General and Comparative Endocrinology 179 (2012) 436–450

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Hypothalamic–pituitary–adrenal (HPA) axis function in the California mouse (*Peromyscus californicus*): Changes in baseline activity, reactivity, and fecal excretion of glucocorticoids across the diurnal cycle

Breanna N. Harris [a,*], Wendy Saltzman [a], Trynke R. de Jong [a,b], Matthew R. Milnes [c,d]

[a] Department of Biology, University of California, Riverside, CA 92521, USA
[b] Department of Behavioral and Molecular Neurobiology, University of Regensburg, Regensburg, Germany
[c] San Diego Zoo Institute for Conservation Research, Escondido, CA 92027, USA
[d] Department of Natural Science, Mars Hill College, 100 Athletic St., Mars Hill, NC 28754, USA

ARTICLE INFO

Article history:
Received 6 April 2012
Revised 7 July 2012
Accepted 13 August 2012
Available online 28 September 2012

Keywords:
HPA axis
Peromyscus californicus
Stress
Corticosterone
Fecal glucocorticoid metabolites
Diurnal rhythm

ABSTRACT

The California mouse, *Peromyscus californicus*, is an increasingly popular animal model in behavioral, neural, and endocrine studies, but little is known about its baseline hypothalamic–pituitary–adrenal (HPA) axis activity or HPA responses to stressors. We characterized plasma corticosterone (CORT) concentrations in *P. californicus* under baseline conditions across the diurnal cycle, in response to pharmacological manipulation of the HPA axis, and in response to a variety of stressors at different times of day. In addition, we explored the use of fecal samples to monitor adrenocortical activity non-invasively. California mice have very high baseline levels of circulating CORT that change markedly over 24 h, but that do not differ between the sexes. This species may be somewhat glucocorticoid-resistant in comparison to other rodents as a relatively high dose of dexamethasone (5 mg/kg, s.c.) was required to suppress plasma CORT for 8 h post-injection. CORT responses to stressors and ACTH injection differed with time of day, as CORT concentrations were elevated more readily during the morning (inactive period) than in the evening (active period) when compared to time-matched control. Data from ^{3}H-CORT injection studies show that the time course for excretion of fecal CORT, or glucocorticoid metabolites, differs with time of injection. Mice injected in the evening excreted the majority of fecal radioactivity 2–4 h post-injection whereas mice injected during the morning did so at 14–16 h post-injection. Unfortunately, the antibody we used does not adequately bind the most prevalent fecal glucocorticoid metabolites and therefore we could not validate its use for fecal assays.

1. Introduction

The hypothalamic–pituitary–adrenal (HPA) stress response has been characterized in many vertebrates [31,38] and is activated when an organism is presented with an actual or potential threat, resulting in the release of glucocorticoids (GCs) from the adrenal cortex. The principal glucocorticoid produced by humans and other primates is cortisol, whereas many rodents, including *Peromyscus californicus*, primarily produce corticosterone [64]. Despite the fact that GCs are often referred to as "stress hormones", the HPA axis is continuously active and GCs at baseline levels have important daily functions [72]. Baseline levels of GCs exhibit a sinusoidal pattern over the course of the day, with the highest concentrations occurring just prior to the onset of waking in most species [reviewed by 33,54,57,58]. Glucocorticoids help organisms respond to and recover from stressors, and aid in regulation of inflammation and immune function, gluconeogenesis, brain function, cardiovascular activity, various behaviors, and numerous other processes [19,24,25,60,69,72,77,78].

The California mouse, *P. californicus*, is a monogamous, biparental rodent that is becoming an increasingly popular animal model. Not only is this species useful for studying parental behavior and its neural and hormonal correlates [4,7,12,29,30,41,55,84], but it is also used in studies on aggression [6,76,85,86] and immune function [36,61,62], as well as in studies investigating relationships between stress and behavior [3,15,46,87]. California mice survive and breed well in captivity, and parental behavior by both sexes has been well characterized [6,12,29,30,39]. Since both males and females invest heavily in their offspring [40,42], *P. californicus* provides a valuable model for studying the effects of stress on parental care, as well as the effects of parental status on stress responsiveness in both mothers and fathers.

Despite the increasing use of California mice in behavioral, neural, and endocrine studies, little is known about their baseline HPA activity or their HPA response to stressors. Although

* Corresponding author. Fax: +1 951 827 4286.
E-mail address: bharr002@ucr.edu (B.N. Harris).

0016-6480/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2012.08.026

many aspects of the HPA axis are conserved across vertebrates, several parameters, including circulating GC concentrations, circadian dynamics, and temporal responses to and recovery from stressful stimuli, differ markedly among species [9,28,34,54,68,95] and even within strains of a single species [32,73]. Thus, before we are able to fully understand and interpret behavioral or physiological data in the context of stress in California mice, we must first characterize normative activity and reactivity of the HPA axis.

Determination of circulating GC concentrations via plasma or serum assay provides the most direct measure of GC levels available to the tissues and can provide important information on immediate HPA responses to perturbations; however, this approach presents several problems. Handling and blood collection are stressful to many animals and can alter GC levels, as well as other physiological and behavioral measures, over the short-term and potentially the long-term, which complicates characterization of basal GC levels. Collection of repeated blood samples is compromised by the small body size of many animals and the limited volume of blood that can be removed without adverse effects. To avoid these problems, many researchers are turning to fecal hormone assays, especially in small and/or free-living animals. Fecal hormone levels are not as sensitive to minor disturbances as are hormone concentrations measured in the circulation, and reflect circulating hormone levels over a period of hours due to the processes of metabolism and excretion [81–83]. Fecal measures can thus provide more time-integrated hormone data, thereby yielding a more comprehensive measure of hormone levels over a period of time [see 63]. Glucocorticoids or their metabolites have been measured from fecal samples in numerous mammals, birds and reptiles [13,37,52,63,82,92]; however, proper and extensive assay validation for each species is essential to confirm that biologically relevant GCs or GC metabolites can be measured accurately [63,66,81–83]. Although fecal GC measures in California mice have been reported previously [3], a fecal GC assay has not been validated for this species, and GC or GC metabolite excretion patterns have not been characterized.

The primary objectives of this study were to characterize plasma corticosterone (CORT) concentrations in *P. californicus* (1) under baseline conditions across the diurnal cycle, (2) in response to pharmacological manipulation of the HPA axis, and (3) in response to a variety of stressors at different times of day. In addition, we explored the use of fecal samples as a means to monitor adrenocortical activity non-invasively in this species.

2. Materials and methods

2.1. Animals

We used California mice that were born and maintained in our breeding colony at the University of California, Riverside. They were descended from individuals purchased from the Peromyscus Genetic Stock Center, University of South Carolina (Columbia, SC) in 2007. The colony was kept on a 14:10 light:dark cycle, with lights-on at 0500 h and lights-off at 1900 h. Ambient temperature was approximately 23 °C with humidity of about 65%. Mice were housed in standard shoe-box-style, polycarbonate cages ($44 \times 24 \times 20$ cm) lined with aspen shavings; cotton wool was provided for nesting material. Food (Purina 5001 rodent chow) and water were provided *ad libitum*. Cages were cleaned once per week unless otherwise noted. In our colony, siblings are never mated with one another, and first-cousin matings are avoided whenever possible. Animals were weaned at 27–32 days of age (prior to the birth of younger siblings), ear-punched for individual identification, and housed in same-sex groups of 2–4 mice. Mice either remained in same-sex groups or were paired with an individual of the opposite sex after 90 days of age.

We used a total of 147 mice (69 males and 78 females) from either same-sex (virgin) or male–female pairs. We were very interested at obtaining basic information about the HPA axis in a representative sample of California mice as this species is becoming increasingly popular animal model. Additionally, data from our lab show that baseline CORT concentrations do not differ between males and females (see Section 3.1), and that baseline and stress-induced CORT levels do not vary across differing reproductive conditions [15;unpub.data] or adult ages [unpub. data]. Therefore, mice of both sexes and of various ages and reproductive conditions were used for our HPA-characterization experiments; whenever possible, however, we avoided the use of pregnant and possibly pregnant females (6 out of 78 females were housed with a reproductively mature adult male, and might have been pregnant during testing). Additionally, some mice were used for more than one data set (diurnal rhythms, stress tests, pharmacological manipulation, or fecal collection); the mean ± SE number of data sets to which each mouse contributed data was 1.02 ± 0.05, range 1–3. Different experiments on the same animal were separated by at least 1 week to allow recovery. The sex, number, and housing condition of mice used for each experiment are listed in the description of each experiment, below. UCR has full AAALAC accreditation, and all procedures were approved by the UCR IACUC and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*.

2.2. Blood collection and analysis

Mice were anesthetized with isoflurane, and blood (70–140 μl) was collected from the retro-orbital sinus using heparinized microhematocrit tubes. Time from disturbance or end of the preceding test to collection of the blood sample was always less than 4.6 min (mean ± SE: 89 ± 2.61 s; range 22–279 s); 97% of samples were collected in under 3 min and 84% in 2 min or less. Blood samples were centrifuged for 12 min (13,300 rpm, 4 °C), and plasma was removed and stored at −80 °C until assay.

Plasma was assayed in duplicate for CORT using an ^{125}I double-antibody radioimmunoassay (RIA) kit (#07–120102, MP Biomedicals, Costa Mesa, CA) previously validated for this species [15]. Samples from each experiment were analyzed in the same assay if possible, or balanced evenly across multiple assays; however, an individual mouse's samples from a given experiment were always analyzed in a single assay run. The standard curve ranged from 12.5 ng/ml (91% bound) to 1000 ng/ml (20% bound), and plasma samples were assayed using dilutions ranging from 1:50 to 1:1600 depending on anticipated CORT concentrations. Inter- and intra-assay coefficients of variation (CVs) were 11.2% and 4.7%, respectively ($N = 45$ assays).

2.3. Characterization of diurnal rhythm

A total of 6 plasma samples (1200, 1600, 2000, 2400, 0400, 0800 h) were collected from 8 virgin male and 7 virgin female California mice (all housed in same-sex pairs) under undisturbed conditions to characterize the diurnal pattern of circulating CORT concentrations. At least 7 days elapsed between collection of successive samples from the same animal, and the order of sampling time points was approximately balanced across individuals within each sex.

2.4. Pharmacological stimulation of adrenal activity

We used adrenocorticotropic hormone (ACTH) to stimulate the production of CORT. Synthetic ACTH (Cosyntropin, Penn Veterinary, Lancaster, PA) was diluted to 25 μg/ml with sterile saline

(0.9%) immediately prior to use. Injection volumes ranged from 0.13 to 0.35 ml. The volume of saline injections (vehicle control) was adjusted for body mass in the same manner as ACTH. All injections were administered i.p. using a 27G needle.

In the first ACTH challenge, male mice ($n = 6$ per injection condition), either housed in same-sex or male–female pairs, were injected with either 100 μg/kg ACTH or an equivalent volume of sterile saline at 0900 h, and a blood sample was collected 1 h later. These mice were not used in any other experiments. The relatively high dose of ACTH was chosen to maximally or near-maximally stimulate the adrenal gland and was based off previous studies in rats [2,56]. We conducted a second ACTH challenge to elucidate temporal differences in the adrenocortical response to exogenous stimulation at times near either endogenous peak (evening) or nadir (morning) HPA activity. Based on response from the 100 μg/kg dose (see Section 3.2.1), mice in this experiment received two consecutive injections of either saline or ACTH (150 μg/kg). We chose this higher dose and double-injection protocol because we aimed to maximally stimulate the adrenal glands, and visual comparison with our stressor data (see Section 3.5.1) the 100 μg/kg does was not sufficient to produce maximal response. In order to maintain comparable groups, we repeated the AM injections with the new protocol. All mice ($n = 48$) used in the 150 μg/kg ACTH double-injection protocol were virgin females housed in same-sex pairs and had not been used in any prior experiments; however, 8 of them were later used in the DEX (see Section 2.5) experiments. The first set of mice was injected with either ACTH ($n = 8$) or saline ($n = 8$) at 0915 h and again at 0945 h. Half of the mice from each group underwent blood sampling 30 min after the second injection (1015 h; $n = 4$ per group), and the others were sampled 60 min after the second injection (1045 h; $n = 4$ per group).

The double-injection procedure was repeated in a different set of animals 3 h prior to lights-off to determine if the response to ACTH injection changed throughout the day. A total of 32 animals were injected with ACTH (150 μg/kg) or saline at 1600 h and then again at1630 h. Half of the mice in each injection group (saline or ACTH; $n = 8$ per group) underwent blood sampling at 1700 h (30 min after the second injection) and the remaining animals ($n = 8$ per group) had samples collected at 1730 h (60 min after the second injection). Plasma from all ACTH challenge trials was assayed for CORT as described above (see Section 2.2).

2.5. Pharmacological suppression of adrenal activity

Dexamethasone sodium phosphate (DEX, a synthetic glucocorticoid; American Regent, Inc., Shirley, NY) was injected i.p. using a 27G needle at 0.5, 5, or 10 mg/kg. The stock solution of DEX (4 mg/ml) was diluted with sterile saline prior to injection to concentrations of 0.1 mg/ml or 1 mg/ml, or left undiluted, respectively. A total of 24 mice housed in either same-sex (12 females and 10 males) or male–female pairs (2 males) were used for the DEX injection study; reproductive conditions were balanced across dosages when possible. Sixteen of the 24 animals were used in other experiments. On day 1 all mice underwent blood sampling at 1700 h for determination of baseline CORT levels, and were then weighed. On day 2 each animal received a DEX injection at one of the three doses ($n = 4$ males and 4 females per dose; injection volume ranged from 0.09 to 0.33 ml) at 0900 h. Blood was collected from each animal at 1700 h on day 2 (8 h post-injection), day 3 (32 h post-injection) and day 4 (56 h post-injection). Thus, each mouse underwent blood collection at each of the four time points, but was treated with only a single dose of DEX. Equipment failure occurred during processing of one male's 8 h post-injection sample for the 10 mg/kg group; therefore, that animal's data were omitted from the analyses.

2.6. Physical and environmental stimulation of adrenal activity

To quantify adrenal activity in response to a variety of challenges, we exposed mice to four commonly used laboratory stressors [47]: predator urine, forced swim, restraint, and shaking. All four stimuli were presented once in the morning at 0755–0945 h (lights-on, inactive period) and once in the evening at 1930–2100 h (lights-off, active period), in order to determine how the HPA response to stressors changes throughout the day. For forced swim, restraint, and shaking stress data, some mice were used in two of the three experiments; all mice were given at least 7 days to recover between testing.

2.6.1. Predator-urine exposure

Predator urine (fox and bobcat) was purchased from Maine Outdoor Solutions (Hermon, ME). Pilot studies and previous research from our lab suggest that fox and bobcat urine both elicit a rise in plasma CORT and that response magnitude does not differ between odor types [15; unpub. data]. Odor testing is described in detail elsewhere [15]. Briefly, mice were taken to a procedure room and placed individually in clean cages identical to their home cage but containing no nesting cotton, food or water. A cotton ball soaked with 1 ml predator odor was placed in a shallow plastic cup, and the cup was placed in the cage for the allotted time. The cup and urine-soaked cotton ball were then removed from the cage and from the testing room. A time-course study was conducted following 8 min of fox-urine exposure beginning at 0755 h (during lights-on). Blood samples were collected from different mice ($n = 4$ per time point; 12 females and 4 males, housed in same-sex (8 females) or male–female pairs (4 females, 4 males)) at 1, 15, 30, and 60 min following the end of urine exposure; 0800 h samples from the diurnal-rhythm study (see Section 2.3) were used as undisturbed control values for comparison. These mice were not used in any other experiments.

A lights-off trial was conducted following 5 min of bobcat-urine exposure beginning at 1930 h. A blood sample was collected from each mouse ($n = 8$; 5 males (all pair-housed with females) and 3 females (pair-housed with a male ($n = 2$) or female ($n = 1$)) immediately following exposure to the urine; 2000 h samples from the diurnal-rhythm study (see Section 2.3) were used as undisturbed control values for comparison. These mice were not used in any other experiments.

2.6.2. Forced swimming

Containers for swimming (1500 ml Vac-Rite beakers, Baxter, Deerfield, IL) were filled with approximately 850 ml of water (24–25 °C); this volume prevented mice both from touching the bottom of the beaker and from climbing out of the top. Mice ($n = 4$ per time point; all males, housed in either same-sex ($n = 6$) or male–female ($n = 10$) pairs, housing conditions balanced across time points) were transferred to a procedure room and placed in individual swim containers for 5 min, during which they were monitored closely. After the swimming session the mice were towel-dried and either underwent blood collection immediately (1–2 min post-stress) or were placed back in their home cage, which remained in the procedure room for 30 min, after which a blood sample was collected (30 min post-stress). The lights-on session began at 0845 h and the lights-off session at 2050 h; different groups of mice were used for the two times of day.

2.6.3. Restraint

Mice ($n = 4$ per time point; all males, housed in either same-sex ($n = 5$) or male–female ($n = 11$) pairs, with housing conditions balanced across time points) were taken to a procedure room and placed in a plastic DecapiCone (MDC-200, Braintree Scientific, Braintree, MA) for 5 min. The back portion of the DecapiCone

was folded around the tail and secured with a binder clip, or was held in place manually, in order to prevent movement. Upon termination of the procedure mice either underwent blood sampling immediately (1–2 min post-stress) or were placed back in their home cage, which remained in the procedure room, for 30 min and then sampled (30 min post-stress). The restraint stressor was performed during both lights-on (beginning at 0840 h) and lights-off (beginning at 2050 h), and different groups of mice were used for each time of day.

2.6.4. Shaking

Mice ($n = 4$ per time point; all males, housed in either same-sex ($n = 10$) or male–female ($n = 6$) pairs, with housing conditions balanced across time points) were placed individually in small plastic containers (approximate volume 700 ml), transferred to a procedure room and shaken on a lab rotator (Unico, Dayton, NJ) at a speed of 200 rpm for 5 min. Mice were then immediately taken for blood sampling (1–2 min post-stress) or placed back in their home cage, which remained in the procedure room, until blood collection (30 min post-stress). The stressor was administered both during lights-on, beginning at 0825 h, and during lights-off, beginning at 2030 h; different groups of mice were used for each time of day.

2.7. Corticosterone metabolism and excretion

2.7.1. ^{3}H-Corticosterone Injections

To examine the metabolism and time course of excretion of CORT in *P. californicus*, we followed the methods previously reported by Touma and colleagues (2003) for house mice (*Mus musculus domesticus*). Eight mice (4 males and 4 females; all virgin animals housed in same-sex pairs that had not been part of any other experimental procedure) were injected i.p. with ^{3}H-CORT (NET399250UC, Perkin Elmer, Waltham, MA). Each mouse received 100 μl of ^{3}H-CORT in saline, yielding a dose of 40 μCi (~37,000,000 cpm per injection). Beginning 3 h prior to injection, mice were individually housed in polycarbonate cages ($44 \times 24 \times 20$ cm) designed to facilitate collection of feces and urine. The cages did not contain bedding or cotton, and the cage bottom was lined with filter paper to absorb urine. A wire mesh grate (23G galvanized steel, 0.64 cm mesh) raised 2.5 cm above the bottom of the cage was placed on the filter paper so that fecal pellets would fall to the bottom of the cage. In order to determine if the time course of CORT metabolism and/or excretion changes over the course of the activity cycle, the 8 mice were split into two groups ($n = 2$ males and 2 females per group): one group was injected with ^{3}H-CORT at 0600 h (1 h after lights-on) and the second group was injected at 2000 h (1 h after lights-off); the two groups were injected on separate days.

For both groups, all fecal pellets and the filter paper that absorbed urine were collected every 2 h for the first 24 h following injection, and then every 12 h until 84 h post-injection. Collections that occurred during the dark phase were done using a red-light headlamp in order to minimize disturbance. During sample collection each mouse was placed individually into a plastic container while all fecal pellets were gathered from the cage and filter paper was replaced. Following the return of each mouse to its cage, fecal pellets collected from the cage and the temporary holding container were counted and combined as a single time-point sample. Fecal pellets and urine samples (filter paper) were stored at −20 °C until extraction and analysis. At the end of the experiment (84 h post-injection), mice were euthanized by pentobarbital overdose; thus, each mouse was used for only one injection.

2.7.2. Preparation and extraction of ^{3}H-corticosterone samples

All urinary and fecal steroid extractions, high-performance liquid chromatography, and radioimmunoassays were performed at the San Diego Zoo Institute for Conservation Research (Escondido, CA). Individual sheets of filter paper containing absorbed urine samples were placed on a UV light table to allow visualization of urine spots. Spots were traced with a pen, cut from the sheet, placed into scintillation vials along with 5 ml of scintillation cocktail, and then counted in a LS 6500 liquid scintillation counter (Beckman Coulter, Brea, CA). The percentage of ^{3}H-CORT and ^{3}H-CORT metabolites excreted in urine at each time point was calculated as the total amount of radioactivity recovered from the filter paper at each time point divided by the total radioactivity recovered in the urine across the entire 84-h collection period.

All fecal pellets collected from each mouse at a single time point were extracted and analyzed together in order to determine the total amount of ^{3}H-CORT and ^{3}H-CORT metabolites excreted in feces at each collection time point. Fecal samples were transferred to 150×16 mm glass tubes and extracted in 20 ml of 80% methanol by shaking for 60 min. After allowing the fecal pellets to settle to the bottom of the tube, the extract was drawn off and stored at −20 °C until further analysis. Total radioactivity at each time point was determined by transferring 1 ml of fecal extract to a scintillation vial along with 5 ml of scintillation cocktail, counting on a liquid scintillation counter, and multiplying the counts per minute by 20. The percentage of ^{3}H-CORT and ^{3}H-CORT metabolites excreted in feces at each time point was calculated as the total amount of radioactivity recovered at each time point divided by the total radioactivity recovered in the feces across the entire 84-h collection period.

2.7.3. High-Performance Liquid Chromatography (HPLC)

In an effort to characterize the major glucocorticoid metabolites present in *P. californicus* feces, fecal extracts from the ^{3}H-CORT-injected mice were analyzed by HPLC. A total of 8 fecal extract samples were chosen that corresponded to the time points that yielded the greatest amount of recovered radioactivity: the 4 and 14-h post-injection samples from the 2000 and 0600 h injections, respectively. Fecal extracts (1 ml) were dried down, reconstituted in water, and then passed through C18 cartridges (WAT051910 Sep-Pak, Waters, Milford, MA) that had been conditioned with 10 ml each of 100% methanol and then water. The samples were then washed with 10 ml of water, eluted from the cartridges with 5 ml of 100% methanol, dried down under vacuum, and reconstituted in 0.2 ml of 100% methanol.

Following solid-phase extraction, 20 μl of each fecal extract sample was injected into a Beckman System Gold 3-piece unit (Programmable Solvent Modules 125/406 and Diode Array Detector Module 186, Beckman Coulter) and separated on a Nova Pak C18 column (WAT086344, 3.9×150 mm, Waters). Samples were separated along an acetonitrile gradient beginning with 2:98 (acetonitrile:water, v/v) and increasing to 75:25 over 75 min at a flow rate of 1 ml per min. Fractions were collected at 1 min intervals; 250 μl of each fraction was added to 5 ml of scintillation cocktail and counted to determine the radioactivity of each fraction. The elution times of ^{3}H-labeled fractions were compared to the elution times of several commercially available (Steraloids, Newport, RI) steroids (corticosterone, cortisol, testosterone, estrone sulfate, and progesterone) and steroid metabolites (5β-androstane-3α-ol-11–17-dione and 5α-pregnane-3β,11β,21-triol-20-one) similarly prepared and fractionated by the same HPLC protocol.

2.8. Fecal glucocorticoid immunoreactivity

To determine the feasibility of noninvasive adrenal activity monitoring, we tested a fecal glucocorticoid RIA based on a commonly used [e.g., 5,14,37,45,92] and commercially available polyclonal antibody (rabbit anti-corticosterone, 07120113, MP Biomedicals, Costa Mesa, CA). Fecal samples were collected from two

female mice at 1600 h by gently holding the animals by the scruff until fecal pellets were produced. The fecal samples were immediately place in 1.5 ml vials and stored at −20 °C until extraction and assay. Non-radioactive fecal pellets were extracted as previously described (Section 2.7.2) with the following exceptions. Individual fecal pellets were lyophilized overnight and extracted in 1 ml of 80% methanol in 12 × 75 mm borosilicate tubes. Following 60 min of shaking, 0.9 ml of the extract was transferred to a clean tube, dried down under vacuum, and reconstituted in 1 ml of phosphate-buffered saline (PBS, pH 7.0). Duplicate aliquots of 100 µl were set aside from each sample for direct analysis by RIA, while the remaining extracts were combined and prepared for HPLC by solid-phase extraction as described above (Section 2.7.3). Duplicate aliquots of the combined and concentrated extract were fractionated by HPLC, resulting in 75 duplicate 1 ml fractions. Duplicate HPLC fractions were dried down and reconstituted in 1 ml PBS prior to analysis by RIA.

Serially diluted standards of 0.19–24 ng/ml corticosterone were prepared in PBS and run in duplicate in each RIA, along with quality control samples of approximately 0.4 and 4.0 ng/ml. To reduce nonspecific binding, 0.4 ml of 0.4% bovine serum albumin (Fraction V; Fisher Scientific, Pittsburgh, PA) in PBS was added to each tube. Tritiated CORT (1,2,6,7-^{3}H; Perkin Elmer, Waltham, MA) was added at 10,000 cpm per 0.1 ml. Finally, CORT antiserum was added in 0.1 ml of PBS at a dilution of 1:1200 to bring the final volume to 0.7 ml. Following an overnight incubation at 4 °C, bound-free separation was performed by adding 250 ml of 5% charcoal/0.5% dextran in PBS, incubating at 4 °C for 30 min, and centrifugating at 2000 x g for 15 min. The supernatant was combined with 3.5 ml of scintillation cocktail and counted on a Beckman LSC6500 scintillation counter. The concentrations of corticosterone standards were plotted against the log–logit transformation of the % ^{3}H-CORT bound. Immunoreactivity was expressed as mean pg/fraction.

2.9. Statistical analysis

All analyses were performed using SPSS 17.0 for Windows (IBM Corporation, Armonk, NY). Data were tested for normality and were visually inspected to ensure that results were not being driven by a small number of extreme values. All plasma CORT data were $\log_{10}$-transformed to meet normality assumptions; however, data are presented as non-transformed values, in the text and the figures, for ease of interpretation. Plasma CORT data were analyzed via independent-samples *t*-test, Pearson's correlation, ANOVA, or repeated-measures ANOVA. Post-hoc tests following ANOVA were either Fisher's LSD (for resolution of simple main effects, and for diurnal rhythm data), or were Sidak-corrected comparisons (all other post hoc analyses). All tests were two-tailed, and $P \leqslant 0.05$ was considered significant. ^{3}H-CORT results were analyzed using a mixed-model ANOVA so that animals with missing data (no fecal samples produced) could be included in the analysis. Akaike Information Criterion was used to determine which covariance structure provided the best fit [43,91]; compound symmetry, correlative compound symmetry, and the first autoregressive covariance structures were tested.

3. Results

3.1. Diurnal rhythm in plasma CORT concentrations

CORT data were analyzed via repeated-measures ANOVA, and Fisher's LSD post hoc tests were used to determine which time points differed from one another, due to our prediction that CORT would follow a daily cycle with highest levels occurring just prior to the onset of activity. Plasma CORT concentration (Fig. 1) varied dramatically throughout the day ($F_{5,65} = 75.840$, $P < 0.001$) but did not differ between the sexes ($F_{1,13} = 0.025$, $P = 0.876$), nor was there a time∗sex interaction ($F_{5,65} = 0.527$, $P = 0.755$). The highest plasma CORT levels occurred shortly after lights-off (1818.01 ± 159.63 ng/ml at 2000 h) and were significantly elevated compared to all other time points ($P \leqslant 0.007$ for each post hoc comparison), whereas the lowest CORT levels occurred at the beginning of the inactive period (39.94 ng/ml at 0400 h and 41.69 ng/ml at 0800 h). Plasma CORT concentration did not differ between the 0400 h and 0800 h samples ($t_{14} = 0.19$, $P = 0.853$), or between the 1600 h and 2400 h samples ($t_{14} = 1.04$, $P = 0.320$); as with 2000 h samples, the 1200 h time point (207.31 ± 65.18 ng/ml) was different from all other time points ($P \leqslant 0.001$ for each post hoc comparison).

To determine whether time elapsed from cage disturbance until sample collection had an effect on plasma CORT levels, Pearson's correlations were performed using seconds until sample collection and $\log_{10}$-transformed CORT concentration, for both the nadir (0800 h) and peak (2000 h) time points. Time from disturbance of cage until collection of blood sample (range: 60–168) was not significantly correlated with plasma CORT concentration at either time point (0800 h: $r = 0.036$, $P = 0.898$, $n = 15$; 2000 h: $r = 0.198$, $P = 0.480$, $n = 15$).

3.2. Plasma CORT response to ACTH challenge

3.2.1. 100 µg/kg vs. saline

Injection of 100 µg/kg ACTH i.p. at 0900 h significantly increased plasma CORT levels 1 h later when compared to saline injection (1255.57 ± 217.24 vs. 26.92 ± 7.03 ng/ml CORT, respectively; $t_{10} = 12.597$, $P < 0.001$; independent-samples *t*-test). Additionally, post-saline-injection CORT levels were lower than 0800 h baseline values from the diurnal-rhythm study (26.92 ± 7.03 vs. 41.69 ± 3.84 ng/ml, respectively; $t_{19} = 2.146$, $P = 0.045$).

3.2.2. 150 µg/kg vs. saline

CORT data (Fig. 2. A&B) were analyzed by two 2 × 2 ANOVAs, one for each time of day, with time post-injection (30 or 60 min) and treatment (ACTH or saline) as fixed factors.

3.2.2.1. Morning (0900h) injections. Plasma CORT levels were significantly higher at 30 min than at 60 min following the second ACTH injection ($F_{1,12} = 5.632$, $P = 0.035$). Additionally, ACTH-injected animals had higher plasma CORT levels than did saline-injected animals ($F_{1,12} = 74.541$, $P < 0.001$). There was no interaction between treatment and time ($P = 0.695$).

3.2.2.2. Afternoon (1600h) injections. In contrast to morning CORT levels, afternoon CORT levels did not differ significantly between ACTH- and saline-treated animals ($P = 0.234$) or between the 30- and 60-min time points post-injection ($P = 0.514$). Additionally, there was no interaction between treatment and time of sampling ($P = 0.877$).

3.3. Plasma CORT Response to DEX Challenge

Data were analyzed using repeated-measures ANOVA with day of sample (day 1, 2, 3, and 4) as a within-subjects factor and dose (0.5, 5, and 10 mg/kg) as a between-subjects factor (Fig. 3). Day of sample had a significant effect on plasma CORT ($F_{3,60} = 7.941$, $P < 0.001$), as did dose of DEX ($F_{2,20} = 10.554$, $P = 0.001$), and a day∗dose interaction ($F_{6,60} = 4.279$, $P = 0.001$) was observed.

Within treatment groups, 0.5 mg/kg DEX did not suppress plasma CORT levels at any time point measured, as plasma CORT levels did not differ across time ($P > 0.794$ for all post hoc comparisons). A

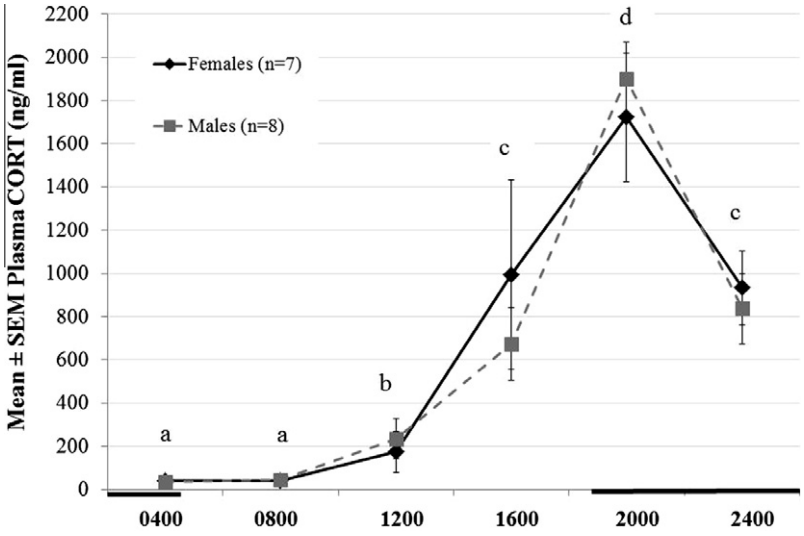

Fig. 1. Diurnal rhythm of plasma CORT concentrations in adult, same-sex-housed, virgin male ($n = 8$) and virgin female ($n = 7$) California mice. Data were $\log_{10}$-transformed for analysis but are presented as non-transformed values for ease of interpretation. CORT levels changed across time but did not differ between the sexes; main effect of time for both sexes combined is displayed on the graph. Points with different letters differ significantly from one another ($P < 0.007$). Horizontal black bars correspond to lights-off.

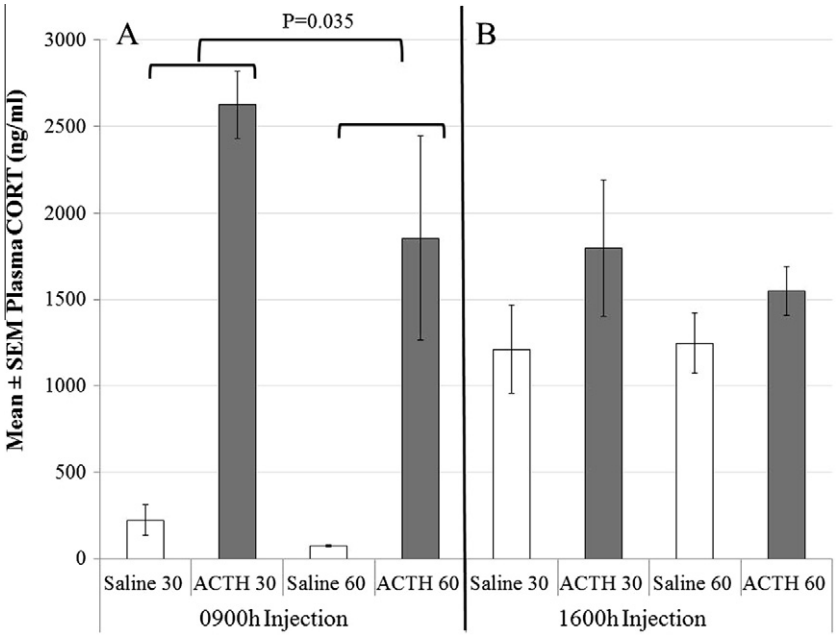

Fig. 2. Plasma CORT concentrations of adult, virgin, same-sex-housed female California mice at 30 or 60 min following the second of two successive injections of ACTH (150ug/kg) or saline administered at either 0900 h and 0930 h (A; $n = 4$ per time point) or 1600 h and 1630 h (B; $n = 8$ per time point) (lights-on: 0500–1900 h). Data were $\log_{10}$-transformed prior to analysis, but non-transformed data are presented for ease of interpretation. Injection of ACTH in the morning (A) significantly elevated plasma CORT levels at both 30 and 60 min compared to saline injection ($P < 0.001$), and CORT levels 30 min post-injection were higher than those 60 min post-injection ($P = 0.035$). ACTH injection did not produce a significant increase in CORT when administered in the afternoon (B).

dose of 5 mg/kg DEX caused a decrease in CORT 8 h post-injection compared to baseline levels ($t = 3.672$, $P = 0.009$); however, CORT concentrations at 32 h and 56 h post-DEX did not differ from baseline levels ($P = 0.929$ and $P = 0.105$, respectively). The 10 mg/kg DEX dose suppressed CORT at 8 h post-injection when compared to baseline ($t = 4.864$, $P = 0.001$), but, as seen with the 5 mg/kg dose, neither 32 h ($P = 0.992$) nor 56 h post-DEX values differed from baseline ($P = 0.999$).

On day 2 (8 h post-DEX) the 0.5 mg/kg DEX group had higher plasma CORT compared to the 5 mg/kg DEX group ($t = 3.778$, $P = 0.004$) and the 10 mg/kg DEX group ($t = 5.303$, $P < 0.001$); CORT levels did not differ between the 5 mg/kg and 10 mg/kg DEX groups ($t = 1.656$, $P = 0.303$). CORT concentrations did not differ significantly among the groups on day 1 (baseline; all pairwise P values > 0.8), day 3 (32 h post-DEX; $P > 0.6$), or day 4 (56 h post-DEX; $P \geqslant 0.25$).

3.4. Plasma CORT response to predator odor

Plasma CORT levels over time following exposure to predator odor during lights-on were analyzed using a one-way ANOVA (Fig. 4.). Time of sample post-exposure (baseline, 1, 15, 30, or 60 min) affected plasma CORT levels ($F_{4,26} = 17.883$, $P < 0.001$). CORT concentrations were significantly elevated above baseline at each time point following predator-odor exposure (1 min: $t = 4.570$, $P = 0.001$; 15 min: $t = 7.282$, $P < 0.001$; 30 min: $t = 4.361$,

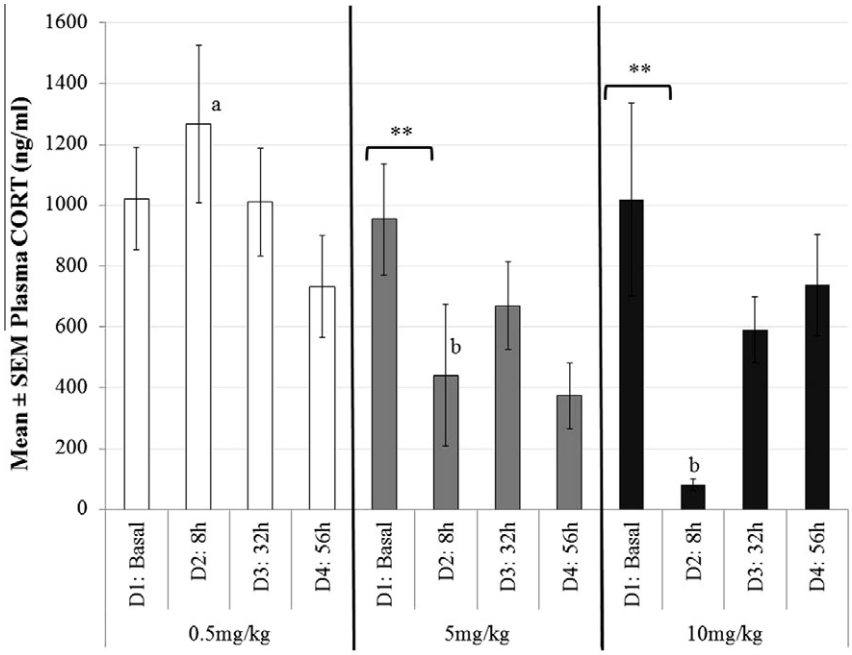

Fig. 3. Plasma CORT concentrations at 1900 h of adult male and female California mice at baseline (D1), and 8 (D2), 32 (D3) and 56 (D4) hours after DEX injection (0.5, 5 or 10 mg/kg) at 0900 h on D2. Data were $\log_{10}$-transformed for analysis, but non-transformed values are presented for ease of interpretation; CORT data from males and females are pooled (4 males, 4 females per dose except for the 10 mg/kg which had 3 males and 4 females). Both the 5 mg/kg and 10 mg/kg doses of DEX suppressed plasma CORT at 8 h after injection as compared to baseline, but not at 32 h or 56 h post-injection. CORT levels 8 h post-DEX did not differ between the 5 and 10 mg/kg doses, but both were significantly lower than CORT values 8 h after the 0.5 mg/kg dose (a vs. b; $P < 0.004$ after Sidak-corrected comparison). CORT concentrations did not differ among the three groups on D1, D3, or D4. $^{**}P < 0.01$ following Sidak-corrected comparisons after repeated-measures ANOVA.

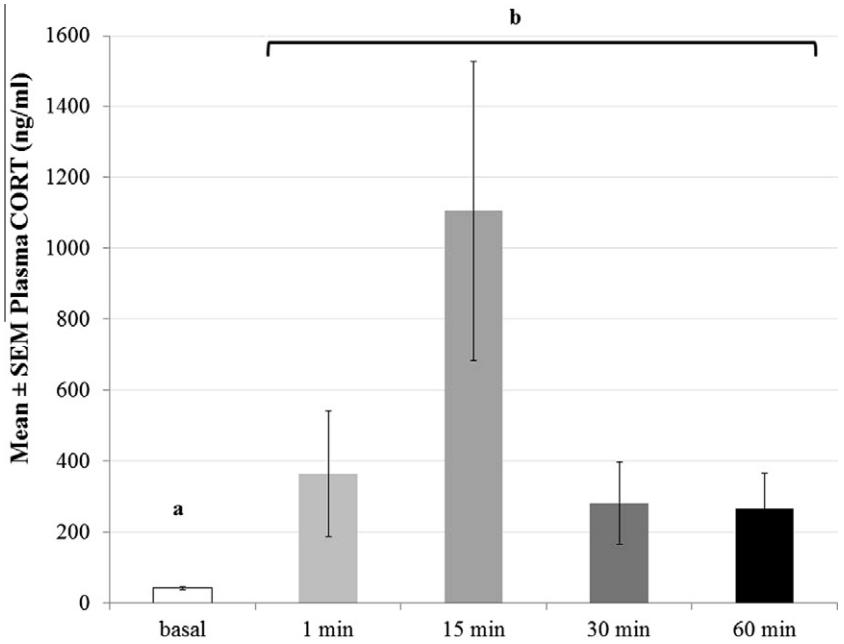

Fig. 4. Plasma CORT response of adult male and female California mice to predator odor exposure at 0800 h. Data for each post-stress time point comprise values from 4 individual mice, and sexes were pooled; basal values are from the diurnal-rhythm data set. Data were $\log_{10}$-transformed prior to analysis, but non-transformed values are presented for ease of interpretation. CORT concentrations did not differ across post-stress time points, but all post-stress values (b) were higher than baseline levels (a). Baseline vs. 1 min, $P = 0.001$; baseline vs. 15, min $P < 0.001$; baseline vs. 30 min, $P = 0.002$; baseline vs. 30 min, $P = 0.003$.

$P = 0.002$; 60 min: $t = 4.175$, $P = 0.003$). Plasma CORT levels peaked at 15 min post-stress and then declined at 30 and 60 min post-stress; however, CORT levels did not differ significantly between any of the post-stress samples ($P > 0.186$ for all post hoc comparisons).

A different set of animals was exposed to predator odor during lights-off (~2000 h). Data were analyzed via independent-samples *t*-test. Exposure to 1 ml of bobcat urine did not significantly elevate plasma CORT, 1–2 min post-stress, above baseline values collected from control mice at the same time of day (1818.01 ± 159.63 vs.

2281.14 ± 271.38 ng/ml CORT, respectively; $t_{21} = 1.36$, $P = 0.189$). Evening post-stress CORT levels were significantly elevated compared to morning (0800 h) baseline levels (independent-samples *t-test;* $t_{21} = 22.142$, $P < 0.001$) and, samples collected 1 min following evening predator-urine exposure contained higher levels of CORT than samples collected 1 min following morning predator-urine exposure (2281.14 ± 271.38 vs. 365.123 ± 177.17 ng/ml; $t_{3.32} = 4.096$, $P = 0.022$). Additionally, CORT concentrations following morning urine exposure (all time points combined, $n = 16$) were lower than evening (2000 h) baseline concentrations (504.36 ± 140.35 vs. 1818.02 ± 159.63 ng/ml; $t_{29} = 5.916$, $P < 0.001$). These data show that predator odor elicits a significant rise above baseline CORT concentrations in the morning, when endogenous CORT is low, but does not elevate CORT above baseline levels in the evening, when baseline levels are high. Additionally, post-exposure values in the evening were significantly higher than post-exposure values in the morning, and morning post-exposure levels did not reach evening baseline concentrations.

3.5. Plasma CORT responses to stressors during lights-on and lights-off

Plasma CORT responses to each stressor (restraint, shaker, forced swim) were compared between lights-on and lights-off using a 2 (time of day; 0800 h or 2000 h) × 3 (blood sample time; baseline, 1–2 min or 30 min) ANOVA; baseline values from the diurnal-rhythm data set (see Section 3.1) were used for comparison (Fig. 5). Analysis for each stressor type revealed significant effects of blood sample time ($F_{2,40} = 63.658$, restraint; $F_{2,40} = 56.369$, shaker; $F_{2,40} = 104.306$, forced swim; $P < 0.001$ for each analysis), time of day ($F_{1,40=}24.865$, restraint; $F_{1,40=}54.789$, shaker; $F_{1,40=}76.297$, forced swim; $P < 0.001$ for each analysis), as well as an interaction between the two ($F_{2,40=}126.925$, restraint; $F_{2,40=}83.562$, shaker; $F_{2,40=}63.187$, forced swim; $P < 0.001$ for each analysis). Samples collected in the evening contained higher CORT concentrations than those collected in the morning ($P < 0.001$ for each analysis). The results of the interaction between time of day and blood sample time for each stressor are presented below.

3.5.1. Forced swim

Morning post-stress CORT levels were higher than morning baseline values (1–2 min vs. baseline, $t = 12.48$, $P < 0.001$; 30 min vs. baseline, $t = 15.45$, $P < 0.001$). CORT levels did not differ significantly between the two morning post-stress time points, but tended to be higher 30 min post–stress than 1–2 min post-stress ($P = 0.068$). In the evening, CORT levels were significantly elevated above baseline at 1–2 min ($t = 2.83$, $P = 0.022$) but not at 30 min post-stress ($P = 0.666$); CORT concentrations did not differ between the two post-stress time points ($P = 0.412$). Samples taken 1–2 min following forced swim in the evening contained more CORT than those taken 1–2 min following forced swim in the morning ($t = 3.09$, $P = 0.004$); however, there was no difference between plasma CORT levels 30 min following forced swim in the morning and evening ($P = 0.491$).

To determine whether CORT levels following evening post-swim were indicative of maximal CORT output, we compared CORT concentrations from 1–2 min post-evening swim to those obtained 30 min following the second AM 150 μg/kg ACTH injection, as this was a very high dose of ACTH and maximal adrenal output may have been achieved. CORT levels 1–2 min post-swim in the evening were higher than those obtained by pharmacological manipulation (3729.27 ± 370.51 vs. 2625.62 ± 193.63, respectively; independent-samples *t*-test; $t_6 = 2.859$, $P = 0.029$).

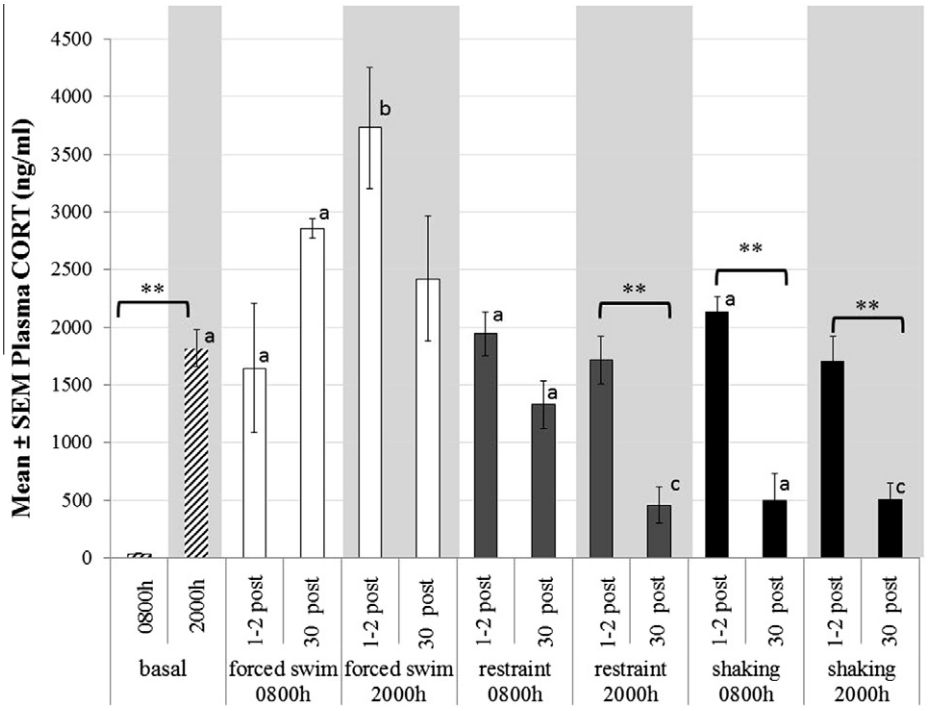

Fig. 5. Plasma CORT concentrations of adult male California mice ($n = 4$ per sample time) 1–2 and 30 min following exposure to a forced-swim, restraint, or shaking stressor at either 0800 h or 2000 h. Basal levels from the diurnal-rhythm study were used for comparison. Data were $\log_{10}$-transformed and analyzed via three 2x3 ANOVAs; however, non-transformed data are presented for ease of interpretation. Vertical gray bars signify lights-off time periods. Several post-stress means were significantly higher than 0800 h baseline levels (denoted by a), only one post-stress sample time point was higher than 2000 h baseline levels (denoted by b), and two post-stress samples were lower than baseline at the same time of day (denoted by c). Baseline CORT levels at 0800 h were lower than baseline levels at 2000 h; CORT levels decreased rapidly following shaking at both times of day, and following restraint at 2000 h. $^{**}P < 0.01$ following Sidak-corrected comparisons. In addition to differences shown on the graph, samples taken 1–2 min post forced-swim at 2000 h contained more CORT than did samples collected 1–2 min post forced-swim at 0800 h; and samples collected 30 min post-restraint at 0800 h had higher corticosterone than did samples from 2000 h.

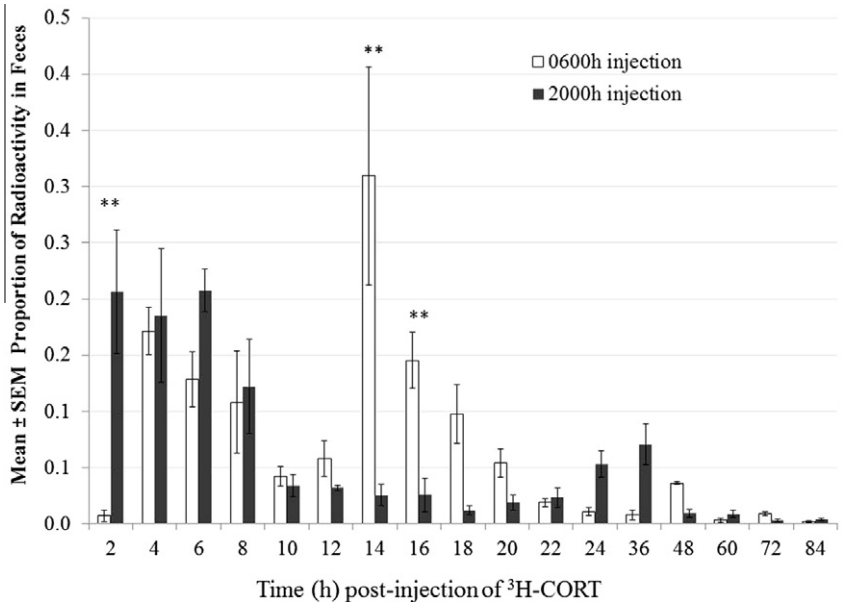

Fig. 6. Mean proportion of total fecal radioactivity excreted by adult male and female California mice (4 male, 4 female; half of which were injected at 2000 h and the other half at 0600 h) per time point; data from the sexes were pooled. Animals injected at 2000 h excreted more radioactivity 2 h post-injection but less radioactivity at 14 h and 16 h post-injection, compared to animals injected at 0600 h. $^{**}P < 0.01$, $^{*}P < 0.05$.

3.5.2. Restraint

In the morning, CORT was elevated above baseline at both 1–2 ($t = 15.28$, $P < 0.001$) and 30 min post-restraint ($t = 13.70$, $P < 0.001$), but did not differ between the two post-stress times ($t = 1.25$, $P = 0.521$). In the evening, CORT levels 1–2 min post-restraint did not differ from baseline ($P = 0.999$). Unexpectedly, CORT levels 30 min after restraint were lower than CORT levels both at the evening baseline ($t = 5.84$, $P < 0.001$) and at 1–2 min post-stress ($t = 4.65$, $P < 0.001$). CORT levels 30 min following restraint were lower in the evening than in the morning ($t = 3.79$, $P < 0.001$), whereas plasma CORT levels 1–2 min following restraint did not differ between the morning and evening ($P = 0.693$).

3.5.3. Shaking

In the morning, plasma CORT concentrations differed at all three time points (baseline, 1–2 min- and 30 min-post stress), with post-stress values being higher than baseline (baseline vs. 1–2 min: $t = 15.01$, $P < 0.001$; baseline vs. 30 min: $t = 8.52$, $P < 0.001$) and 1–2 min post-stress levels being higher than 30 min post-stress (1–2 min vs. 30 min: $t = 5.16$, $P < 0.001$). In the evening, CORT levels 1–2 min post-stress did not differ from baseline ($P = 0.999$), but CORT levels 30 min post-stress were significantly lower than both baseline ($t = 4.84$, $P < 0.001$) and 1–2 min post-stress levels ($t = 3.84$, $P = 0.001$). CORT levels 1–2 min ($P = 0.484$) and 30 min ($P = 0.546$) following shaking did not differ between the morning and evening.

3.6. Route of ^{3}H-labelled corticosterone excretion

Route-of-excretion data were analyzed using a multivariate ANOVA with sex and time of injection (0600 or 2000 h) as fixed factors, and total proportion of injected radioactivity excreted, proportion of excreted radioactivity present in fecal samples, and proportion of excreted radioactivity present in urine samples as dependent variables. Sex did not affect any of these measures ($P > 0.276$ for all measures), so data from males and females were pooled. Animals injected at 0600 h excreted a lower proportion of total injected radioactivity than did mice injected at 2000 h (0.160 ± 0.005 vs. 0.211 ± 0.008; $F_{1,6} = 28.955$, $P = 0.002$). The proportion of injected radiation excreted in feces (0600 h vs 2000 h: 0.599 ± 0.064 vs. 0.557 ± 0.053; $F_{1,6} = 0.254$, $P = 0.632$) or urine (0600 h vs 2000 h: 0.401 ± 0.064 vs. 0.443 ± 0.053; $F_{1,6} = 0.254$, $P = 0.632$) did not differ based on injection time. Paired-samples *t*-tests revealed that the proportion of excreted radioactivity contained in feces and in urine did not differ following ^{3}H-CORT injection at either 0600 h (0.599 vs. 0.401, respectively; $t_3 = 1.536$, $P = 0.222$) or 2000 h (0.526 vs. 0.443, respectively; $t_3 = 1.075$, $P = 0.361$).

3.7. ^{3}H-labelled CORT excretion time course

The proportion of total excreted radiation in either feces or urine was calculated for each time point and was analyzed using two separate mixed-model ANOVAs (one for each form of excreta) to account for missing data points (which occurred when no fecal or urine samples were excreted in a given collection period). The covariance structure used for the analysis of fecal CORT was compound symmetry, and the first-order autoregressive structure was used for urine data. Time of injection (0600 or 2000 h) did not affect the total proportion of radioactivity excreted in urine ($F_{1,57.55} < 0.01$, $P = 0.999$) or feces ($F_{1,2.89} = 0.944$, $P = 0.405$). Time of collection post-injection affected urinary radioactivity (Supplemental Table A; $F_{16,27.17} = 52.512$, $P < 0.001$) as well as fecal radioactivity (Supplemental Table B; $F_{16,77.47} = 6.747$, $P < 0.001$). When injection was administered at 2000 h, a greater amount of urinary radioactivity was excreted at 2 h post-injection than at any other time point; when injection occurred at 0600 h, however, urinary radioactivity did not differ between the 2 and 4 h post-injection time points, but radioactivity at both of these times did differ from all other time points (Supplemental Table A). Excretion of fecal radioactivity was most prominent in the first 8 h following injection at 2000 h, but showed a more bimodal pattern of excretion when injection of radioactivity occurred at 0600 h, with peaks at both 2–4 h and 14–16 h post-injection (Supplemental Table B). Additionally, the interaction between time of injection and time of collection was significant for both fecal ($F_{16,77.474} = 5.00$,

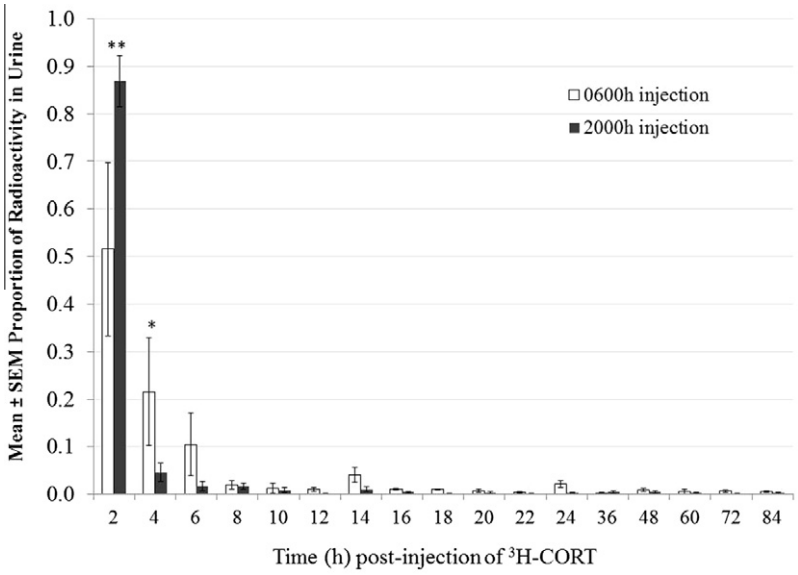

Fig. 7. Mean proportion of total urinary radioactivity excreted by adult male and female California mice (4 male and 4 female; half of which were injected at 2000 h and the other half at 0600 h) per time point; data from the sexes were pooled. Animals injected at 2000 h excreted more radioactivity 2 h post-injection but less radioactivity at 4 h post-injection, compared to animals injected at 0600 h. $^{**}P < 0.01$, $^{*}P < 0.05$.

$P < 0.001$) and urine ($F_{16,\ 27.174} = 2.895$, $P = 0.007$) samples; those results are discussed below.

Animals injected with ^{3}H-CORT at 2000 h excreted a higher proportion of fecal radioactivity in the first 2 h post-injection ($t = 4.81$, $P < 0.001$), but excreted a lower proportion of fecal radioactivity at 14 h ($t = 6.06$, $P < 0.001$) and 16 h ($t = 2.86$, $P = 0.005$) post-injection, when compared to animals injected at 0600 h (Fig. 6.). Proportion of fecal radioactivity excreted did not differ between the two groups at any other time point.

For urine samples, animals injected at 2000 h excreted a higher proportion of urinary radioactivity in the first 2 h post-injection ($t = 5.45$, $P < 0.001$), but a lower proportion of urinary radioactivity 4 h post-injection ($t = 2.62$, $P = 0.014$; Fig. 7), when compared to animals injected at 0600 h. Proportion of urinary radioactivity did not differ between the two groups at any other time points.

3.8. HPLC results

3.8.1. Elution time of known hormones and radioactive fractions

Fecal extract containing the highest proportion of radioactivity from mice injected with ^{3}H-CORT at 0600 h (14 h post-injection) and 2000 h (4 h post-injection) were separated by HPLC. The resulting 75 HPLC fractions were analyzed for presence of radioactivity; radioactivity in each HPLC fraction was divided by the total radioactivity per sample, and results are displayed as average percentage of radioactivity per fraction (Fig. 8). Five of the most radioactive fractions from the 4 h post-injection samples eluted during or after fraction 18, whereas the 5 most radioactive fractions from the 14 h post-injection samples eluted prior to or during fraction 18 (Table 1). The majority of radioactivity from both injection times (67% and 78% for the 4 and 14 h, respectively) eluted prior to fraction 31 which is the elution time for pure CORT (Fig. 8). No two mice displayed the exact same ranking of the top five radioactivity containing fractions, and no single fraction fell in the top five for all mice (data not shown).

3.8.2. Presence of immunoreactive fractions

HPLC-partitioned fecal extract from unmanipulated California mice contained several fractions that reacted with the MP Biomedicals antibody; however, none of the major immunoreactive peaks corresponded to the 5 most radioactive fractions following ^{3}H-CORT injections (Fig. 8, Table 1). The two highest values for immunoreactivity from unmanipulated fecal extract fractions corresponded to elution times of known hormones (estrone sulfate and progesterone), but the concentration and cross-reactivity or the MP antibody for these steroids was not investigated.

4. Discussion

Over the course of this study we characterized plasma CORT concentrations in *P. californicus* (1) under baseline conditions across the diurnal cycle, (2) in response to pharmacological manipulation of the HPA axis (ACTH and dexamethasone challenges), and (3) in response to four different stressors at different times of day. In addition, we described steroid hormone metabolism and excretion time course in these mice. Our results suggest that the HPA axis of California mice exhibits several unusual features, including (1) extremely pronounced changes in circulating CORT levels from trough to peak of the diurnal cycle, (2) high diurnal peak CORT levels that were only surpassed following one of the applied stressors (but several stressors produced CORT levels higher than diurnal trough), (3) reduced DEX-induced negative feedback (as compared to most other rodents that have been studied), (4) decreased response to exogenous ACTH near the diurnal peak of the endogenous CORT rhythm, and (5) a decrease, as opposed to an increase, in circulating CORT concentrations following several stressors near the time of the diurnal peak.

4.1. Diurnal rhythm in plasma corticosterone levels

One of our most striking findings was the high concentrations of baseline CORT experienced throughout the day by California mice. The range of basal CORT values observed in this study, ~40–1800 ng/ml, is markedly higher than those in many other species, including several laboratory rodents and other organisms [e.g., house mice ~4–65 ng/ml, [60]; Sprague–Dawley rats ~50–360 ng/ml, [80]; Gambel's white-crowned sparrows ~6–18 ng/ml, [11]; marine iguanas ~2–8 ng/ml, [96]; for review on birds [see

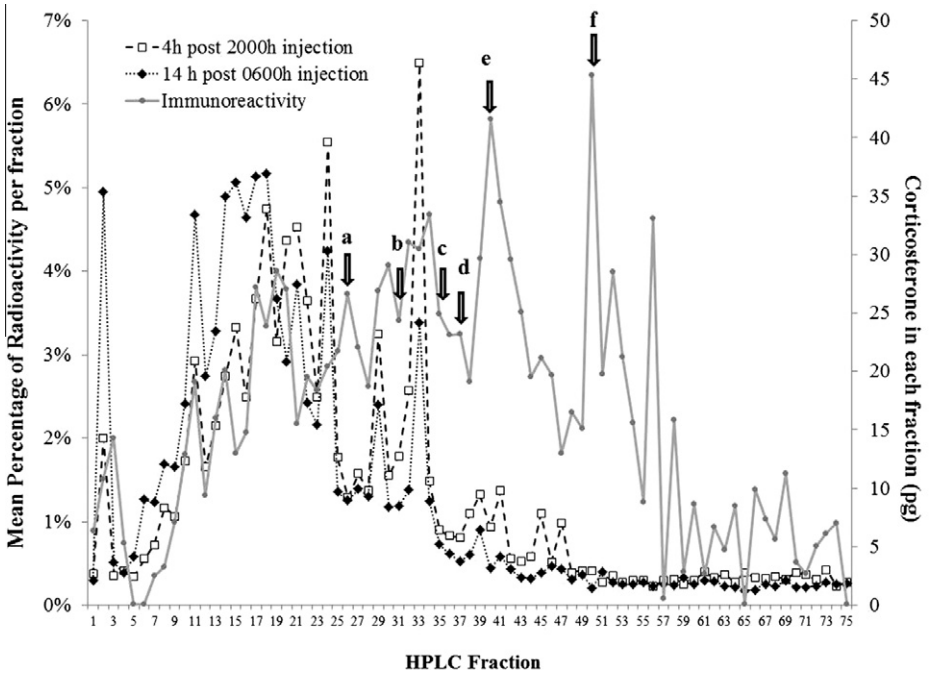

Fig. 8. Immunochromatogram displaying percent of radioactivity contained in each post-HPLC fecal extract, and the immunoreactivity of each fraction (displayed as amount of CORT), following injection of ^{3}H-CORT. The identity of the most radioactive fractions differed between the 4 h and 14 h post-injection samples. Additionally, none of the most radioactive fractions were the same as the most immunoreactive. Arrows represent elution times for known hormones (cortisol, a; corticosterone, b; 5α-pregnane-3β,11β,21-triol-20-one, b; 5β-androstane-3α-ol-11–17-dione, c; testosterone, d; estrone sulfate, e; progesterone, f).

Table 1
The HPLC elution times of the 5 most prevalent ^{3}H-CORT metabolites in California mouse fecal extracts and the elution times of the five most immunoreactive fractions as determined by CORT radioimmunoassay (RIA). Percentages of total radioactivity or immunoreactivity for each fraction are given in parentheses. Fecal extracts were prepared from samples collected either 4 or 14 h after ^{3}H-CORT injections at 2000 or 0600 h, respectively.

	Average rank in order of radioactivity (4 and 14 h) or immunoreactivity (RIA)				
	1	2	3	4	5
4 h	33 (6.50%)	24 (5.55%)	18 (4.75%)	21 (4.53%)	20 (4.37%)
14 h	18 (5.17%)	17 (5.13%)	15 (5.06%)	2 (4.95%)	14 (4.89%)
RIA	50 (3.66%)	40 (3.36%)	41 (2.79%)	34 (2.70%)	56 (2.67%)

8]]. It should be noted, however, that prairie voles also have high circulating baseline glucocorticoid levels [~475–1100 ng/ml; 79], as do guinea pigs [~100–400 ng/ml; 51] and some New World primates and human lineages [16–18,20,70,98], so California mice are not entirely unique in this regard. Baseline CORT values from other studies of California mice [27,36,65] are consistent with values obtained here, and the assay that we used has been validated specifically for use in this species [15], indicating that the high CORT values observed in this study are not artifacts of our methodology.

California mice also show a very dynamic diurnal CORT profile, with plasma CORT concentrations increasing approximately 45-fold from trough to peak (inactive to active periods). For comparison, Sprague Dawley rats experience diurnal changes in CORT concentrations on the order of 8.5-fold [80], while Wistar rats experience a change of approximately 20-fold in males and 7.3-fold in estrous females [1]. House mice show about a 5-fold change from trough to peak [26], marine iguanas show an approximate 4-fold change [96] and White-crowned sparrows experience about a 3-fold change over the course of the day [11]. How differences in fold increase over the diurnal cycle relate to differences, if any, in glucocorticoid function is not known.

In our study, peak plasma CORT levels occurred around the onset of the active period (2000 h, 1 h after lights-off), and the lowest values were obtained during the inactive period, consistent with HPA function in other species [23]; for an exception see 11]. Surprisingly, we found no differences in CORT levels between the sexes; in other species, females have generally been found to have elevated CORT concentrations compared to males, due to interactions between sex hormones and the HPA axis [1,44,53,74,90].

4.2. Plasma CORT responses to adrenocorticotropic hormone (ACTH) injection

In an attempt to maximize adrenal response we chose a dose of ACTH that has been used in rats [100 μg/kg; 2,56] but is typically higher than doses used in other species [2 μg/kg in guinea pigs, 51,59; 0.0075 or 0.015 μg (total) in rats, [22]; 10 μg/kg in marmosets, 71]. ACTH at a dose of 100 μg/kg produced a marked increase in plasma CORT 1 h following a morning (0900 h) injection, when compared to saline injection. A CORT increase within this time frame post-injection is consistent with reports from other species. Additionally, we found that CORT levels 1 h following saline injection were significantly lower than those obtained under baseline conditions, suggesting that our handling and injection procedures elicit, at most, a very transient CORT elevation.

As with a single dose of ACTH, two injections, separated by 30 min, of a higher ACTH dose (150 μg/kg), in the morning (0900 h) elicited a robust CORT elevation at both 30 and 60 min following the second injection. Notably, even two consecutive injections of 150 μg/kg ACTH did not produce maximal CORT out-

put, as CORT values following evening forced-swim were significantly higher than those following morning ACTH injection.

Surprisingly, when the double-injection paradigm was repeated in the afternoon (injections at 1600 h and 1630 h), plasma CORT concentrations did not differ between ACTH-injected and saline-injected animals at either 30 or 60 min after the second injection. This does not seem to be a result of the adrenals being maximally stimulated, as both morning ACTH injections and other stressors resulted in higher CORT values than those achieved following afternoon ACTH injection (visual comparison of the data). These results suggest that adrenal responsiveness to ACTH changes across the diurnal cycle in California mice. One interpretation is that when baseline CORT concentrations are increasing around the time of the diurnal peak (evening), the adrenal gland cannot increase CORT output when stimulated with exogenous ACTH, whereas when basal CORT concentrations are low (morning) ACTH injection stimulates the adrenals and increases CORT concentration.

Differential responses of the adrenal glands based on time of day have also been noted in other species [reviewed by 10,49]. Two studies found that rats showed greater CORT responses to ACTH administered near the peak of endogenous CORT production (evening) as compared to the trough [morning; 22,50]. Splanchnic innervation of the adrenal gland is thought to partly mediate the diurnal rhythm of CORT secretion in rats by increasing adrenal sensitivity to ACTH around the time of the diurnal peak [88] and/or by decreasing adrenal sensitivity to ACTH during the diurnal trough [48]. In contrast, California mice are more responsive to ACTH near the trough, not the peak, of the diurnal CORT rhythm. The mechanism underlying the change in adrenal responsiveness to ACTH across the diurnal cycle in California mice is unknown, but further studies investigating splanchnic innervation, adrenal ACTH-receptor binding, adrenal ACTH receptor density, and intra-adrenal CORT synthesis would be illuminating.

4.3. Plasma CORT responses to dexamethasone (DEX) injection

DEX is commonly administered to test the responsiveness of the HPA axis to negative feedback: the longer CORT remains suppressed following DEX treatment, or the lower the DEX dose that suppresses CORT secretion, the more sensitive the axis. DEX acts primarily at the anterior pituitary, as it does not readily cross the blood–brain barrier [21]; therefore effects are expected to be driven primarily by a decreased synthesis or secretion of ACTH, leading to reduced CORT production. Due to the high levels of endogenous circulating CORT in this species, we tested the response to a range of DEX doses (0.5, 5 and 10 mg/kg). Both the 5 and 10 mg/kg doses suppressed CORT 8 h following injection, whereas the 0.5 mg/kg dose did not; no dose suppressed CORT until the 32 h mark. In comparison, 0.1 mg/kg is sufficient to suppress CORT for 12 h in rats [79], and roughly 0.02 mg/kg suppresses cortisol for at least 9 h in humans [reviewed by 75]. The finding that 5 mg/kg of DEX was needed to suppress baseline CORT activity 8 h post-injection, and that even a 10 mg/kg dose could not suppress CORT to 32 h post-injection, suggests that California mice might be somewhat glucocorticoid-resistant at the level of the pituitary. Alternatively, it is possible that DEX did suppress ACTH release in our animals, but that decreased ACTH levels did not translate to an observable decrease in plasma CORT due a non-linear relationship between ACTH and CORT (as may be suggested from the ACTH-injection data, see Section 3.2). Another possibility is that California mice might clear DEX very rapidly, thus preventing long-term CORT changes. We did not analyze pituitary GC receptor affinity or binding dynamics, nor did we measure plasma ACTH levels or DEX clearance in this study, but characterizing these factors would be an important future step to further elucidating negative feedback in the HPA axis of California mice.

4.4. Effects of experimental stressors on plasma CORT

4.4.1. Predator odor

Exposure to predator urine in the morning resulted in a significant elevation of plasma CORT when compared to time-matched baseline samples, consistent with previous data from our lab [15]. When comparing CORT concentrations at different times post-exposure (1, 15, 30 or 60 min), all values were elevated above baseline but no one time point was significantly higher than any other. These results suggest that CORT increased rapidly and then was slow to clear from the system, or that the stressor elicited a prolonged increase in CORT secretion, even beyond the period of acute exposure. Additionally, morning post-stress CORT concentrations were not significantly higher than evening baseline values, so despite the post-urine-exposure rise, achieved levels were not higher than concentrations that occur on a daily basis.

When mice were exposed to predator odor in the evening and blood sampled 1 min later, no difference between post-stress and time-matched baseline CORT concentrations was observed. However, post-urine-exposure CORT levels in the evening were significantly higher than post-urine levels in the morning, and were also significantly higher than morning baseline levels. The differential responses seen across the day in our mice could be mediated by inputs to the HPA axis, as it is possible that mice do not perceive the odor stimulus in the same manner. In rats, for example, olfactory sensitivity to both fox urine and mineral oil, measured by fos activation in several olfactory-related brain regions (e.g. main olfactory bulb, primary olfactory cortex) is higher in the evening than in the morning [35]. On the other hand, it is possible that predator odor is perceived identically at both time points, but that the high levels of circulating CORT already present in the blood stream during the evening result in mechanistic constraints at some level of the HPA axis. In addition to plasma CORT, other HPA hormones (e.g. corticotropin-releasing hormone) are important in behavioral and physiological responses to stressors, and measurement of these hormones would therefore be valuable to determine if the upstream, intracerebral response to stress differs throughout the day. Unfortunately, we cannot be certain that using a different predator odor in the morning vs. evening (bobcat vs. fox, respectively) did not account for the differential CORT response, although data from our lab suggest that California mice show similar CORT responses to urine from several different predators [15], unpub.data].

4.4.2. Morning vs. evening stress tests

It is clear from the double ACTH-injection studies and the predator-odor trials that time of day, at least broadly defined as morning vs. evening, markedly affects the plasma CORT response to both pharmacological and psychological stimulation in California mice. The difference in CORT response to acute stress based on time of day is not new, as several studies on rats have reported similar findings [67,89]. To investigate this phenomenon further in California mice, we used three common lab stressors to determine if CORT responses to these stressors differ from baseline levels in the morning and in the evening, and if any of these stressors elevate plasma CORT above peak baseline concentrations found across the diurnal cycle.

When mice were stressed in the morning (0800 h), each of the three stressors significantly elevated plasma CORT levels above morning baseline at both 1–2 min and 30-min post-stressor, as expected. When stressors were presented in the evening (2000 h), only forced swimming elevated CORT above the corresponding evening baseline value, and only at 1–2 min post-stress. Thus, plasma CORT concentrations can be elevated above baseline levels occurring at the peak of the diurnal rhythm in response to at least this one stressor, indicating that the diurnal peak is

not maximal CORT output for this species. Therefore, forced swimming, which is both a psychological and a physiological stressor with a thermoregulatory component (water was 24–25 °C), appears to affect the HPA axis differently than restraint or shaking, which are predominantly psychological stressors. Similar results have been found in rats, in that stressors with a thermoregulatory component – i.e., forced-swim [89] or immersion in cold water [67] – result in elevated CORT near both the peak and trough of the diurnal CORT rhythm, whereas open-field testing [89] and foot-shock or restraint [67] produced CORT responses only near the diurnal trough.

In our study, CORT concentrations in evening plasma samples collected 1–2-min after restraint or shaking were not different from baseline at that time of day, and surprisingly, values 30 min after stress were lower than evening baseline levels. Thus, two stimuli that elicit a conventional stress response – i.e., an elevation in CORT concentrations – in the morning elicit a decrease in CORT levels in the evening. The reason for the rapid decrease following either restraint or shaking is unknown, but these findings could suggest an increase in CORT catabolism following certain stressors during the active period. Studies in rats have reported half-lives of injected CORT between 10 and 35 min [93,94,97]. Therefore, increased metabolism might account for the reduced plasma CORT levels observed 30 min post-stress, but probably cannot explain the absence of difference from baseline in the 1–2 min post-stress samples. Data from our ^{3}H-CORT injections show that CORT is cleared more rapidly during the active period when compared to the inactive period, which is in line with the above prediction. Additional data on CORT metabolism and clearance from the plasma would be informative.

4.5. Metabolism and excretion of ^{3}H-CORT

Unfortunately, we were not able to successfully validate a fecal CORT assay for California mice. When comparing the five most prevalent immunoreactive fractions to the 5 most common radioactive fractions, no overlap between the two was observed. These data indicate that the MP Biomedicals antibody was not able to bind the most prevalent metabolites of CORT present in feces and therefore could not provide reliable, biologically relevant data for this species. These results highlight the importance of thorough validation prior to use of a fecal assay. Our data do, however, provide valuable information on the time course and mode of excretion of CORT and its metabolites. Circulating CORT is excreted in urine and feces in almost equal proportions, which suggests that measuring CORT or its metabolites in feces is a plausible method for detection in this species. Additionally, the time course of excretion is dependent on when the elevation of plasma CORT occurred. CORT excretion in feces occurred rapidly (within approximately 2–6 h) when an injection was given during the active phase (lights-off), but occurred more slowly (4–6 and 14–16 h), and did not peak until the 14–16 h mark, following injection during the inactive phase (lights-on). These results are consistent with CORT excretion times from laboratory house mice, as time frames of 4–6 h vs. 8–12 h after radiolabelled CORT injection during lights-off or lights-on, respectively, were observed [81]. However, other species have been shown to differ greatly in their temporal patterns of CORT excretion [see 92]. The activity cycle appeared to be at least partially driving a portion of CORT metabolism in our mice, as mice expelled large amounts of radiation in feces around a specific time of day (2200 h), regardless of injection time. However, this activity-related pattern was not observed in urinary excretion of radioactivity. Knowing the time course of hormone excretion is critical for collection of accurate post-stress fecal hormone data.

5. Conclusions

Understanding differences in HPA dynamics – including changes in baseline circulating CORT concentrations, stress reactivity, adrenal responsiveness to ACTH, as well as metabolism, excretion, and clearance of CORT – over the diurnal cycle will contribute to interpretation of post-stress hormone values in this species. Specifically, having thorough baseline and post-stress data from different times of day will allow us to better understand the biological consequences of elevated CORT in different scenarios. Obtaining species-specific data on HPA function is imperative for proper interpretation of results as we have shown that California mice differ markedly from rats and house mice in several aspects of HPA function, despite the fact they are all rodents. We hope the data presented in this paper will aid in interpretation of CORT concentrations in this species and will allow for more thorough interpretation of interactions between the HPA axis and reproductive behavior.

Acknowledgements

We would like to thank the vivarium staff, Leslie Karpinski, Jim Sinclair and John Kitasako, for their help in maintaining the mouse colony. In addition, laboratory assistance from Corrine Pisacane, Dr. Chris Tubbs, Dr. Kristine Kaiser, Dr. Miyetani Chauke, and Alan Fetter is greatly appreciated, as is statistical advice provided by Matthew Wolak and Dr. Zach Hohman. Several undergraduate assistants aided in the collection of samples; we would like to especially thank Vanessa Yang, Radhika Chandramouli, and Julia Cho. Finally, we thank two anonymous reviewers for constructive comments on an earlier draft of the manuscript. This work was supported by funds from the University of California, Riverside and by NIH grant 1R21MH087806 to W. Saltzman.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2012.08.026.

References

[1] H.C. Atkinson, B.J. Waddell, Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle, Endocrinology 138 (1997) 3842–3848.
[2] E. Bamberg, R. Palme, J.G. Meingassner, Excretion of corticosteroid metabolites in urine and faeces of rats, Lab. Anim. 35 (2001) 307–314.
[3] M. Bardi, C.L. Franssen, J.E. Hampton, E.A. Shea, A.P. Fanean, K.G. Lambert, Paternal experience and stress responses in California Mice (*Peromyscus californicus*), Comp. Med. 61 (2011) 20–30.
[4] E.A. Becker, B.M. Moore, C. Auger, C.A. Marler, Paternal behavior increases testosterone levels in offspring of the California mouse, Horm. Behav. 58 (2010) 385–389.
[5] A.M. Behie, M.S.M. Pavelka, C.A. Chapman, Sources of variation in fecal cortisol levels in howler monkeys in Belize, Am. J. Primatol. 72 (2010) 600–606.
[6] J.K. Bester-Meredith, L.J. Young, C.A. Marler, Species differences in paternal behavior and aggression in *Peromyscus* and their associations with vasopressin immunoreactivity and receptors, Horm. Behav. 36 (1999) 25–38.
[7] J.K. Bester-Meredith, C.A. Marler, The associations between male offspring aggression and paternal and maternal behavior of *Peromyscus* mice, Ethology 109 (2003) 797–808.
[8] V. Bókony, A.Z. Lendvai, A. Liker, F. Angelier, J.C. Wingfield, O. Chastel, Stress response and the value of reproduction: are birds prudent parents?, Am Nat. 5 (2009) 589–598.
[9] F. Bonier, P.R. Martin, I.T. Moore, J.C. Wingfield, Do baseline glucocorticoids predict fitness?, Trends Ecol Evol. 24 (2009) 634–642.
[10] S.R. Bornstein, W.C. Engeland, M. Ehrhart-Bornstein, J.P. Herman, Dissociation of ACTH and glucocorticoids, Trends Endocrinol. Metab. 19 (2008) 175–180.
[11] C.W. Breuner, J.C. Wingfield, L.M. Romero, Diel rhythms of basal and stress-induced corticosterone in a wild, seasonal vertebrate, Gambel's white-crowned sparrow, J. Exp. Zool. 284 (1999) 334–342.
[12] R.E. Brown, Hormonal and experiential factors influencing parental behaviour in male rodents: an integrative approach, Behav. Process 30 (1993) 1–28.

[13] B. Case, G.A. Lewbart, P.D. Doerr, The physiological and behavioural impacts of and preference for an enriched environment in the eastern box turtle (*Terrapene carolina carolina*), Appl. Anim. Behav. Sci. 92 (2005) 353–365.
[14] S.A. Cavigelli, S.L. Monfort, T.K. Whitney, Y.S. Mechref, M. Novotny, M.K. McClintock, Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms, J. Endocrinol. 184 (2005) 153–163.
[15] M. Chauke, J.L. Malisch, C. Robinson, T.R. de Jong, W. Saltzman, Effects of reproductive status on behavioral and endocrine responses to acute stress in a biparental rodent, the California mouse (*Peromyscus californicus*), Horm. Behav. 60 (2011) 128–138.
[16] G.P. Chrousos, D. Renquist, D. Brandon, C. Eil, M. Pugeat, R. Vigersky, G.B. Cutler Jr., D.L. Loriaux, M.B. Lipsett, Glucocorticoid hormone resistance during primate evolution: Receptor-mediated mechanisms, Proc. Natl. Acad. Sci. USA 79 (1982) 2036–2040.
[17] G.P. Chrousos, D.L. Loriaux, M. Tomita, D. Brandon, M.B. Lipsett, The new world primates as animal models of cortisol resistance, in: G.P. Chrousos, D.L. Loriaux, M.B. Lipsett (Eds.), Steroid Hormone Resistance. Mechanisms and Clinical Aspects, Plenum Press, New York, 1986, pp. 129–144.
[18] G.P. Chrousos, S.D. Detera-Wadleigh, M. Karl, Syndromes of glucocorticoid resistance, Ann. Intern. Med. 119 (1993) 1113–1124.
[19] G.P. Chrousos, Editorial: ultradian, circadian, and stress-related hypothalamic–pituitary–adrenal axis activity – a dynamic digital-to-analog modulation, Endocrinology 139 (1998) 437–440.
[20] C.L. Coe, A. Savage, L.J. Bromley, Phylogenetic influences on hormone levels across the Primate order, Am. J. Primatol. 28 (1992) 81–100.
[21] M.A. Cole, P.J. Kim, B.A. Kalman, R.L. Spencer, Dexamethasone suppression of corticosteroid secretion: evaluation of the site of action by receptor measures and functional studies, Psychoneuroendocrinology 25 (2000) 151–167.
[22] M.F. Dallman, W.C. Engeland, J.C. Rose, C.W. Wilkinson, J. Shinsako, F. Siedenburg, Nycthemeral rhythm in adrenal responsiveness to ACTH, Am. J. Physiol. Regul. Integr. Comp. Physiol. 235 (1978) 210–218.
[23] M.F. Dallman, S.F. Akana, C.S. Cascio, D.N. Darlington, L. Jacobson, N. Levin, Regulation of ACTH secretion: variations on a theme of B, Rec. Prog. Horm. Res. 43 (1987) 113–173.
[24] M.F. Dallman, A.M. Strack, S.F. Akana, M.J. Bradbury, E.S. Hanson, K.A. Scribner, M. Smith, Feast and famine: critical role of glucocorticoids with insulin in daily energy flow, Front. Neuroendocrinol. 14 (1993) 303–347.
[25] M.F. Dallman, V.G. Viau, S. Bhatnagar, F. Gomez, K. Laugero, M.E. Bell, Corticotropin-releasing factor, corticosteroids, stress, and sugar: energy balance, the brain and behavior, in: D.W. Pfaff, A.P. Arnold, S.E. Fahrbach, A.M. Etgen, R.T. Rubin (Eds.), Hormones, Brain and Behavior, Academic Press, 2002, pp. 571–631.
[26] S. Dalm, L. Enthoven, O.C. Meijer, M.H. van der Mark, A.M. Karsssen, E.R. de Kloet, M.S. Oitzl, Age-related changes in hypothalamic-pituitary-adrenal axis activity of male C57BL/6J mice, Neuroendocrinology 81 (2005) 372–380.
[27] E.S. Davis, C.A. Marler, The progesterone challenge: steroid hormone changes following a simulated territorial intrusion in female *Peromyscus californicus*, Horm. Behav. 44 (2003) 185–198.
[28] K.B. Davis, N.C. Parker, Plasma corticosteroid stress response of fourteen species of warmwater fish to transportation, Trans. Am. Fish. Soc. 115 (1986) 495–499.
[29] T.R. de Jong, M. Chauke, B.N. Harris, W. Saltzman, From here to paternity: neural correlates of the onset of paternal behavior in California mice (*Peromyscus californicus*), Horm. Behav. 56 (2009) 220–231.
[30] T.R. de Jong, K.R. Measor, M. Chauke, B.N. Harris, W. Saltzman, Brief pup exposure induces Fos expression in the lateral habenula and serotonergic caudal dorsal raphe nucleus of paternally experienced male California mice (*Peromyscus californicus*), Neuroscience 169 (2010) 1094–1104.
[31] R.J. Denver, Structural and functional evolution of vertebrate neuroendocrine stress systems, Ann. N.Y. Acad. Sci. 1163 (2009) 1–16.
[32] F.S. Dhabhar, B.S. McEwen, R.L. Spencer, Stress response, adrenal steroid receptor levels and corticosteroid-binding globulin levels – a comparison between Sprague-Dawley, Fisher 344 and Lewis rats, Brain Res. 616 (1993) 89–98.
[33] T. Dickmeis, Glucocorticoids and the circadian clock, J. Endocrinol. 200 (2009) 3–22.
[34] N.J. Dingemanse, P. Edelaar, B. Kempenaers, Why is there variation in baseline glucocorticoid levels?, Trends Ecol Evol. 25 (2010) 262–263.
[35] D. Funk, S. Amir, Circadian modulation of fos responses to odor of the red fox, a rodent predator, in the rat olfactory system, Brain Res. 866 (2000) 262–267.
[36] E.R. Glasper, A.C. DeVries, Social structure influences effects of pair-housing on wound healing, Brain Behav. Immun. 19 (2005) 61–68.
[37] T. Good, M.Z. Khan, J.W. Lynch, Biochemical and physiological validation of a corticosteroid radioimmunoassay for plasma and fecal samples in oldfield mice (*Peromyscus polionotus*), Physiol. Behav. 80 (2003) 405–411.
[38] N. Greenberg, J.C. Wingfield, Stress and reproduction: reciprocal relationships, in: D.O. Norris, R.E. Jones (Eds.), Hormones and Reproduction in Fishes, Amphibians, and Reptiles, Plenum Press, New York, 1987, pp. 461–503.
[39] D.J. Gubernick, J.R. Alberts, The biparental care system of the California mouse, *Peromyscus californicus*, J. Comp. Psychol. 101 (1987) 169–177.
[40] D.J. Gubernick, S.L. Wright, R.E. Brown, The significance of father's presence for offspring survival in the monogamous California mouse, *Peromyscus californicus*, Anim. Behav. 46 (1993) 539–546.
[41] D.J. Gubernick, K.A. Schneider, L.A. Jeannotte, Individual differences in the mechanisms underlying the onset and maintenance of paternal behavior and the inhibition of infanticide in the monogamous biparental California mouse, *Peromyscus californicus*, Behav. Ecol. Sociobiol. 34 (1994) 225–231.
[42] D.J. Gubernick, T. Teferi, Adaptive significance of male paternal care in a monogamous mammal, Proc. Biol. Sci. 267 (2000) 147–150.
[43] R. Gueorguieva, J.H. Krystal, Move over ANOVA progress in analyzing repeated-measures data and its reflection in papers published in the Archives of General Psychiatry, Arch. Gen. Psych. 61 (2004) 310–317.
[44] R.J. Handa, L.H. Burgess, J.E. Kerr, J.A. O'Keefe, Gonadal steroid hormone receptors and sex differences in the hypothalamo–pituitary–adrenal axis, Horm. Behav. 28 (1994) 464–476.
[45] J.M. Harper, S.N. Austad, Fecal glucocorticoids: a noninvasive method of measuring adrenal activity in wild and captive rodents, Physiol. Biochem. Zool. 73 (2000) 12–22.
[46] B.N. Harris, J.P. Perea-Rodriguez, W. Saltzman, Acute effects of corticosterone injection on paternal behavior in California mouse (*Peromyscus californicus*) fathers, Horm. Behav. 60 (2011) 666–675.
[47] A.S. Jaggi, N. Bhatia, N. Kumar, N. Singh, P. Anand, R. Dhawan, A review on animal models for screening potential anti-stress agents, Neurol. Sci. 32 (2011) 993–1005.
[48] M.S. Jasper, W.C. Engeland, Splanchnicotomy increases adrenal sensitivity to ACTH in nonstressed rats, Am. J. Physiol. Endocrinol. Metab. 273 (1997) E363–E368.
[49] A. Kalsbeek, R. van der Spek, J. Lei, E. Endert, R.M. Buijs, E. Fliers, Circadian rhythms in the hypothalamo–pituitary–adrenal (HPA) axis, Mol. Cell. Endocrinol. 349 (2012) 20–29.
[50] M. Kaneko, K. Kaneko, J. Shinako, M.F. Dallman, Adrenal sensitivity to adrenocorticotropin varies diurnally, Endocrinology 109 (1981) 70–75.
[51] M. Kapoor, S.G. Matthews, Short periods of prenatal stress affect growth, behaviour and hypothalamo–pituitary–adrenal axis activity in male guinea pig offspring, J. Physiol. 566 (2005) 967–977.
[52] J.M. Keay, J. Singh, M.C. Gaunt, T. Kaur, Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review, J. Zoo Wildl. Med. 37 (2006) 234–244.
[53] B.M. Kudielka, C. Kirschbaum, Sex differences in HPA axis responses to stress: a review, Biol. Psychol. 69 (2005) 113–132.
[54] M.M. Landys, M. Ramenofsky, J.C. Wingfield, Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes, Gen. Comp. Endocrinol. 148 (2006) 132–149.
[55] A.W. Lee, R.E. Brown, Medial preoptic lesions disrupt parental behavior in both male and female California mice (*Peromyscus californicus*), Behav. Neurosci. 116 (2002) 968–975.
[56] M. Lepschy, C. Touma, R. Hruby, R. Palme, Non-invasive measurement of adrenocortical activity in male and female rats, Lab. Anim. 41 (2007) 372–387.
[57] R. Levin, S. Levine, Development of circadian periodicity in base and stress levels of corticosterone, Am. J. Phys. 229 (1975) 1397–1399.
[58] S.L. Lightman, C.C. Wiles, H.C. Atkinson, D.E. Henley, G.M. Russell, J.A. Leendertz, M.A. McKenna, F. Spiga, S.A. Wood, B.L. Conway-Campbell, The significance of glucocorticoid pulsatility, Eur. J. Pharmacol. 583 (2008) 255–262.
[59] L. Lui, S.G. Matthews, Adrenocortical response profiles to corticotrophin-releasing hormone and adrenocorticotrophin challenge in the chronically catheterized adult guinea-pig, Exp. Physiol. 84 (1999) 971–977.
[60] J.L. Malisch, C.W. Breuner, F.R. Gomes, M.A. Chappell, T. Garland Jr., Circadian pattern of total and free corticosterone concentrations, corticosteroid-binding globulin, and physical activity in mice selectively bred for high voluntary wheel-running behavior, Gen. Comp. Endocrinol. 156 (2008) 210–217.
[61] L.B. Martin, Z.M. Weil, R.J. Nelson, Seasonal trade-offs between reproduction and immune activity, Philos. Trans. R. Soc. Lond. B Biol. Sci. 363 (2008) 321–339.
[62] L.B. Martin, E.R. Glasper, R.J. Nelson, A.C. De Vries, Prolonged separation delays wound healing in monogamous California mice, *Peromyscus californicus*, but not in polygynous white-footed mice, *P. leucopus*, Physiol. Behav. 87 (2006) 837–841.
[63] E. Möstl, R. Palme, Hormones as indicators of stress, Domest. Anim. Endocrinol. 23 (2002) 67–74.
[64] A.O. Ogunsua, A.F. de Nicola, H. Traikov, M.K. Birmingham, S. Levine, Adrenal steroid biosynthesis by different species of mouselike rodents, Gen. Comp. Endocrinol. 16 (1971) 192–199.
[65] T.O. Oyegbile, C.A. Marler, Weak winner effect in a less aggressive mammal: correlations with corticosterone but not testosterone, Physiol. Behav. 89 (2006) 171–179.
[66] R. Palme, Measuring fecal steroids: Guidelines for practical application, Ann. N.Y. Acad. Sci. 1046 (2005) 75–80.
[67] S. Retana-Márquez, H. Bonilla-Jaime, G. Vázquez-Palacios, E. Domínguez-Salazar, R. Martínez-García, J. Velázquez-Moctezuma, Body weight gain and diurnal differences of corticosterone changes in response to acute and chronic stress in rats, Psychoneuroendocrinology 28 (2003) 207–227.
[68] L.M. Romero, Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates, Gen. Comp. Endocrinol. 128 (2002) 1–24.
[69] B. Roozendaal, Glucocorticoids and the regulation of memory consolidation, Psychoneuroendocrinology 25 (2000) 213–238.
[70] W. Saltzman, N.J. Schultz-Darken, G. Scheffler, F.H. Wegner, D.H. Abbott, Social and reproductive influences on plasma cortisol in female marmoset monkeys, Physiol. Behav. 56 (1994) 801–810.
[71] W. Saltzman, S.L. Prudom, N.J. Schultz-Darken, D.H. Abbott, Reduced adrenocortical responsiveness to adrenocorticotropic hormone (ACTH) in

socially subordinate female marmoset monkeys, Psychoneuroendocrinology 25 (2000) 463–477.
[72] R.M. Sapolsky, L.M. Romero, A.U. Munck, How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions, Endoc. Rev. 21 (2000) 55–89.
[73] F. Sarrieau, V. Chaouloff, P. Lemaire, Morméde, Comparison of the neuroendocrine responses to stress in outbred, inbred and F1 hybrid rats, Life Sci. 63 (1998) 87–96.
[74] J.V. Seale, S.A. Wood, H.C. Atkinson, E. Bate, S.L. Lightman, C.D. Ingram, D.S. Jessop, M.S. Harbuz, Gonadectomy reverses the sexually diergic patterns of circadian and stress-induced hypothalamic–pituitary–adrenal axis activity in male and female rats, J. Neuroendocrinol. 16 (2004) 516–524.
[75] T.E. Seeman, R.J. Robbins, Aging and hypothalmaic–pituitary–adreanal response to challenge in humans, Endocr. Rev. 15 (1994) 233–261.
[76] A.L. Silva, W.H.D. Fry, C. Sweeney, B.C. Trainor, Effects of photoperiod and experience on aggressive behavior in female California mice, Behav. Brain Res. 208 (2010) 528–534.
[77] R.S. Sloviter, G. Valiquette, G.M. Abrams, E.C. Ronk, A.L. Sollas, L.A. Paul, S. Neubort, Selective loss of hippocampal granule cells in the mature rat brain after adrenalectomy, Science 243 (1989) 535–538.
[78] A.M. Strack, R.J. Sebastian, M.W. Schwartz, M.F. Dallman, Glucocorticoids and insulin: reciprocal signals for energy balance, Am. J. Physiol. Regul. Integr. Comp. Physiol. 268 (1995) R142–R149.
[79] S.E. Taymans, A.C. DeVries, M.B. DeVries, R.J. Nelson, T.C. Friedman, M. Castro, S. Detera-Wadleigh, C.S. Carter, G.P. Chrousos, The hypothalamic–pituitary–adrenal axis of Prairie Voles (*Microtus ochrogaster*): evidence for target tissue glucocorticoid resistance, Gen. Comp. Endocrinol. 106 (1997) 48–61.
[80] P.K. Thanos, S.A. Cavigelli, M. Michaelides, D.M. Olvet, U. Patel, M.N. Diep, N.D. Volkow, A non-invasive method for detecting the metabolic stress response in rodents: characterization and disruption of the circadian corticosterone rhythm, Physiol. Res. 58 (2009) 219–228.
[81] C. Touma, N. Sachser, E. Mostl, R. Palme, Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice, Gen. Comp. Endocrinol. 130 (2003) 267–278.
[82] C. Touma, R. Palme, N. Saschser, Analyzing corticosterone metabolites in fecal samples of mice. a non-invasive technique to monitor stress hormones, Horm. Behav. 45 (2004) 10–22.
[83] C. Touma, R. Palme, Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation, Ann. N.Y. Acad. Sci., 1046 (2005) 54–74.
[84] B.C. Trainor, C.A. Marler, Testosterone, paternal behavior, and aggression in the monogamous California mouse (*Peromyscus californicus*), Horm. Behav. 40 (2001) 32–42.
[85] B.C. Trainor, M.S. Finy, R.J. Nelson, Paternal aggression in a biparental mouse: parallels with maternal aggression, Horm. Behav. 53 (2008) 200–207.
[86] B.C. Trainor, K.K. Crean, W.H.D. Fry, C. Sweeney, Activation of extracellular signal-regulated kinases in social behavior circuits during resident-intruder aggression tests, Neuroscience 165 (2010) 325–336.
[87] B.C. Trainor, Stress responses and the mesolimbic dopamine system: social contexts and sex differences, Horm. Behav. 60 (2011) 457–469.
[88] Y.M. Ulrich-Lai, M.M. Arnhold, W.C. Engeland, Adrenal splanchnic innervation contributes to the diurnal rhythm of plasma corticosterone in rats by modulating adrenal sensitivity to ACTH, Am. J. Physiol. Regul. Integr. Comp. Physiol. 209 (2003) R1128–R1135.
[89] P. Verma, K.G.C. Hellemans, F.Y. Choi, W. Yu, J. Weinberg, Circadian phase and sex effects on depressive/anxiety-like behaviors and HPA axis response to acute stress, Physiol. Behav. 99 (2010) 276–285.
[90] V. Viau, Functional cross-talk between the hypothalamic–pituitary–gonadal and –adrenal axes, J. Neruroendocrinol. 14 (2002) 506–513.
[91] Z. Wang, L.A. Goonewardene, The use of MIXED models in the analysis of animal experiments with repeated measures data, Can. J. Anim. Sci. 84 (2004) 1–11.
[92] S.K. Wasser, K.E. Hunt, J.L. Brown, K. Cooper, C.M. Crockett, U. Bechert, J.J. Millspaugh, S. Larson, S.L. Monfort, A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species, Gen. Comp. Endocrinol. 120 (2000) 260–275.
[93] B.D. White, C.B. Corll, J.R. Porter, The metabolic clearance rate of corticosterone in lean and obese male zucker rats, Metab. Clin. Exp. 38 (1989) 530–536.
[94] R.J. Windle, S.A. Wood, N. Shanks, S.L. Lightman, C.D. Ingram, Ultradian rhythm of basal corticosterone release in the female rat: dynamic interaction with the response to acute stress, Endocrinology 139 (1998) 443–450.
[95] J.C. Wingfield, The concept of allostasis: coping with a capricious environment, J. Mammal. 86 (2005) 248–254.
[96] S.K. Woodley, D.L. Painter, M.C. Moore, M. Wikelski, L.M. Romero, Effect of tidal cycle and food intake on the baseline plasma corticosterone rhythm in intertidally foraging marine iguanas, Gen. Comp. Endocrinol. 132 (2003) 216–222.
[97] C.J.H. Woodward, G.R. Hervey, R.E. Oakey, E.M. Whitaker, The effects of fasting on plasma corticosterone kinetics in rats, Br. J. Nutr. 66 (1991) 117–127.
[98] S. Yamamoto, S. Utsu, Y. Tanioka, N. Ohsawa, Extremely high levels of corticosteroids and low levels of corticosteroid binding macromolecule in plasma of marmoset monkeys, Acta Endocrinol. 85 (1977) 398–405.