when looking at each rearing condition separately, the study suggests that supplementary feeding, at least following the practice of the colony studied, had no long-term adverse effects. The young animals may even be better equipped to deal with challenges, which is of great importance for the wellbeing of an individual, especially in a laboratory environment. Although greater controls would be beneficial for future studies, such investigations can help increase our understanding of individual differences in stress responsivity.

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Declarations of interest: none

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Figure legends:

Fig 1: Timeline for each monkey, including their rearing background, housing changes from infancy to adulthood and timings of behaviour/cortisol collection for the study.

Fig 2: Mean (+/- 1 SE) salivary cortisol concentration (nmol/L) in each rearing condition at baseline, post capture 5 mins and post capture 30 mins time points. * Cortisol at baseline was significantly ($P<0.05$) higher than at post capture 5 mins and post capture 30 mins only in 2stays.

Fig 3:

A. Median frequency of occurrence (per 5 minutes) of short duration stress-related behaviours over each time point for family reared twin marmosets. Scratching was significantly ($P < 0.05$) higher at baseline than post capture 5 mins; scent marking was significantly lower at baseline than at post capture 5 mins and post capture 30 mins. Median: solid line; Interquartile range: boxes; Minimum and Maximum value: whiskers; Outliers: stars.

B. Median frequency of occurrence (per 5 minutes) of short duration stress-related behaviours over each time point for family reared 2stay marmosets. There were no significant changes in either behaviour over the time points.

C. Median frequency of occurrence (per 5 minutes) of short duration stress-related behaviours over each time point for supplementary fed triplet marmosets. There were no significant changes in either behaviour over the time points.

Fig 4:

A. Median estimated percentage of the 5 minute observation period spent in long-duration stress-related behaviour over each time point for family reared twin marmosets. Inactive alert was significantly ($P<0.05$) lower at post capture 5 mins than at baseline and post capture 30 mins; agitated locomotion was lower at baseline than at post capture 5 mins; proximity to partner was higher at baseline than post 5 mins. Median: solid line; Interquartile range: boxes; Minimum and Maximum value: whiskers; Outliers: stars.

B. Median estimated percentage of the 5-minute observation period spent in long-duration stress-related behaviour over each time point for 2stay marmosets. Agitated locomotion was significantly ($P<0.05$) lower at baseline than at post capture 30 mins.

C. Median estimated percentage of the 5-minute observation period spent in long-duration stress-related behaviour over each time point for supplementary fed triplet marmosets. Agitated locomotion was significantly ($P<0.05$) lower at baseline than at post capture 5 mins and post capture 30 mins.

Fig 5:

A. Median estimated percentage of the 5 minute observation period spent in long duration positive behaviours over each time point for family reared twin marmosets. Calm locomotion was significantly ($P<0.05$) higher at baseline than post capture 5 mins and post capture 30 mins. Median: solid line; Interquartile range: boxes; Minimum and Maximum value: whiskers; Outliers: stars.

B. Median estimated percentage of the 5-minute observation period spent in long duration positive behaviours over each time point for 2stay marmosets. Foraging was significantly ($P<0.05$) higher at post capture 5 mins than baseline and post capture 30 mins.

C. Median estimated percentage of the 5 minute observation period spent in long duration positive behaviours over each time point for supplementary fed triplet marmosets. Exploration was significantly ($P<0.05$) higher at post capture 5 minutes than at baseline.

Figure 1

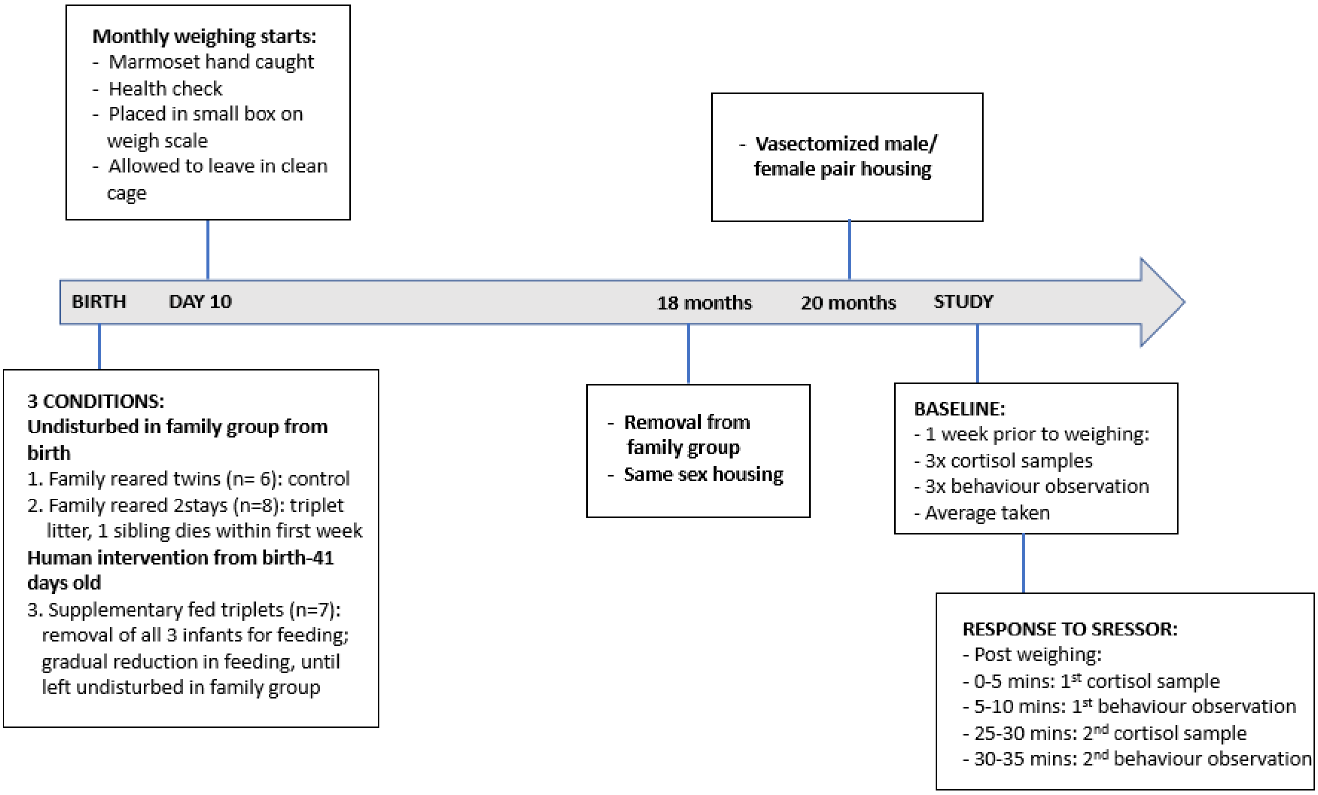

Monthly weighing starts:
- Marmoset hand caught
- Health check
- Placed in small box on weigh scale
- Allowed to leave in clean cage
- Vasectomized male/ female pair housing
BIRTH
DAY 10
18 months
20 months
STUDY
3 CONDITIONS:
Undisturbed in family group from birth
1. Family reared twins (n= 6): control
2. Family reared 2stays (n=8): triplet litter, 1 sibling dies within first week
Human intervention from birth-41 days old
3. Supplementary fed triplets (n=7): removal of all 3 infants for feeding; gradual reduction in feeding, until left undisturbed in family group
- Removal from family group
- Same sex housing
BASELINE:
- 1 week prior to weighing:
- 3x cortisol samples
- 3x behaviour observation
- Average taken
RESPONSE TO SRESSOR:
- Post weighing:
- 0-5 mins: 1st cortisol sample
- 5-10 mins: 1st behaviour observation
- 25-30 mins: 2nd cortisol sample
- 30-35 mins: 2nd behaviour observation

Figure 2

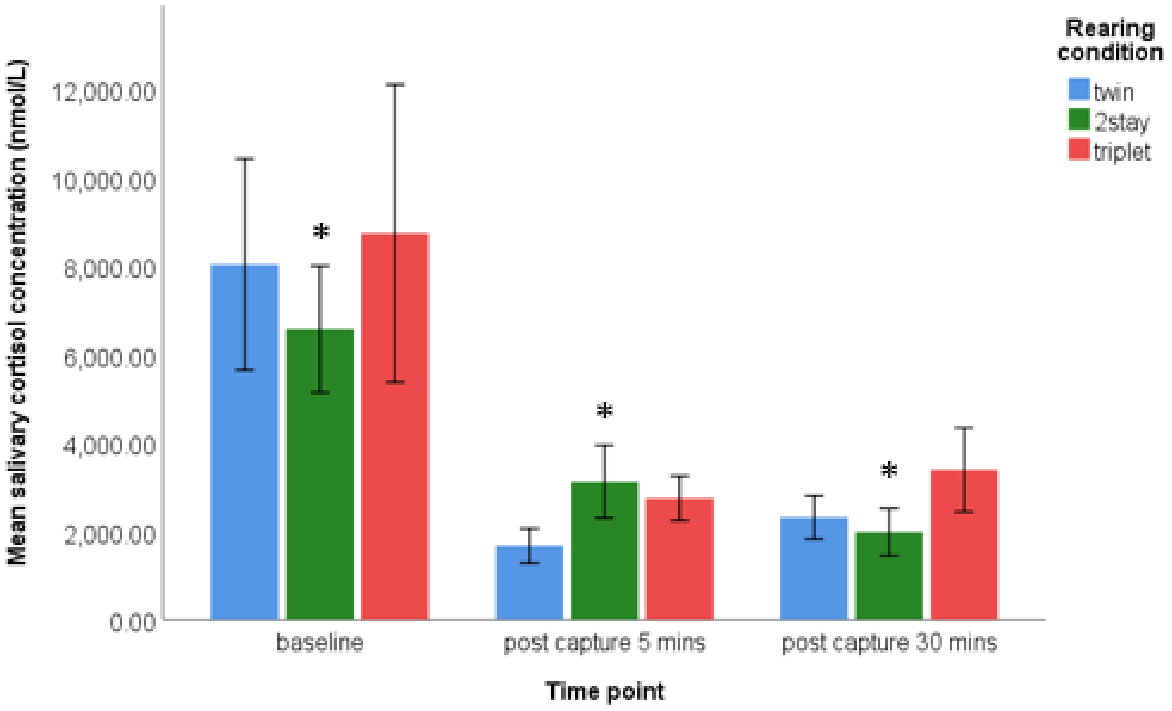
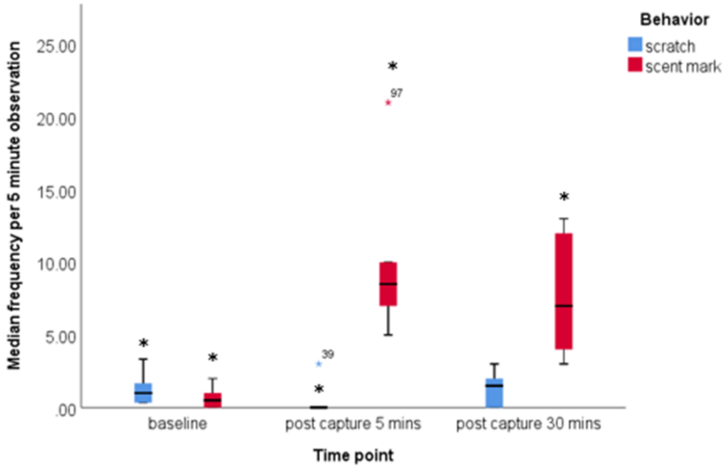
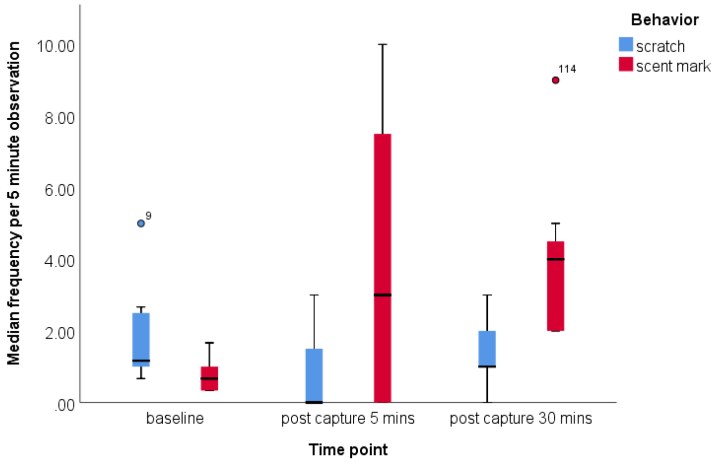

Mean salivary cortisol concentration (nmol/L)
0.00
2,000.00
4,000.00
6,000.00
8,000.00
10,000.00
12,000.00
*
*
*
baseline
post capture 5 mins
post capture 30 mins
Time point
Rearing condition
twin
2stay
triplet

Figure 3

A


Behavior
🟦 scratch
🟥 scent mark
Median frequency per 5 minute observation
25.00
20.00
15.00
10.00
5.00
.00
97
39
baseline
post capture 5 mins
post capture 30 mins
Time point

B


Behavior
🟦 scratch
🟥 scent mark
Median frequency per 5 minute observation
10.00
8.00
6.00
4.00
2.00
.00
9
114
baseline
post capture 5 mins
post capture 30 mins
Time point

C

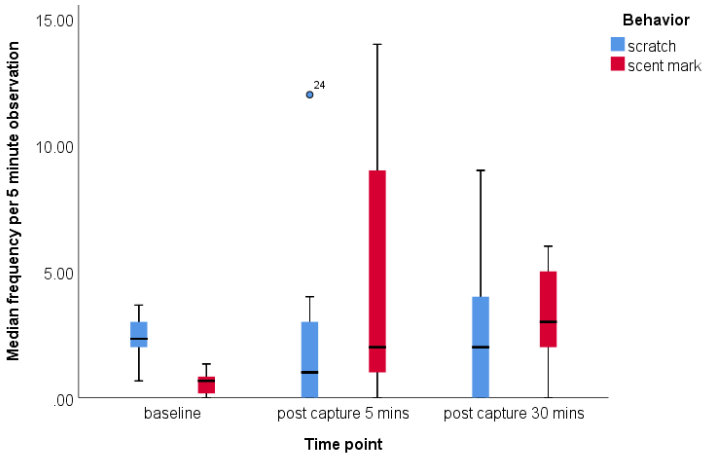
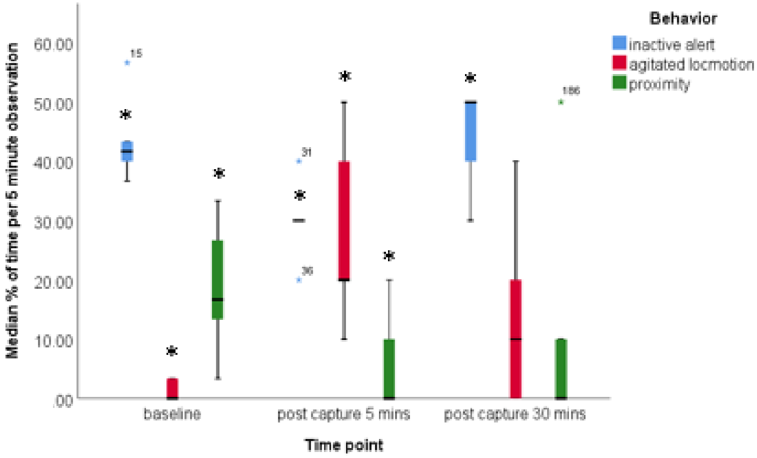
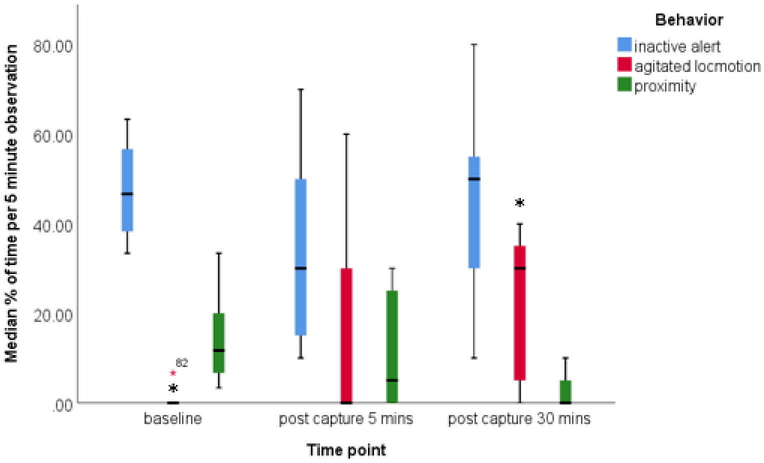

Behavior
🟦 scratch
🟥 scent mark
Median frequency per 5 minute observation
15.00
10.00
5.00
.00
24
baseline
post capture 5 mins
post capture 30 mins
Time point

Figure 4

A


Median % of time per 5 minute observation
60.00
50.00
40.00
30.00
20.00
10.00
.00
15
31
36
186
baseline
post capture 5 mins
post capture 30 mins
Time point
Behavior
🟦 inactive alert
🟥 agitated locmotion
🟩 proximity

B


Median % of time per 5 minute observation
80.00
60.00
40.00
20.00
.00
82
baseline
post capture 5 mins
post capture 30 mins
Time point
Behavior
🟦 inactive alert
🟥 agitated locmotion
🟩 proximity

C

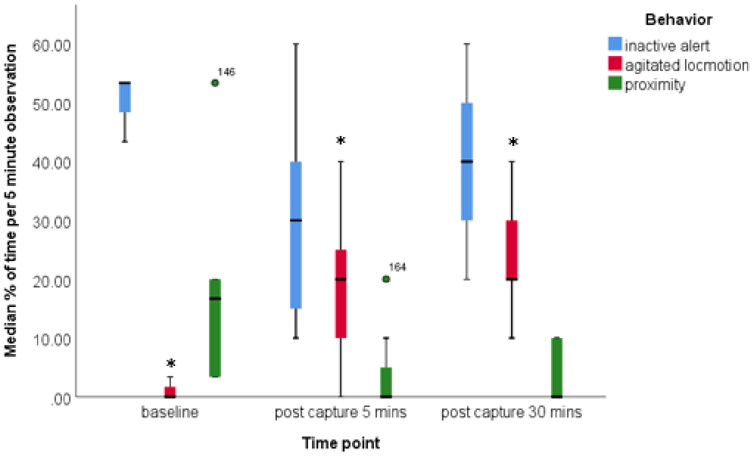
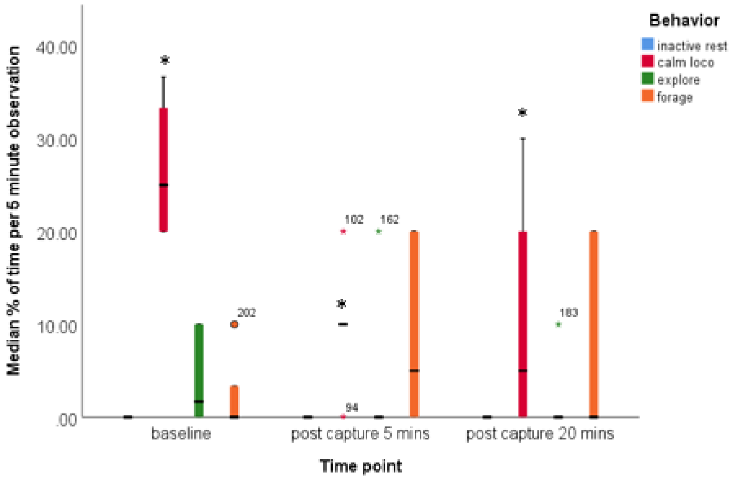
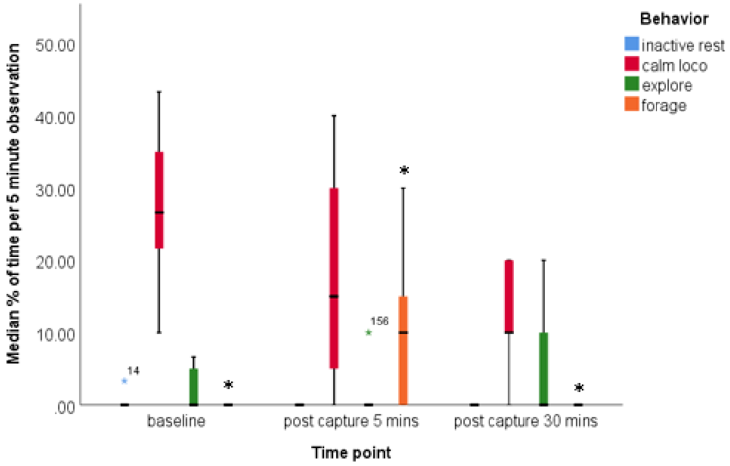

Median % of time per 5 minute observation
60.00
50.00
40.00
30.00
20.00
10.00
.00
146
164
baseline
post capture 5 mins
post capture 30 mins
Time point
Behavior
🟦 inactive alert
🟥 agitated locmotion
🟩 proximity

Figure 5

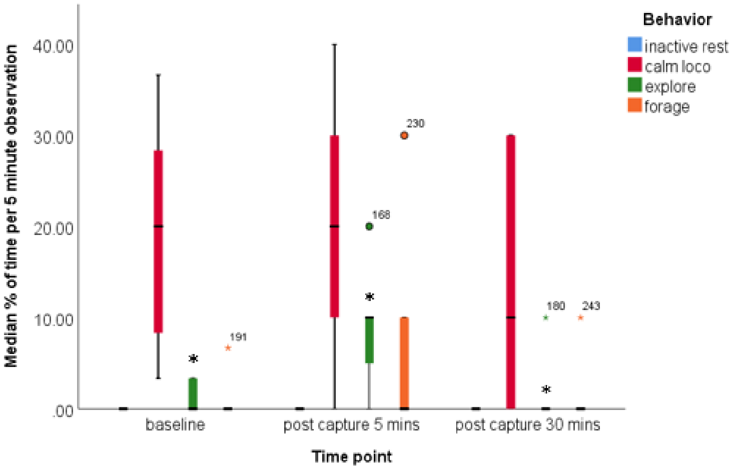
A


Behavior
🟦 inactive rest
🟥 calm loco
🟩 explore
🟧 forage
Median % of time per 5 minute observation
40.00
30.00
20.00
10.00
.00
202
102
162
94
183
baseline
post capture 5 mins
post capture 20 mins
Time point

B


Behavior
🟦 inactive rest
🟥 calm loco
🟩 explore
🟧 forage
Median % of time per 5 minute observation
50.00
40.00
30.00
20.00
10.00
.00
14
156
baseline
post capture 5 mins
post capture 30 mins
Time point

C


Behavior
🟦 inactive rest
🟥 calm loco
🟩 explore
🟧 forage
Median % of time per 5 minute observation
40.00
30.00
20.00
10.00
.00
191
230
168
180
243
baseline
post capture 5 mins
post capture 30 mins
Time point