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Urinary corticosterone measures: Effects of strain and social rank in BKW and CD-1 mice

Ann E. Fitchett [a], Sarah A. Collins [a], Howard Mason [b], Christopher J. Barnard [a], Helen J. Cassaday [c,*]

[a] *School of Biology, University of Nottingham, University Park, Nottingham, NG7 2RD, UK*
[b] *Health & Safety Laboratory, Harpur Hill, Buxton, Derbyshire, SK17 9JN, UK*
[c] *School of Psychology, University of Nottingham, University Park, Nottingham, NG7 2RD, UK*

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Abstract

We used urinary assays as a non-invasive method to examine corticosterone levels in two outbred strains of male laboratory mice (BKW and CD-1). Measures were taken before and after 2 weeks of pair housing, to examine the effects of social stress. We found that CD-1 mice had significantly higher corticosterone levels compared to BKW mice both before and after pairing. Behavioural measures provided evidence that, when paired, both strains of mice polarised into dominants and subordinates, with a higher overall incidence of aggressive acts in the BKW mice.

Some pairings had to be separated to prevent injuries so the pairing procedure introduced a selection for non-aggressive socially tolerant mice. Social status was nevertheless found to be associated with pre-existing differences in urinary corticosterone in the CD-1 strain: mice that later became dominant had overall lower levels of urinary corticosterone compared to subordinates. In conclusion, urinary corticosterone levels indicated clear differences in physiology, likely to be related to the adrenal stress response, dependent on both strain and social status. Thus, this non-invasive measure could help to predict the welfare outcomes of social housing and how these may depend on dominance status, rather than overall levels of aggression, in different strains of mice.

Keywords: BKW; CD-1; Corticosterone; Mouse; Social rank; Strain difference

1. Introduction

Whilst group housing is generally considered beneficial for a range of laboratory animals, social stress can also raise husbandry issues. In general, we need to be able to quantify the likely reproductive costs and benefits of social living before we can evaluate its impact on

* Corresponding author. Tel.: +44 115 951 5124; fax: +44 115 951 5324.
E-mail address: helen.cassaday@nottingham.ac.uk (H.J. Cassaday).

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the welfare of group-housed individuals (Barnard and Hurst, 1996; Hurst et al., 1997). In the present study, we report evidence relating housing conditions to a putative physiological welfare indicator (urinary corticosterone) by using this to complement behavioural measures in drawing conclusions about welfare.

1.1. The costs and benefits of social living

It has been proposed that the stress caused by an animal's social environment could be as severe as any form of experimental stressor. For example, in rodents, compared to electric foot-shocks, restraint stress and food and water deprivation, the greatest plasma corticosterone response was observed in response to social defeat (Koolhaas et al., 1997). Typically, the male mice used in scientific research are individually housed because of problems with aggression levels when group housed. However, single housing may also be problematic and, in the extreme, results in 'the isolation syndrome' (Valzelli, 1973), which includes heavier adrenal glands, higher plasma corticosterone and hypertension and behavioural consequences (on later exposure to other individuals), e.g., compulsive aggressive behaviour and deviated or decreased sexual activity. Isolation-induced aggression is most commonly reported in outbred albino strains (Goldberg et al., 1973) of the kind used here.

Even in the absence of the opportunity to reproduce, there is evidence that mice show preferences for social living. For example, controlled studies have shown that male mice opt to sleep with a familiar cage mate and that the need for social contact increases with age (Van Loo et al., 2004). Similarly, dominant mice have been found to prefer soiled bedding from familiar subordinate males and this preference is greater than that shown for bedding from females (Rawleigh et al., 1993). This shows a preference for cues associated with conspecifics in the absence of any direct reproductive benefit, though clearly dependent on likely advantage in terms of social rank.

1.2. What mediates the detrimental effects of single housing?

One possibility is that single housing constitutes a barren housing environment that blocks natural behavioural responses that depend on environmental features, thus inducing abnormal behaviour such as stereotypies (Würbel, 2001). Secondly, the mismatch between postnatal environment (social with littermates) and adult environment (when animals are separated) is likely to disrupt habitat-dependent adaptation. In other words, the littermate rearing environment does not prepare the animal for adult life in standard laboratory conditions (Würbel, 2001). All this suggests that, notwithstanding any stress that may be caused by the immediate social environment, the welfare of laboratory rodents should be enhanced by the use of group housing.

1.3. How are the benefits of group housing influenced by social rank?

Particularly under laboratory conditions, where the opportunity to escape is limited, social stress is typically assumed to have a greater effect on subordinate compared to dominant animals, presumably due to the constant threat of defeat by the dominant (Creel et al., 1996). Adopting a subordinate social role, and even just the acute experience of social defeat by a conspecific, have both been shown to cause a number of physiological and behavioural changes in mice (Martinez et al., 1998). These include impaired sexual behaviour (D'Amato and Pavone, 1992), a depressed immune response (De Groot et al., 1999) and increased tendency to modulate the secretion of immunodepressive steroid hormones (Barnard et al., 1996), decreased territorial marking and ultrasonic courtship vocalisations (Lumley et al., 1999), an increase in core body temperature (Keeney et al., 2001) and changes in body weight (Bartolomucci et al., 2004). This raises a dilemma for animal husbandry: at what point and for which mice, do the costs of social living outweigh the benefits?

1.4. Corticosterone levels as a measure of stress

Corticosterone is released from the adrenal cortex after activation of the hypothamalamic-pituitary-adrenal axis in response to, among other things, stress (De Kloet, 2000). Elevated corticosterone levels have been seen in subordinates in a number of species: mice (Avitsur et al., 2001; Keeney et al., 2001; Louch and Higginbotham, 1967), rats (Blanchard et al., 1993), trout (Sloman et al., 2002) and lizards (Summers et al., 2003). However, there are some inconsistencies in the

findings with respect to laboratory rodents (Martinez et al., 1998).

Corticosterone is commonly assayed using plasma samples. One potential advantage of this method is that the immediacy of changes detectable in plasma after exposure to a stressor means that the temporal resolution is very good. However, the disadvantage of blood sampling is that the aversive experience of having blood taken can compound with the level of stress, thus confounding the measure. There are also obvious practical problems with this invasive procedure, particularly in small animals such as mice (Touma et al., 2003). To promote refinement in animal experimentation it is important that non-invasive assessment of biological processes are utilised where possible (Van Zutphen, 2000). An alternative non-invasive method for assessing corticosterone is to use urine assays (Dahlborn et al., 1996). Methods for collecting urine are relatively simple and involve some sort of handling or massage (e.g., Drickamer, 1988; Monahan and Yamazaki, 1993; Hurst et al., 1998; Ohl and Fuchs, 1999) or simply placing animals into a novel environment (e.g., Van Loo et al., 2001; Touma et al., 2003). Dahlborn et al. (1996) evaluated three methods: (1) placing into an empty cage without bedding, (2) placing into an empty cage which was put inside a larger cage filled with ice water and (3) gentle handling and bladder region massage. They found that the empty cage and cold stimulation techniques were both more effective than massage. The cold stimulation produced faster results than the empty cage method but had the disadvantage that it caused the mice obvious signs of discomfort. In any event, the alternative methods are sufficiently fast that contamination by any acute response to stress can be avoided since this takes some 40 min to have any detectable effect on urinary corticosterone (Dahlborn et al., 1996).

In the present study, we therefore collected urine samples using the empty cage method to later assay for corticosterone levels. We compared behavioural measures of aggressive tendencies (Mackintosh, 1981) and differences in corticosterone levels between dominant and subordinate individuals in two strains of albino outbred male laboratory mice. Outbred strains were chosen because there is evidence to suggest that they are more likely to develop polarised (dominant versus subordinate) relationships (Nevison et al., 1999). To check for any pre-existing differences, we took two urine samples. The first sample was taken before pairing (when the mice were singly housed) and a second sample was taken 2 weeks after pairing (when the mice were housed in dyads and allowed to form polarised dominance relationships).

This allowed us to determine how urinary corticosterone levels were affected by social rank, as well as to compare across the two strains in use.

2. Materials and methods

2.1. Animals

In study 1, 40, 8-week-old male mice of the outbred albino strain BKW were used (B & K Ltd., UK). In study 2, mice were 40, 6-week-old male mice of the outbred albino strain CD-1 (Harlan, UK). All were maintained on a 12:12 reverse light:dark cycle (white lights on 20:30–08:30 h), with all testing being carried out during the dark phase under dim red light. Food and water were freely available throughout. Initially, all mice were singly housed in NPK M3 cages (48 cm × 15 cm × 13 cm). Mice were marked on the back with black eyelash dye (Colorsport 30 day Mascara, Brodie and Stone Plc, London, UK), to enable identification when later housed in pairs.

2.2. Pairing and behavioural observations

Mice were randomly assigned to pairs, and housed in clean cages, either NPK M3 (48 cm × 15 cm × 13 cm) or NPK MB1 (45 cm × 28 cm × 13 cm). Study 1 used cages of both dimensions. Study 2 used only the NPK M3 cages. In cases where aggression levels escalated to the point of possible injury (i.e., biting that might break the skin), then the mice in that pairing were separated. These mice were then randomly re-paired with other mice. In study 1, six dyads were successfully paired at the first attempt, four dyads were successful at a second attempt and only one dyad required three attempts at pairing. In study 2, 13 dyads were successful at first pairing and 2 dyads were successful at the second pairing. In study 1, 22 mice were successfully paired and in study 2, 30 individuals were successfully paired. The remaining mice could not be successfully paired and could not therefore be included. In study 1 that used two sizes of cage, placement in the different cage sizes was confounded

Table 1
Aggressive, defensive and submissive behavioural categories used during 30 min observation sessions of dyads carried out over the 2-week pairing period (based on Mackintosh, 1981)

Aggressive behaviours	Defensive behaviours	Submissive behaviours
Threat	Offensive sideways	Evade
Aggressive groom	Offensive upright	Retreat
Bite	Sideways posture	Flee
Over	Upright posture	On back
Chase	Defensive sideways	Oblique posture
Rattle	Defensive upright	Kick
Circle		Crouch
Zigzag		Straight legs
Walk round		On bars
		Off bars freeze

with re-pairing in that those mice that had to be re-paired were systematically placed in the smaller cages. However, this reassignment factor did not interact with effects of interest and this methodological difference is therefore irrelevant.

The ages at pairing were 13 weeks (BKWs) and 8 weeks (CD-1s). Mice were then given a 2-week period in which to establish a polarised (dominant or subordinate) relationship. During this time the dyads were observed for aggressive, defensive and submissive behaviours to identify the dominant and subordinate member of the pair. Behaviours were recorded and classified according to defined categories (Mackintosh, 1981). This was done in 30 min observation sessions (one per day), averaged over 4 days' observations in study 1 and 12 days' observations in study 2 (see Table 1). There was no a priori criterion for rank definition.

Methodological variations between studies 1 and 2 are discussed below.

2.3. Urine collection

The first urine collections were taken after an initial settling period, before mice were paired. The settling period was 2 weeks in study 1 and 5 days in study 2. The mice were placed into an empty cage for 30 min during the mid point of the dark phase and any urine produced was collected with a syringe and stored in eppendorf tubes at −20 °C until analysis (Dahlborn et al., 1996). This method was repeated daily and the urine samples were pooled for each mouse until a minimum level of 0.2 ml was reached. This took 9 days' collections in study 1 and 6 days' collections in study 2. A second urine collection was made using the same method 2 weeks after mice had been pair housed. This took 7 days to collect in study 1 and 6 days to collect in study 2.

2.4. Urine assays

The samples were assayed by the Health & Safety Laboratories (Buxton, UK). Urinary corticosterone was measured by adaptation of commercial enzyme immunoassay kits (Correlate—EIA, Assay Designs, MI, USA). The suitability of using the commercial immunoassays with a simple dilution of urine samples was investigated prior to use for the experimental samples. Serial dilutions of mouse urine samples ($n = 4$) showed good parallelism with standard curves for corticosterone over 20–80% of the assay standard range. Mean recoveries of added corticosterone to mouse urine were 93% (range = 79–108%, $n = 4$). Mouse urine samples ($n = 4$) were extracted with ethyl acetate (1 + 1 volume) and diethyl ether (1 + 1 volume), respectively. After separation, the organic layers were blown down under nitrogen and samples reconstituted with 1 volume of kit assay buffer. Serial dilutions of solvent extracted urines and unextracted urine showed good parallelism over the standard range of the assay. Recoveries of corticosterone from 'spiked' urine, subsequently extracted by solvent, were similar to recoveries found in unextracted, diluted urine samples.

Experimental samples were then assayed (as above) after dilution 1/50 with assay buffer, using a ROSYS PLATO system for automatic pipetting, incubation and measurement stages for the assays. Urinary creatinine levels were analysed by an automated, modified Jaffe reaction, using a COBAS MIRA clinical anal-

yser (ABX UK). Quality control samples were run with each batch of urine samples for creatinine at the beginning and end of each immunoassay microtitre plate for the corticosterone assays. Urine corticosterone levels can thus be reported corrected for creatinine content to correct for differences in urine production rate and hydration status. The detectable levels are expressed as mg/mol creatinine. The between batch coefficients of variation for the creatinine and corticosterone assays were 6.9% at 2 mmol/l creatinine and 8.8% at 20 ng/ml corticosterone.

Samples from four animals (two BKW and two CD-1 mice) were excluded because one of the samples (pre- or post-) was too low in volume. Thus from the sub-set of mice that were successfully paired, the analyses were based on the following group sizes: BKW $n = 20$ and CD-1 $n = 28$.

2.5. Statistical analyses

All data analysis was carried out using the SPSS statistical package, version 11.0. Aggressive, defensive and submissive behaviours were compared between the two strains using univariate analysis of variance (ANOVA) on the average number of aggressive, defensive and submissive acts recorded per observation, with strain and social status as between subjects factors. Corticosterone levels were analysed as mg/mol of creatinine. A repeated measures ANOVA was used to compare corticosterone levels pre- and post-pairing, again with strain and social status as between subjects factors. Finally, the corticosterone analyses were repeated separately for each of the strains, with the additional between subjects factor of reassignment (to new cage mates and cages) for the BKW mice. In each case, significant main effects and interactions were explored with independent samples *t*-tests, with corrected degrees of freedom as appropriate (using Levene's test for equality of variance).

3. Results

3.1. Aggressive behaviours

There was a significant main effect of strain, $F(1, 44) = 6.76$, $p = 0.01$. This arose because of the overall higher number of aggressive acts in BKW relative to CD-1 dyads, $t(29.07) = 2.22$, $p < 0.05$ (see Fig. 1).

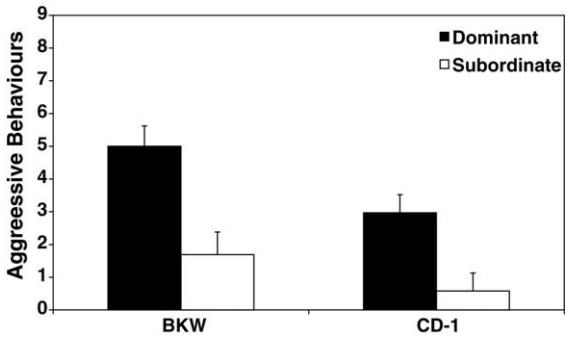

Fig. 1. Mean number of aggressive behaviours recorded per 30 min observation session for dominant and subordinate BKW and CD-1 mice. Error bars are standard errors about the mean for approximate between groups comparisons.

There was also a significant main effect of social status, $F(1, 44) = 22.11$, $p < 0.01$. By definition, dominants produced significantly more aggressive behaviours than subordinates, $t(18) = 2.74$, $p = 0.01$ and $t(16.62) = 4.25$, $p < 0.01$, for BKW and CD-1 mice, respectively (see Fig. 1). However, there was no interaction between social status and strain, $F(1, 44) = 0.57$. Thus, BKW mice of both ranks produced more aggressive behaviours than the CD-1 dyads.

3.2. Defensive behaviours

There was no main effect of strain, $F(1, 44) = 0.74$, or of social status, $F(1, 44) = 2.13$, neither was there any strain × social status interaction, $F(1, 44) = 1.56$.

3.3. Submissive behaviours

There was no main effect of strain, $F(1, 44) = 0.54$. However, there was a significant main effect of social status, $F(1, 44) = 26.42$, $p < 0.01$. As would be expected, in both strains, subordinates produced significantly more submissive behaviours than dominants (BKW subordinates mean = 2.44 (±0.41), dominants mean = 0.61 (±0.37), $t(11.15) = 3.18$, $p = 0.01$; CD-1 subordinates mean = 2.21 (±0.33), dominants mean = 0.32 (±0.33), $t(15.21) = 3.99$, $p < 0.01$). There was no strain × social status interaction, $F(1, 44) = 0.01$.

3.4. Corticosterone levels

There was a clear main effect of strain on corticosterone levels, $F(1, 44) = 55.25$, $p < 0.01$. This arose because CD-1 mice had significantly higher corticosterone levels at both the pre-, $t(30.91) = 5.84$, $p < 0.01$ and post-pairing, $t(46) = 6.39$, $p < 0.01$, urine assay compared to BKW mice (Fig. 2).

There was no overall effect of pairing on corticosterone levels, $F(1, 44) = 1.24$, no pairing × strain interaction, $F(1, 44) = 2.69$ and no pairing × social status interaction, $F(1, 44) = 0.62$. Neither was there was any main effect of social status, $F(1, 44) = 2.45$. However, as there was a marginal social status × strain interaction, $F(1, 44) = 3.45$, $p = 0.07$ and there were some methodological differences between the studies (discussed below), there were grounds to test for rank effects separately in each of the strains. This also allowed us to test for any differences that might arise in consequence of the reassignment to cage mates and cages in study 1 (that arose because initially high levels of aggression meant that some of the BKW mice had to be re-paired).

When this was done, the BKW mice showed no overall effect of pairing, $F(1, 16) = 1.05$, social status: $F(1, 16) = 0.15$ or reassignment, $F(1, 16) = 0.17$. Neither were there any interactions: for pairing × social status interaction, $F(1, 16) = 1.19$ or pairing × reassignment interaction, $F(1, 16) = 0.46$, or social status × reassignment interaction, $F(1, 16) = 0.001$.

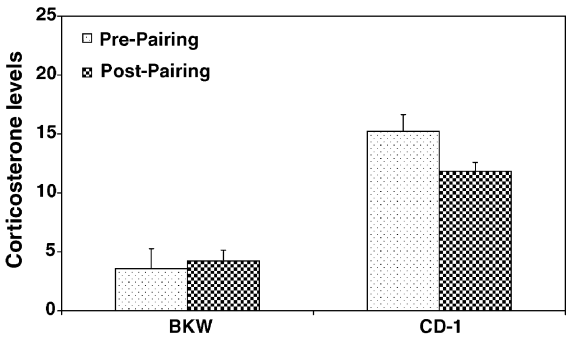

Fig. 2. Mean corticosterone levels (mg/mol of creatinine) for BKW and CD-1 mice from the pre- and post-pairing urine assays. Error bars are standard errors about the mean for approximate between groups comparisons.

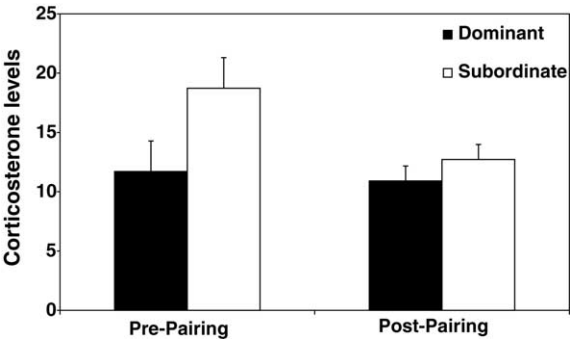

Fig. 3. Mean corticosterone levels (mg/mol creatinine) for dominant and subordinate CD-1 mice from the pre- and post-pairing urine assays. Error bars are standard errors about the mean for approximate between groups comparisons.

By contrast, the CD-1 mice showed a main effect of social status, $F(1, 26) = 4.68$, $p < 0.05$. This arose because dominants had overall lower corticosterone levels at both the pre- and post-pairing urine assay compared to subordinates. Although Fig. 3 suggests that corticosterone levels dropped in subordinate mice after pairing, there was neither significant effect of pairing, $F(1, 26) = 2.84$, nor any pairing × social status interaction, $F(1, 26) = 1.67$.s

4. Discussion

The present studies demonstrate that mouse urinary corticosterone levels can be successfully measured by adaptation of commercial enzyme immunoassay kits. We found a strain difference in that CD-1 mice showed higher corticosterone levels compared with BKW mice. This difference was apparent both before and after being paired with a conspecific. To our knowledge, this is the first direct comparison between these two strains of mice. CD-1 mice are a commonly used laboratory strain. However, there is relatively little behavioural data on BKWs, although they are a strain used in this laboratory to study relationships between social status, immune function and learning (Barnard and Luo, 2002; Barnard and Behnke, 2001).

In addition, there was a social status effect within the CD-1 strain in that males classed as dominant had overall lower levels of urinary corticosterone compared to subordinates. We consider possible limitations on

the comparison between the strains before going on to discuss the implications of these findings.

4.1. Methodological issues

We took care that the duration of the urine collection time used in these studies was too short for the pre- and post-baseline measures of corticosterone to be confounded by any acute response to being placed in the collection chamber (Dahlborn et al., 1996).

However, during study 1 (with the BKW mice) some husbandry issues arose, due to unexpectedly high levels of aggression when pairing males. This meant that we had to further refine the methodology used in study 2 (with the CD-1 mice). The changes introduced (reducing the settling period and the number of attempted pairings) were intended to minimise the time that mice spent singly housed as single housing has been found to increase later aggressive behaviour (Valzelli, 1973). In addition, the urine sampling period did not need to be as long. These changes contributed to the age difference at which the mice were paired.

This age difference between the strains could in principle contribute to the difference in corticosterone levels found in studies 1 and 2. There is some evidence for a gradual increase in glucocorticoids throughout ageing (De Kloet, 1992). However, the age difference in the present studies (total 5 weeks) is unlikely to provide a complete account of the large difference in corticosterone levels observed between the strains. Moreover, the CD-1 mice (in study 2) were relatively younger yet showed higher corticosterone levels, rather than the lower levels that would be predicted if the difference were mediated by age-related changes.

Statistically, the need to reassign some of the pairings made no difference whatsoever to the results of study 1 so we have no evidence that this factor contributed to the observed strain difference. In study 2, only a couple of dyads had to be reassigned so this factor was not an issue.

In any event, the methodological variation that was required could be argued to be a consequence rather than a cause of the apparent strain difference. This interpretation is supported by the fact that differences in corticosterone were apparent before the mice were paired and therefore before methodological differences between the studies introduced.

4.2. How was the strain difference mediated?

In rats, there is evidence that circulating glucocorticoids can increase aggressive behaviours (Kruk et al., 1998; Mikics et al., 2004). Therefore, differences in aggression levels between the two strains could in principle account for the higher corticosterone levels observed in the CD-1 mice, as this strain is known to have one of the highest rates of inter-male attack (Kudryavtseva et al., 2002). Against this interpretation of the strain difference, BKW mice are also known to be somewhat aggressive (Barnard and Luo, 2002) and in the present study 1 they fought more than the CD-1 mice (hence the need to separate some of the pairings).

One possibility is that corticosterone levels are related not to levels of aggression as such but to some other correlate of the development of a polarised dominance relationship, perhaps the lability of the adrenal stress response. Behaviourally, both strains showed rank-related differences (e.g., in the number of aggressive acts). However, only the CD-1 mice showed a rank effect on corticosterone. The fact that the CD-1 mice also had higher levels of corticosterone overall suggests that the influence of rank may be dependent on baseline adrenal function. A series of post-pairing urinary assays would be needed in order to directly relate (changes in) corticosterone levels to the development of polarised dominance relations.

4.3. How was the rank difference mediated?

As might be expected the social rank effect found in CD-1 mice took the form that the subordinates had higher corticosterone levels than the dominants, both before and after pairing, with no evidence that pairing accentuated this difference. Previous studies have found that (later) social rank correlates with (earlier) characteristics such as suckling position and rate of weight gain (Barnard et al., 1998), as well as the level of scent marking (Collins et al., 1997). The difference in corticosterone levels found in present study (prior to pairing and the development of a polarised relationship) also provides evidence that there are pre-existing differences between mice that later assume different social ranks.

The number of defensive behaviours was not sensitive to the development of dominance relations. However, behaviourally, there were effects of social status

in both strains, with dominant mice making a significantly greater number of aggressive acts compared to subordinates. This effect of social status was also confirmed by the number of submissive behaviours. Despite this evidence for the development of dominance, we found no differences in corticosterone levels as a function of rank in the BKW strain. The slightly higher number of re-pairings that were necessary with the BKW strain could in principle have resulted in less polarised dyads. In other words, the BKW mice may have been paired with conspecifics that they were better able to tolerate and so less likely to develop an extreme dominant/subordinate relationship. However, there was no evidence for this interpretation of the lack of rank-related differences in corticosterone levels as the average number of aggressive behaviours was higher in the BKW strain (compared with CD-1 mice, see Fig. 1). It therefore seems unlikely that the lack of difference in corticosterone levels was due to the absence polarised dominance relationships in the BKWs.

4.4. Conclusions and implications

We find evidence for physiological differences, likely to be related to the stress response, between two outbred laboratory strains of mice. Such differences have implications for comparison across strains in laboratory experiments that involve mild stress, specifically social stress. We found elevated corticosterone levels in CD-1 mice which would later become subordinate suggesting that the cost of social living might depend on social status. However, there was no indication that corticosterone levels were increased (relative to this pre-existing difference) when the mice were paired with a conspecific. On the contrary, although the CD-1 mice had overall higher levels of corticosterone, these levels showed some tendency in fact to drop (in the subordinate mice which are more likely to lose fights) when the mice were paired. Thus, although there is very good evidence that social housing can result in stress, in so far as this is measured by corticosterone levels, there is no evidence that the negative effects of social defeat outweigh the benefits of social living.

Finally, the fact that the CD-1 strain showed both an effect of rank on urinary corticosterone levels and the overall highest levels of corticosterone may suggest a relationship between the predisposition to polarise into dominant and subordinate mice and the lability of the adrenal response. Moreover, within the CD-1 strain we find evidence that high corticosterone levels can predict the later adoption of subordinate status. Further controlled experiments are needed to identify which factors mediate these effects and to identify the relative contributions of exposure to visual cues and the odours of conspecifics, in the absence of any physical contact. In general, urinary corticosterone assays provide a non-invasive method objectively to predict the welfare implications of group housing and potentially to predict other behavioural outcomes known to be influenced by social status (Barnard and Luo, 2002).

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