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Hormonal stress response of laboratory mice to conventional and minimally invasive bleeding techniques

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Abstract

*Conventional bleeding of small laboratory animals is often associated with stress and injuries that can cause haematomas, inflammation and ultimately the death of animals under investigation. Here, we used faecal glucocorticoid metabolites as an indicator of stress imposed on laboratory mice (*Mus musculus domesticus*) when bled in three different ways: puncture of the tail vein following mechanical immobilisation; puncture of the retro-orbital plexus following chemical immobilisation; and a minimally invasive technique using blood-sucking bugs (*Reduviidae, Heteroptera*) without any immobilisation. We hypothesised that blood-sucking bugs provoke a lower hormonal stress response than conventional bleeding techniques because laboratory animals are not handled and because the mechanical stimulus of an insect sting is supposedly weak. Each of the 16 mice was bled using one of the three methods at a time in a random order with seven days of recovery between subsequent bleeding events. To monitor the stress hormones, we determined corticosterone metabolites in faecal samples of mice collected one day before, 8 h after and one day after the bleeding event. Concentrations of faecal glucocorticoid metabolites increased in all three treatment groups compared with baseline values. However, average concentration of stress hormone metabolites after bleeding was higher by a factor of about 1.5 when conventional bleeding techniques were applied than when bugs were used. We conclude that blood-sucking bugs may offer a gentle alternative for obtaining blood samples from small animals such as mice.*

Keywords: *animal welfare, blood samples, corticosterone,* Dipetalogaster maximus, *faecal samples,* Mus musculus

Introduction

Animal welfare requirements necessitate that pain imposed on laboratory vertebrates should be minimised, particularly during bleeding (Monash University Animal Welfare Committee 2008; GV Solas 2009). This is most often accomplished by adjusting the specific technique of bleeding to the species in question, individual animal characteristics (eg age, sex), the minimum volume of blood needed for analysis and the requirements of the experimental design. Yet, during vein puncturing with conventional needles, it is inevitable that skin tissue and blood vessels are damaged. This may lead to haematomas or even more severe complications (Hoff 2000). Delicate veins or arteries of small animals, such as mice and birds, may complicate bleeding (Voigt *et al* 2006). As a consequence, small animals may have to be punctured repeatedly, which may even increase the risk of injuries. Also, animals have to be either mechanically or chemically immobilised for bleeding, which often causes stress (Gärtner *et al* 1980; Vahl *et al* 2005). Stressful handling may activate the sympathetic nervous system and the hypothalamus-pituitary-adrenal (HPA) axis and this may lead to increased levels of circulating stress hormones. Generally, the acute release of catecholamines and glucocorticoids should help organisms to respond quickly to new situations for immediate survival. In case of chronic stress, however, the release can lead to significant negative effects such as suppression of the immune system, reproduction and vitality of animals (Riley 1981; Holst 1998; Kloet *et al* 1999; Touma & Palme 2005). In laboratories, glucocorticoids of domestic and wild animals have been validated as indicators for an animal's body condition, health and welfare, making them an important tool for ecological and laboratory studies. The use of this tool, however, requires accurate measurement of baseline levels for unstressed animals (Arnold *et al* 2008). Unfortunately, the determination of baseline corticosterone

levels using conventional bleeding techniques is very difficult, since glucocorticoid concentrations increase rapidly when bleeding is stressful for the animals. In addition, endocrinological studies have shown that stress caused by conventional bleeding may alter the blood parameters of interest (Romero & Romero 2002; Becker *et al* 2005). Therefore, it is widely assumed that baseline corticosterone levels can only be measured when animals are captured and bled within 3 min (Romero & Reed 2005; Vahl *et al* 2005). Beyond this time limit, glucocorticoid secretion may already alter whole or differential blood cell counts, and blood chemistry (Gärtner *et al* 1980; Riley 1981; Wells *et al* 1984; Stefanski 1998; Marco & Lavin 1999). Consequently, it is difficult to obtain accurate baseline values of blood parameters by using conventional bleeding techniques (Sheriff *et al* 2011), and minimally invasive bleeding techniques are in high demand to avoid stress-induced biases of blood parameters, injuries caused by conventional needles or an impairment of animal health.

In this study, we investigated the glucocorticoid response of laboratory mice after being bled conventionally and after being bled in a presumably minimally invasive way, ie we used blood-sucking bugs to collect blood from mice (Helversen & Reyer 1984). Blood-sucking bugs (*Triatominae; Reduviidae*) are highly adapted for consuming blood from terrestrial mammals, such as marsupials, rodents, bats, and carnivores, and also from birds and reptiles (Lent & Wygodzinsky 1979). Depending on the larval stage, reduviid bugs ingest between 0.1 and 4.0 ml blood during a 2- to 30-min blood meal (Voigt *et al* 2004; Thomsen & Voigt 2006).

A variety of studies have focused on the advantages of this technique over the conventional approach using sterile medical needles (Helversen *et al* 1986; Voigt *et al* 2004; Thomsen & Voigt 2006; Arnold *et al* 2008). One important aspect is that the animals in question do not necessarily have to be captured, restrained or anaesthetised for blood sampling when blood-sucking bugs sting the animals from a hide. Past validation experiments have confirmed that this bleeding technique is suitable for serological measurements (Voigt *et al* 2006; Vos *et al* 2010), for doubly labelled water experiments (Voigt *et al* 2003, 2005), for endocrinological measurements (Voigt *et al* 2004; Arnold *et al* 2008) and partly also for the analysis of haematological parameters and blood chemistry (Markvardsen *et al* 2012; Klöckner *et al* 2013). In almost all cases, the less-invasive method provided reliable and accurate measurements of the blood parameters of interest. Furthermore, Arnold *et al* (2008) showed that mean corticosterone levels in blood samples taken from trapped birds via a needle within 3 min of capture were strongly correlated with those of samples taken via the bug method. In addition, a study with rabbits (*Oryctolagus cuniculus domesticus*) demonstrated that the bug method induced a lower level of stress on animals than a conventional bleeding method (Voigt *et al* 2004).

The first goal of our study was to test for a potential higher secretion rate of glucocorticoids shortly after bleeding mice conventionally or minimally invasively using bugs. Our second goal was to compare the adrenocortical response of animals among all three blood collection techniques. To this end, we compared the hormonal stress response after applying one of three bleeding techniques in laboratory mice: two conventional methods using surgical instruments (scalpel and medical needle) and the minimally invasive technique using larval instars of blood-sucking *Dipetalogaster maximus*. We used corticosterone metabolite concentrations in faecal samples as an indicator for the stress response of study animals. The determination of hormone levels in faecal samples offers a completely non-invasive method for monitoring stress hormones because faeces can be collected repeatedly without causing disturbance to the animals (Palme 2005; Touma & Palme 2005). The faecal metabolites reflect the release and elimination of hormones over several hours. We expected to measure a lower corticosterone metabolite concentration in faeces of laboratory mice when exposed to the bug method than when exposed to the conventional bleeding methods.

Materials and methods

Study animals and general housing conditions

We used 36 adult female C57BL/6CrlN mice (*Mus musculus f. domesticus*), aged 4–6 months, obtained from Charles River GmbH (Sulzfeld, Germany) and maintained them at the animal facilities of the Institute for Farm Animal Research of the Humboldt University, Berlin. The mice were taken from their mothers at 21 days of age and housed in groups of five in standard Macrolone type III cages (380 × 220 × 150 mm [length × width × height], EBECO, Castrop-Rauxel, Germany) with sawdust as bedding material (Lignocel, J Rettenmaier & Söhne GmbH & Co KG, Rosenberg, Germany). During the experiment, mice were separated and housed individually in Macrolone type II cages (265 × 205 × 140 mm). Each cage was equipped with a house (EBECO, Castrop-Rauxel, Germany) and bedding material (Nestlets EBECO, Castrop-Rauxel, Germany). The house was fixed by placing it on top of a sealable EuroPlus Basic18 plastic box (180 × 149 × 40 mm), Allit AG Rotaly-Mühle, Germany (see Figure 1). The lid of this box had a window the size of the basal surface of the house and this window was covered with metal gauze (mesh size: 2 mm). The plastic box served as a hide for the blood-sucking bugs from which they could sting with their proboscis (Voigt *et al* 2006) and suck blood when mice used the house for resting and sleeping. Food (Sniff M-Z Spezialdiäten GmbH, Soest, Germany) and water was available *ad libitum*.

The blood-sucking bugs (larval instar 2, *D. maximus*, *Reduviidae*, Heteroptera) derived from our laboratory-bred colony at the Leibniz Institute of Zoo and Wildlife Research (Berlin, Germany). Bugs were maintained on a diet of rabbit blood that was offered from plastic dispensers at 3–4 week intervals. To avoid contamination with bugs' haemolymph we used exclusively 'empty' bugs which had been starved for a period of eight weeks prior to the start of the experiment (Lehane 2005; Thomsen & Voigt 2006). After the end of the experiments, both bugs and mice were used for breeding.

Figure 1

Modified house in a standard rodent cage. The house was put on top of a plastic box that was covered by a screen of metal gauze. A blood-sucking bug was placed in the plastic container under the screen so that it could sting and suck blood from the mouse (female C57BL/6CrlN, *Mus musculus f. domesticus*) when it used the house for resting and sleeping.

Bleeding techniques

We started to bleed the mice four days after they were introduced to the experimental cages. Blood was collected by two conventional and one alternative method. Each day around 0800h experiments began. During conventional bleeding, we took blood samples either from the orbital sinus (OS) of anaesthetised mice or from the tail vein (TV) of mice without anaesthesia, in accordance with the rules and regulations of the German Society for Laboratory Animal Science (GV Solas 2009). During alternative bleeding, we took blood samples using a blood-sucking bug (B). The sequential order of the bleeding techniques was chosen randomly for each individual and approximately the same amount of blood volume was taken from each individual. The mice were allowed to recover from bleeding by maintaining them for seven days without any handling.

To collect blood from the orbital sinus, a mouse was placed under an inhalation mask (Jorgen Kruuse A/S, Langeskov, Denmark) with an influx of the anaesthetic gas isoflurane (5% volume isoflurane in pure oxygen, Linde AG, Munich, Germany) that caused a brief general anaesthesia. The anaesthetised mouse was fixed by neck grip and a Pasteur pipette (diameter: 1 mm) was introduced under slight pressure and rotating movements into the corner of the eye socket underneath the eyeball. To collect blood from the tail vein, a mouse was fixed mechanically into a restraining tube (diameter: 4 cm). The tail was stretched and the ventral tail vein scratched open with a small cut using a sterile scalpel. By gentle manual pressure on the tail, droplets of blood were extracted from the vein.

To collect blood from the mouse using reduviid bugs we placed two starved bugs into the plastic box. We left the bugs in the plastic container for a period of up to 5 h and checked at 15-min intervals, from a distance, whether one of the bugs obtained blood during this period. More than 80% of the successful bugs finished their blood meal within 2 h of introduction. Reduviid bugs were weighed before and after their blood meal to estimate the amount of blood ingested.

Table 1 Mean (± SD) concentration of corticosterone metabolites (ng 0.05 g^{-1} faeces) of all three days (–1, 0, 1) and all three bleeding methods.

Method	Day	Corticosterone metabolites (ng 0.05 g^{-1} faeces)	SW-test	*F*-value	*P*-value	Bonferroni test	*P*-value
OS	–1	98.9 (± 42.6)	0.154			–1 vs 0	< 0.0001
	0	141.4 (± 53.2)	0.464	17.2	< 0.0001	–1 vs 1	0.928
	1	99.4 (± 45.6)	0.063			1 vs 0	0.001
TV	–1	102.6 (± 56.1)	0.625			–1 vs 0	0.008
	0	147.0 (± 56.7)	0.202	10.7	< 0.0001	–1 vs 1	0.552
	1	95.3 (± 40.6)	0.826			1 vs 0	< 0.0001
Bug	–1	74.4 (± 25.0)	0.194			–1 vs 0	0.007
	0	95.9 (± 47.9)	0.074	10.5	< 0.0001	–1 vs 1	0.101
	1	63.3 (± 28.7)	0.083			1 vs 0	0.001

OV: puncture of orbital veins; TV: tail-vein puncture; B: bug method. Comparison of the concentrations from the days within each method (rm-ANOVA: F_2, *P*-values). *P*-values for the Shapiro-Wilk normality tests are given in the column marked SW-test.

Comparison of stress response among treatments

We quantified the level of stress by measuring the concentration of corticosterone metabolites in faecal samples collected one day prior to bleeding (day –1), 8–11 h after bleeding (day 0), and one day after bleeding (day 1) always at the exact same time of day. For collection of faecal samples, mice were placed in extra cages with less bedding material 8 h after blood sampling. After 3 h, samples were collected and stored at –20°C overnight and at –80°C until further analysis. Faecal samples were extracted using an 80% methanol solution (0.05 g faeces in 1 ml 80% methanol) as described in Touma *et al* (2004). Extracted samples were sent to the Institute of Medical Biochemistry at the University of Veterinary Medicine in Vienna, Austria.

The amount of faecal corticosterone metabolites was determined using a previously established 5α-pregnane-3β,11β,21-triol-20-one EIA, which proved to be suitable to assess adrenocortical activity in mice (Touma *et al* 2003, 2004). This EIA utilises an antibody measuring steroids with a 5α-3β,11β-diol structure. The antibody has been shown to cross-react with various steroids (Steraloids, Wilton, NH, USA) as follows: 5α-pregnane-3β,11β,21-triol-20-one (100%); 5α-pregnane-3β,11β,20β,21-tetrol (110%); 5α-pregnane-3β,11β,17α,21-tetrol-20-one (45%); 5α-androstane-3β,11β-diol-17-one (230%). Cortisol, corticosterone or metabolites, which differed at one of the three recognised positions (5α, 3β- or 11β-ol), showed cross-reactivities of less than 1%. If more positions differed, the cross-reactivity was even less (< 0.1%). All tested gonadal steroids, such as progesterone, androstenedione and dehydroepiandrosterone, or their reduced metabolites (eg 5α/β-pregnane-3β-ol-20-one; 5α/β-androstane-3β,17β-diol; 5α/β-androstane-3β-ol-17-one), cross-reacted less than 1%. In our study, the intra- and inter-assay coefficients of variation were 9.6 and 14.4%, respectively.

Statistical analysis

To exclude any possible bias from age-related changes in glucocorticoid baseline and stress responses and from repeated but unsuccessful bleeding events by the bug, we only considered and compared data that were taken within a three-week period. This decision was based on the observation that some mice did not become accustomed to the house in their cage, ie they would not rest in the house where the bug was waiting in the hide. Since the bugs' repeated attempts to consume blood from these mice delayed the procedure and since repeated stinging and time delay can cause age-related and/or sequential effects on chronic stress in mice, we introduced a limit of three weeks for conducting all three bleeding methods within the same individual. Consequently, we reduced the overall dataset to those of 16 mice where all bleeding methods were successful within three weeks.

To test for normal distribution we used the Shapiro-Wilk test. To test for sphericity we applied the Mauchly test. All tests were two-tailed with alpha set at 0.05 and run in SPSS (version 8.0 for windows) and SYSTAT. All data sets were normally distributed. Thus, we used an analysis of variance with repeated measures design (rm-ANOVA), followed by pair-wise *t*-tests to test whether the faecal corticosterone values differed among day –1, day 0 and day 1 and if bleeding techniques had an effect on absolute corticosterone secretions on day 0. In order to control for possible difference in baseline corticosterone values, we also calculated relative changes in faecal corticosterone metabolite values based on the difference between day –1 and 0. Relative increases in faecal corticosterone metabolites' concentrations were compared using paired Student's *t*-tests.

Results

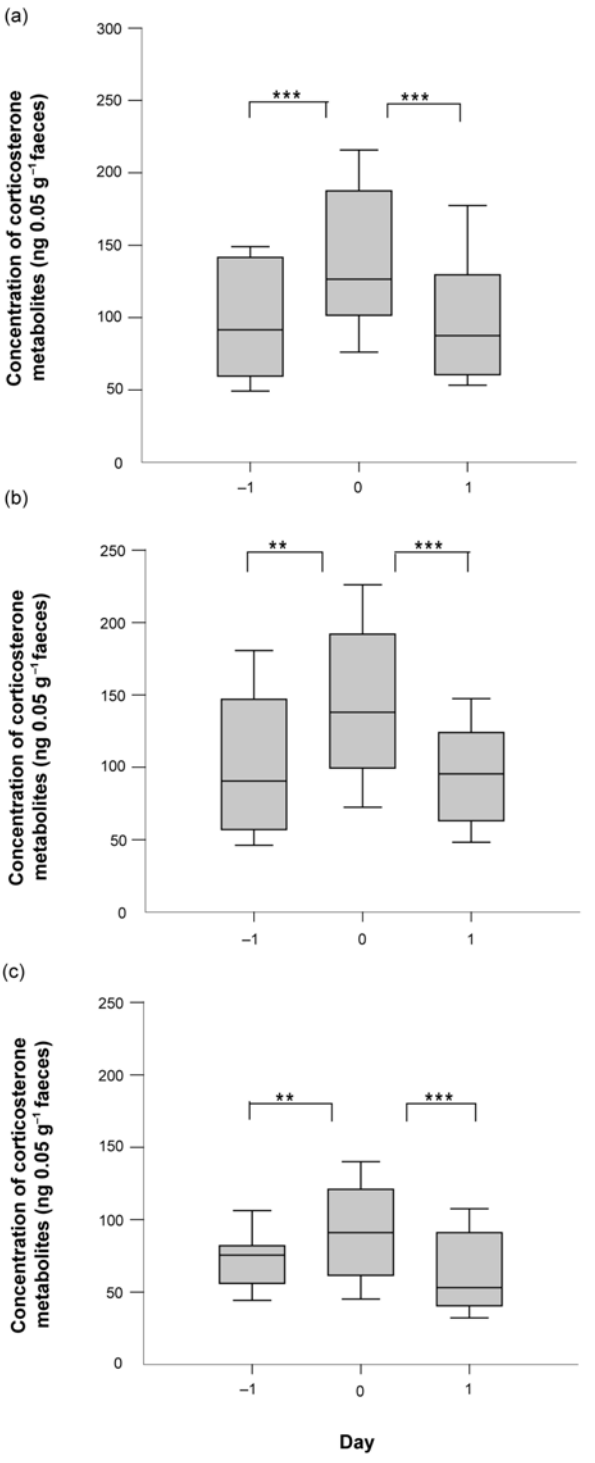
Using the retro-orbital bleeding technique, we obtained approximately 50 µl of blood from each of the 16 mice. The duration of the retro-orbital bleeding averaged 2.9 (± 1.2) min. Tail-vein puncturing yielded, on average, only 10 µl of blood and required 1.9 (± 1.0) min. On average, bugs each ingested about 90 µl of blood. In order to assess the hormonal stress response, we collected faecal samples from all mice and then compared corticosterone metabolite concentrations one day before, shortly after and one day after the bleeding events. Faecal corticosterone metabolite concentrations differed among the three subsequent days (day –1, day 0, day 1) in each of the treatments (puncture of orbital sinus [OS]: $F_{2, 30} = 17.2$, $P < 0.0001$; tail-vein puncture [TV]: $F_{2, 30} = 10.7$, $P < 0.0001$; bug method [B]: $F_{2, 30} = 10.5$, $P < 0.0001$; Table 1, Figure 2), ie from day –1 to day 0 we observed a significant increase and from day 0 to day 1 a significant decrease in faecal glucocorticoids. Between days –1 and 1, we could not detect any significant changes ($P > 0.05$; Table 1, Figure 2).

Comparisons of the absolute faecal corticosterone metabolite concentrations of day 0, the day of bleeding, revealed significant differences in stress responses among the bleeding techniques ($F_{2, 30} = 8.1$; $P = 0.001$), ie average corticosterone metabolite concentrations were lower when mice were bled with bugs than when bled by OS puncture ($P = 0.012$) or TV ($P = 0.001$). After bleeding at day 0, the concentrations of corticosterone metabolites were not significantly different between the two conventional bleeding techniques ($P = 0.673$). Although baseline values of faecal corticosterone metabolites at day –1 did not differ between treatments (Kruskal Wallis test: KW = 3.46, $P = 0.1772$), we calculated relative changes in faecal corticosterone metabolites between treatments to compare relative increases in faecal corticosterone metabolites among the three treatments. Pair-wise comparisons of relative differences between treatments showed no difference in stress hormone increase between conventional methods ($t_{15} = 0.12$, $P = 0.19$), and trends for differences in pair-wise comparisons between the conventional methods and the bug method (both $t_{15} = 1.8$, $P = 0.095$).

Discussion

The improvement and refinement of bleeding small laboratory animals is actively pursued in animal welfare research (Parasuraman *et al* 2010). In this study, we compared the intensity of the hormonal stress response in laboratory mice when exposed to three different bleeding techniques. Blood sample collection was carried out by either puncturing the orbital sinus, incision of the tail vein or through the use of blood-sucking bugs. To monitor adrenocortical activity, we applied the non-invasive technique of measuring corticosterone metabolites in faecal samples (Touma & Palme 2005). Firstly, we found an increase in glucocorticoid secretion from day –1 to day 0 followed by a decrease from day 0 to day 1 in all three treatments, indicating that mice were exposed to some stressor. Secondly, we found no differences in concentrations of absolute faecal corticosterone metabo-

Figure 2



Corticosterone metabolite concentration in faecal samples (ng 0.05 g^{-1} faeces) before (day –1), 8–11 hours after (day 0) and 24 h (day 1) after applying one of three bleeding techniques to 16 laboratory mice for (a) puncturing the orbital sinus following chemical immobilisation, (b) puncturing of the tail vein following mechanical immobilisation and (c) blood-sucking of reduviid bug from unrestrained mice. The upper and lower margins of the box plots indicate the 25th and 75th percentiles and the whiskers the 5th and 95th percentiles. The solid line within the box represents the median value. Horizontal lines above boxes indicate significant differences: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

lites between the two conventional methods, but significant differences in absolute faecal corticosterone metabolites between the conventional methods and the bug method, ie absolute concentrations of corticosterone metabolites following bleeding with conventional techniques were significantly higher than after bleeding mice by using bugs. For relative faecal corticosterone metabolites (relative increases of faecal corticosterone metabolites between day –1 and 0 of treatment), we found a trend for differences between conventional techniques and the bug method, but not between the two conventional methods. The absence of a significant difference in stress hormone response between conventional techniques and the bug method may have been caused by introducing additional variation of baseline values into the data set, when subtracting values of day 0 from those of day –1. Alternatively, baseline samples may have been slightly lower, albeit not statistically detectable, during the bug treatment than during the conventional treatment. Yet, this is unlikely, since faecal glucocorticoid concentrations are known to show diurnal fluctuations (Touma *et al* 2004), ie an increase towards the end of the day. Since we had to accept delays in the bug bleeding event compared with the conventional bleeding treatment, we would have expected a higher faecal glucocorticoid concentration in the bug treatment because of the additive effects of experimental stressor (bug) and increased baseline levels of faecal glucorticoid levels caused by diurnal fluctuation. Yet, the hormonal stress response of the bug treatment was lower than the response after the conventional treatments. Therefore, our conclusion that bugs cause a milder hormonal stress response in mice than the conventional OS and TV bleeding techniques is conservative and most parsimonious.

Previous work in rodents utilising conventional bleeding techniques have shown behavioural and physiological stress responses occur not only due to the (probably painful) bleeding procedure itself but also to catching, restraining and anaesthesia (Gärtner *et al* 1980; Vahl *et al* 2005). Therefore, the unavoidable necessity of handling the mice in order to apply conventional bleeding techniques should have imposed most stress on the mice in our study as well. However, bleeding animals with bugs may also cause a degree of stress to the animals in question. For example, the experimenter manipulated the rodent cage during the experiment when lifting the lid of the cage, placing the bug into the hide and retrieving it after the blood meal. Therefore, even though mice were not handled directly when bled by blood-sucking bugs, they may have been exposed to a stressor of some description due to these manipulations. This might have caused a mild stress response in the experimental animals as indicated by the slight increase in faecal glucocorticoid levels between day –1 and day 0 when bugs were introduced to the mice cage. Overall, we cannot exclude the possibility that the observed hormonal stress response in mice bled via bugs is solely attributable to the disturbance inside the home cages (Gärtner *et al* 1980) and not necessarily to the bleeding, ie to the sting and blood sucking of the bug, itself. In order to disentangle the effect of bleeding via bugs and the handling of cages on the hormonal stress response of laboratory animals, future tests should focus on whether laboratory animals exhibit the same hormonal stress response when cages are only handled or cleaned, without the involvement of any bleeding via blood-sucking bugs.

A disadvantage of the alternative bug method is the extended duration required to obtain a significant amount of blood from animals in question. Occasionally, bugs required several attempts to suck blood successfully, for example when mice did not rest inside their house for an extended period. Then, bugs may require up to or beyond 20 min to finish a blood meal (Voigt *et al* 2004; Thomsen & Voigt 2006). An improved hide, however, could increase the efficiency of bleeding via bugs. For example, the level of disturbance could be reduced when bugs are introduced and removed from the outside without manipulating the cage. This would be easily achievable with a modified cage containing a retractable drawer at one of the side walls. Such applications are already successfully in use to bleed various animal species, eg in mouse lemurs (*Microcebus murinus*) (Thomsen & Voigt 2006), lynx (*Lynx pardinus*) (Braun *et al* 2009) and domestic cats (*Felis sylvestris catus*) (Thomsen unpublished data) and should, thus, be applicable to mice as well.

Besides the benefit of minimising stress for the animals in question, two main advantages of the bug method are the avoidance of injuries and scars, allowing repeated bleeding from the same individuals over a prolonged period of time and the possibility of defining precisely the amount of blood obtained with a blood-sucking bug by choosing the adequate larval instar (Helversen *et al* 1986; Thomsen & Voigt 2006). The latter benefit is of particular significance since small animals may easily die from overly bleeding.

Animal welfare implications

Summarising, from the perspective of hormonal stress response and animal welfare, we consider the bug method as an appropriate alternative for collecting blood from laboratory animals in a minimally invasive way if adequate cages are developed and appropriate validation experiments are performed for the type of measurements of interest.

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