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Evidence for a mechanism of phenotypic integration of behaviour and innate immunity in a wild rodent: implications for animal personality and ecological immunology

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If a single mechanism influences multiple traits, it may facilitate functional integration or impede optimal trait expression to produce consistent individual differences and correlations among those traits. The fields of animal personality and ecological immunology each aim to understand variation and covariation of behavioural and immune traits. Studying these traits together may provide additional insight into patterns of (co)variation than studying behaviours or immunity in isolation, as trade-offs between behaviour and immunity are likely. Hormonal mechanisms may be involved in the variation and covariation between behavioural and immune traits, and the role of receptors in particular has rarely been tested in wild animals. In wild-caught Belding's ground squirrels, *Urocitellus beldingi*, we delivered mifepristone to experimentally block the actions of glucocorticoid receptors (GRs), a component of the stress response. Then we evaluated whether cortisol binding with GRs affects the plasticity of behavioural and immune traits, consistent individual differences and phenotypic integration of exploratory behaviour, activity, antipredator behaviour, response to restraint and bacteria-killing ability, a measure of innate immunity. Mifepristone treatment abolished relationships between faecal glucocorticoid metabolite levels and both exploratory behaviour and bacteria-killing ability. This result indicates that cortisol binding with GRs is a mechanism of plasticity of those traits. Mifepristone also affected relationships among traits. Specifically, mifepristone treatment significantly modulated the relationships between bacteria-killing ability and two behaviours, exploration and activity. This result supports the hypothesis that the GR–cortisol binding is a mechanism of phenotypic integration. Together, these results suggest that GR–cortisol binding balances the often observed trade-off between behaviour and immunity to produce patterns of (co)variation of behavioural and immune traits seen in nature.

An individual's behavioural traits can vary from moment to moment in response to environmental change (i.e. phenotypic plasticity; Pigliucci, 2001; West-Eberhard, 1989). This has led to extensive investigation of the reasons why these traits are often correlated and show consistent individual differences over time and across environments (Dingemanse, Kazem, Réale, & Wright, 2010; Réale, Reader, Sol, McDougall, & Dingemanse, 2007; Sih, Bell, & Johnson, 2004). Such correlations (i.e. behavioural syndromes) and consistent individual differences in behaviour (i.e. animal personality) correspond to similar patterns of variation of immune traits described by the field of ecological immunology (Ardia, Parmentier, & Vogel, 2011; Schmid-Hempel, 2003; Sheldon & Verhulst, 1996). Researchers have questioned whether physiological mechanisms are responsible for these patterns of (co)variation, but the extent to which such mechanisms influence behavioural and immune variation and covariation remains unresolved (Ardia et al., 2011; Demas, Adamo, & French, 2011; Duckworth & Sockman, 2012; Garamszegi et al., 2012; Koolhaas, 2008; Krams et al., 2013; Sih et al., 2004). If multiple traits share a single mechanism, then that shared mechanism can facilitate functional integration of those traits or impede their independent expression, analogous to the pleiotropic effects that a single gene may have on multiple traits (Duckworth & Sockman, 2012; Garamszegi et al., 2012; Ketterson & Nolan, 1999; Krams et al., 2013). By this reasoning, a number of traits may be relevant, but in this study we focus on relationships between behaviour and

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immunity because immunity is relatively understudied with respect to animal personality (but see Kluen, Siitari, & Brommer, 2014; Krams et al., 2013; Sild, Sepp, & Hõrak, 2011) and both are central to other hypotheses of trait covariation and maintenance of variance (e.g. Ezenwa, Stefan Ekernas, & Creel, 2012; L. B. Martin, Brace, Urban, Coon, & Liebl, 2012; Rubenstein & Hauber, 2008). Here we investigated whether a single physiological mechanism influences behaviour and immunity of Belding's ground squirrels, *Urocitellus beldingi*, to produce phenotypic integration (i.e. a behavioural syndrome involving immunity). This will help clarify whether a mechanism accounts for the variability in behaviour and immunity that the fields of animal personality and ecological immunology aim to explain.

The stress response is part of the physiological reaction of individuals to environmental and social challenges, making it a likely mechanism of change in many traits, including behavioural and immune traits. When addressing the physiological stress response, researchers often manipulate and measure glucocorticoids, usually corticosterone or cortisol depending on the species, which are steroid hormones produced by the adrenal glands (Cockrem, 2007; Reeder & Kramer, 2005). Glucocorticoids mobilize energy, regulate immune and reproductive systems, and influence behaviour (Cockrem, 2007; Demas et al., 2011; Reeder & Kramer, 2005; Sapolsky, Romero, & Munck, 2000). By this account, it seems inevitable that environmental stimulation of glucocorticoid release will have wide-ranging effects. However, many of these studies have focused on one type of trait, while research on glucocorticoid-associated phenotypic integration of suites of traits reveals that phenotypic independence is not uncommon (e.g. Buehler et al., 2012; Garamszegi et al., 2012; Koolhaas, De Boer, Coppens, & Buwalda, 2010). This may be because the actions of glucocorticoids have multiple pathways (Sapolsky et al., 2000). After release in response to a real or perceived environmental challenge, glucocorticoids can exert nongenomic effects, but most often bind with two types of receptors that initiate transcription (Groeneweg, Karst, de Kloet, & Joëls, 2011; Sapolsky et al., 2000). Mineralocorticoid receptors (MRs) bind to glucocorticoids with high affinity and are nearly saturated at baseline levels, whereas glucocorticoid receptors (GRs) bind to glucocorticoids with a 10-fold lower affinity (Sapolsky et al., 2000). Both receptors are ligand-driven transcription factors, meaning that when unbound they primarily reside in the cytoplasm and after binding with cortisol (CORT) they migrate to the nucleus to directly and indirectly affect gene transcription (Groeneweg et al., 2011). These genomic effects comprise many common aspects of the stress response, but the specific genes affected by GRs and MRs are largely different (Datson, van der Perk, de Kloet, & Vreugdenhil, 2001).

We focus on GRs because their activation covaries with fluctuations in glucocorticoid levels (Stavreva et al., 2009), and while a few studies have investigated their impact on behaviour or immunity in wild animals (Landys, Piersma, Ramenofsky, & Wingfield, 2004; Landys, Ramenofsky, Guglielmo, & Wingfield, 2004; Lattin, Waldron-Francis, & Romero, 2013), it is not known whether they act as a mechanism of phenotypic integration of those traits in free-ranging animals. Experimental evidence suggests that although acute stress can downregulate GRs, bioavailability of GRs does not substantially vary over the course of a month under chronic stress (Paskitti, McCreary, & Herman, 2000). Furthermore, the developmental causes of variation in GR levels have been explored in detail, and indicate that differences in GR expression are stable into adulthood (Weaver et al., 2004). In turn, manipulating or blocking GRs should interfere with the effects that fluctuations in glucocorticoid levels (which change on the order of minutes, hours and days) produce via binding with the GR (Stavreva et al., 2009). This motivation is based on studies of rats (*Rattus norvegicus*) in laboratory conditions, but the evolutionarily conserved nature of the stress response supports applying it in other rodents (Ellis, Jackson, & Boyce, 2006). Manipulating a single pathway may shed light on the role that mechanisms play in consistent individual differences and correlations of behavioural and immune traits.

A wealth of research has elucidated relationships between glucocorticoids and behaviour (Cockrem, 2007; Downs et al., 2012; L. B. Martin et al., 2012; Reeder & Kramer, 2005) as well as glucocorticoids and immunity (Bourgeon & Raclot, 2006; Brooks & Mateo, 2013; Demas et al., 2011; Downs et al., 2012; L. B. Martin et al., 2012). Laboratory research on rodents has shown that acute and chronic elevation of glucocorticoids can also have differing effects on behaviour (Sapolsky et al., 2000). Likewise, in the short term, acute increases in glucocorticoids can help activate inflammation, but glucocorticoids are primarily anti-inflammatory, particularly when elevated chronically (Sorrells & Sapolsky, 2007). Long-term inflammation can produce glucocorticoid resistance (i.e. insensitivity), which can counter the dynamics described by Sorrells and Sapolsky (2007). Glucocorticoid resistance is often associated with major pathophysiology in humans (Gross, Lu, & Cidlowski, 2009; Pace, Hu, & Miller, 2007). It can also be caused by a week of social defeat in rats (Avitsur, Stark, Dhabhar, Padgett, & Sheridan, 2002). In wild alpine marmots, *Marmota marmota*, and eastern chipmunks, *Tamias striatus*, glucocorticoid measures positively correlate with open-field behaviour (Costantini et al., 2012; Ferrari et al., 2013; Montiglio, Garant, Pelletier, & Réale, 2012). These studies demonstrate that the stress response plays a key role in both behavioural and immune variation, suggesting that glucocorticoids can affect the relationship between behaviour and immunity. In essence, such an effect of glucocorticoids would be a three-way interaction among those factors, but this has rarely been tested in wild animals. L. B. Martin et al. (2012) found evidence of allocation trade-offs between flight performance and innate immunity in response to the stress of captivity in wild-caught house sparrows, *Passer domesticus*, but to our knowledge the effect of glucocorticoids on the relationship between behaviour and immunity has not been tested in a wild mammal, and no study on wild animals has evaluated the effect of glucocorticoids on multiple behaviours and immunity. Furthermore, GRs may play a key role in relationships between behaviour and immunity. However, few studies have directly addressed the impact of GRs on natural patterns of variation in either behaviour or immunity (e.g. Landys, Piersma, et al., 2004; Landys, Ramenofsky, et al., 2004; Lattin et al., 2013), with a particular lack of studies on the effect of GR–glucocorticoid binding on both behaviour and immunity to determine whether GR–glucocorticoid binding is responsible for relationships between those traits. The stress response's joint relationship with behaviour and immunity may be key to explaining the variation of each of those traits.

The stress response may cause behaviours and immunity to covary at multiple levels, both within and between individuals (Dingemanse & Dochetermann, 2013; Downs & Dochtermann, 2014). At one level, traits may covary within an individual as traits change together when individuals encounter differing conditions. At another level, the stress response may cause between-individual covariance, in which the individual average responses of two traits are correlated. Ferrari et al. (2013) found evidence that marmots' between-individual covariance among behavioural and physiological traits differed from the within-individual covariance pattern. Interestingly, glucocorticoids did not correlate with behaviour, heart rate or breathing rate at the between-individual level as predicted by the 'coping styles model', but rather showed correlations at the within-individual level in support of the recent 'two-axes model' (see Koolhaas et al., 2010 for details on models). In house mice, *Mus domesticus*, selected for high voluntary activity, corticosterone

showed a correlated response to selection (Downs et al., 2012). However, a within-individual trade-off between behaviour and immunity persisted in both selected and nonselected lines. Despite independence between the evolution of behavioural and immune traits, both control and artificially selected lines exhibited trade-offs between behavioural and immune responses. These results contradict the hypothesis that hormonal pleiotropies dictate evolutionary trajectories of behaviour and immunity, yet they provide evidence that even with independent evolutionary responses to differing selection pressures, hormonal pleiotropies dictate trade-offs between behaviour and immunity. Field studies in western bluebirds, *Sialia mexicana*, and collared flycatchers, *Ficedula albicollis*, also did not find evidence for hormonal pleiotropies explaining animal personality (Duckworth & Sockman, 2012; Garamszegi et al., 2012), calling for additional tests of the hypothesis that assess other endocrine elements such as receptors.

Belding's ground squirrels exhibit a behavioural syndrome (Dosmann, Brooks, & Mateo, 2015), and relationships among CORT, behaviours and immunity in the species open questions about whether GR–CORT binding is a mechanism accounting for patterns of (co)variation of behaviour and immunity. CORT levels of squirrels vary with environmental variables such as habitat features and predation threat (Mateo, 2007, 2010), meaning that phenotypic responses to changes in CORT can be interpreted in terms of plasticity. For example, because predation threat increases CORT (Mateo, 2010), we expect antipredator responses to threat to be positively related to CORT levels. CORT also has an inverted U-shaped relationship with associative learning of antipredator responses such as vigilance and escape behaviour, and decreases innate immunity of squirrels when chronically elevated (Brooks & Mateo, 2013; Mateo, 2008), making the species an excellent one in which to evaluate the role of GR–CORT binding in the plasticity and (co)variation of behaviour and immunity. In this study, we measured four behavioural traits of squirrels: activity, exploration, antipredator behaviour and response to restraint, as well as a functional measure of constitutive innate immunity, bacteria-killing ability (BKA). Activity, exploration and response to restraint comprise a behavioural syndrome in free-ranging Belding's ground squirrels, with activity and exploration positively correlating at the between-individual and within-individual level while both behaviours negatively correlate with response to restraint at the between-individual level (Dosmann et al., 2015). In addition, CORT and body condition have an interactive effect on squirrels' activity, with squirrels in good condition showing a positive relationship between CORT and activity (Dosmann, Brooks, & Mateo, n.d.). Brooks and Mateo (2013) treated *U. beldingi* with exogenous CORT and found that it decreases BKA while also reducing squirrels' ability to mount immune responses to a lipopolysaccharide challenge; therefore, blocking the actions of GRs are expected to have an anti-inflammatory effect, similar to that found in other species (Sorrells & Sapolsky, 2007). Experimentally elevated glucocorticoids also affect the activity behaviour of wild-caught squirrels, inhibiting the decrease of squirrels' activity over the course of a test occasion (Dosmann, n.d.). These data, paired with the expectation of a trade-off between behaviour and immunity (L. B. Martin et al., 2012) support hypotheses that binding of GRs to glucocorticoids produces patterns of (co)variation among behaviour and immunity. Prior research on the effects of glucocorticoids and GRs on behaviour and immunity (e.g. Gross et al., 2009; Landys, Piersma, et al., 2004; Landys, Ramenofsky, et al., 2004; Lattin et al., 2013; Sorrells & Sapolsky, 2007) also raises the issue that the expected patterns of (co)variation differ depending on the timescale. The prior results of CORT's effect on behaviour and immunity in *U. beldingi* (Brooks & Mateo, 2013; Dosmann et al., n.d.; Mateo, 2008) and the nature of our experiment in this study indicate that we are testing the hypothesis of GR-driven integration of CORT, behaviour and innate immunity on a relatively long-term timescale, but not one involving glucocorticoid resistance (see Avitsur et al., 2002 for timescale of glucocorticoid resistance in rats).

In this study, we delivered mifepristone, a glucocorticoid receptor antagonist, to Belding's ground squirrels and measured multiple traits to test a series of hypotheses regarding individual variation in behaviour and immunity. We wanted to determine whether GR binding with CORT is a mechanism for change of these traits, but we were especially interested in determining whether GR–CORT binding affects the relationships between multiple traits. To answer these questions, we evaluated three nonmutually excluding hypotheses using experimental data from squirrels temporarily brought into captivity from the wild and treated with mifepristone. First, we tested the hypothesis that GR–CORT binding is a mechanism of plasticity for each of the measured traits (Hypothesis 1). If GR–CORT binding is a mechanism of plasticity in *U. beldingi*, blocking GRs could have two effects. First, the slope of the relationship between the trait and CORT could be significantly diminished by mifepristone treatment. Also, mifepristone could have a main effect that counteracts the effect of CORT. In other words, the direction of mifepristone's treatment effect would be opposite of the relationship between the trait and CORT. Second, we evaluated the hypothesis that GR–CORT binding is a mechanism of consistent individual differences (Hypothesis 2). If among-individual differences in GRs cause animal personality, then experimentally blocking GRs will decrease behavioural variance by cancelling out the effects of differences in GRs. Finally, we tested the hypothesis that GR–CORT binding is a shared mechanism that dictates the relationship among behavioural and immune traits (Hypothesis 3). If GRs are part of a mechanism of phenotypic integration, blocking them will significantly change the relationships between traits. Testing these hypotheses together will provide insight into whether glucocorticoid receptors play a role in the phenotypic (co)variation of behaviour and immunity in *U. beldingi*.

METHODS

Study Subjects

Belding's ground squirrels are diurnal rodents that live in the Sierra Nevada and southern Cascade mountains. They are active between April and August and hibernate the remainder of each year. To address whether glucocorticoid receptors affect phenotypic plasticity, animal personality, and phenotypic integration, we trapped 18 squirrels from a population located in Rock Creek Canyon near Mammoth Lakes, California, U.S.A. (37°27′56″N, 118°43′30″W) in July and August of 2011. We used both male ($N = 7$) and female ($N = 11$) squirrels weighing over 150 g, weighed using an Ohaus balance (Ohaus Corp., Parsippany, NJ, U.S.A.). These included adults ($N = 7$), yearlings ($N = 5$) and older juveniles (~2 months old; $N = 6$). We assigned sex based on anogenital distance and discriminated age classes by a combination of reproductive status, fur condition and weight (Sauer & Slade, 1987). We housed squirrels at Sierra Nevada Aquatic Research Laboratory (37°36′51″N, 118°49′47″W), kept them in individual plastic cages ($38 \times 33 \times 11$ cm; solid sides and bottom, wire lid) and provided them with sunflower seeds and four to five pieces of Mouse Diet 5015 (LabDiet, Richmond, IN, U.S.A.) each morning and water ad libitum. We maintained a 13:11 h light:dark cycle. We tested squirrels over the course of 1 month (11 July 2011–18 August 2011), giving roughly 1 week for acclimation to captivity before testing. After testing, we released squirrels at their site of capture.

Variables

Exploration

We measured exploration behaviour of squirrels in a holeboard test (J. G. Martin & Réale, 2008), which is a 122 × 122 cm modified open-field apparatus. The arena had 61 cm tall walls and a wire mesh top to prevent escape. The testing apparatus contained four false burrows evenly spaced 40 cm apart, which provided species-relevant spaces to investigate. We released squirrels into the arena from a Tomahawk trap (Tomahawk Live Trap, Hazelhurst, WI, U.S.A.) via a door in the arena wall, and we counted the number of head dips into the false burrows during a 5 min trial as our dependent variable measuring exploration. Although this measure of exploration requires an individual to be somewhat active, File and Wardill (1975) showed that exploration and activity are not necessarily correlated. To prevent the odours of previously tested squirrels from affecting results we lined the arena with acrylic and cleaned it with ethanol following every trial. We videotaped this test, along with the test of activity and antipredator behaviour, to score later blind to treatment and squirrel identity.

Activity

We measured locomotor activity of squirrels in the holeboard apparatus during the same trial in which we measured exploration. We marked the holeboard into 16 quadrants and counted the number of lines crossed during the 5 min trial as our dependent variable measuring activity.

Antipredator behaviour

In this experiment we used refuge use as a measure of antipredator behaviour (Dosmann & Mateo, 2014). The refuge use test was conducted as a 10 min trial separate from the activity and exploration measurements. We augmented the holeboard test described above with an additional apparatus that covered the false burrows and provided a usable burrow system whose openings were evenly spaced around the arena. We released squirrels into the arena from a Tomahawk trap via a door in the arena wall. We included a food odour stimulus in the test arena outside the burrow system to recreate the natural situation in which squirrels forage outside the burrow but take refuge from predators inside their burrow. We smeared a small amount of peanut butter on a 3 cm^3 polypropylene cube surrounded by wire mesh. Odours could disseminate but squirrels could not access the cube. Approximately 5 min after releasing the squirrel into the arena, and when it was outside the artificial burrow system, we threw a frisbee over the top of the arena from a hidden location to simulate an attacking raptor. Squirrels responded as if they had experienced a predation attempt, often fleeing into the burrow. We then measured the proportion of the remaining test (10 min minus the pre-frisbee time of roughly 5 min) that the squirrel spent inside the safe refuge as our dependent variable for antipredator behaviour.

Behavioural response to restraint stress

Our last behavioural measure consisted of restraining squirrels in a small canvas bag (28 × 22 cm) to measure the proportion of time they spent immobile during 1 min of restraint. This test was not videotaped. One observer (A.D.) pulled squirrels from a Tomahawk trap by hand, placed them into the bag and then suspended the bag in the air for 1 min. We measured the amount of time spent immobile with a stopwatch. This behaviour is similar to tonic immobility, an important antipredator response (Réale et al., 2007). We interpret this test as a measure of docility, defined as the response to human handling (J. G. Martin & Réale, 2008).

Innate immunity

We measured constitutive innate immune function with a bacteria-killing ability (BKA) assay. This assay measures the ability of plasma proteins to kill *Escherichia coli*. Results from this assay represent a functional assessment of individuals' ability to clear a bacterial infection, as individuals that kill more *E. coli* ex vivo should be able to better kill *E. coli*, or other invading bacteria, in vivo (Tieleman, Williams, Ricklefs, & Klasing, 2005). We collected blood from squirrels in capillary tubes via a toenail clip, chilled the blood and then centrifuged it to separate the plasma. We diluted plasma samples 1:30 with CO_2-independent media and added 3 μl of *E. coli* solution (EPower Microorganisms *#0483E7*, MicroBioLogics, St Cloud, MN, U.S.A.) to each diluted plasma sample to obtain 150–200 bacteria colonies per sample. Bacteria–plasma cocktails were incubated at 37 °C for 30 min to allow killing to occur. Next, we added 50 μl to agar plates and incubated them upside down at 37 °C overnight. The following day, we counted colony-forming units (CFUs) on each plate. We performed this assay in duplicate. In addition, we made positive and negative control plates. Positive controls contained 200 μl of media and 3 μl of *E. coli* solution, and negative controls contained 200 μl of media and 3 μl of phosphate-buffered saline solution. The proportion of bacteria colonies on the duplicate sample plates compared to the positive control was used as the measure of BKA. We conducted every part of the BKA assay (except incubation) under laminar flow conditions to avoid contamination. Contaminated plates were excluded from analysis. No negative control plates produced any CFUs.

Cortisol. Cortisol (CORT) is the primary glucocorticoid hormone in *U. beldingi* (Mateo & Cavigelli, 2005). Since the actions of GRs are dependent on binding with CORT, we obtained a measure of CORT on each test occasion to evaluate treatment effect statistically with respect to GR–CORT binding specifically. CORT metabolites excreted in the faeces are an effective proxy for circulating CORT levels in squirrels. In *U. beldingi* serum CORT levels and faecal glucocorticoid metabolite levels are positively and significantly correlated, and they reflect both baseline and stress-induced levels of CORT (Mateo & Cavigelli, 2005). Faecal glucocorticoid metabolites respond to adrenocorticotropin hormone (which provokes a release of CORT into the bloodstream) within 6–30 h (Mateo & Cavigelli, 2005). We obtained a faecal sample on each test occasion, which we froze in a commercial freezer (−20 °C) before mailing back to The University of Chicago, where samples were stored at −80 °C until analysis. We quantified the amount of faecal glucocorticoid metabolites in each sample using the extraction protocol and ^{125}I-cortisol Corticote® radioimmunoassay kit (MP Biomedicals, Irvine, CA, U.S.A.) described in Mateo and Cavigelli (2005). We dried and homogenized faecal samples before weighing 0.2 g of dried faecal material, from which we extracted faecal glucocorticoid metabolites by adding 1.5 ml of 80% ethanol, briefly vortexing (~3 s) and immediately centrifuging at 2500 *g* for 20 min. For the radioimmunoassay, we ran duplicates of samples and reassayed any sample with a coefficient of variation over 20%. We analysed two control samples, created by pooling samples from five individuals with high binding and five individuals with low binding, at the beginning and end of each assay. The mean intra- and inter-assay coefficients of variation were 11.28% and 9.98%, respectively, for the low control and 7.66% and 11.50% for the high control.

Experimental Manipulation

We used mifepristone, also known as RU-486 (Sigma-Aldrich, St Louis, MO, U.S.A.), to block the actions of GRs experimentally. Mifepristone selectively and antagonistically binds to GRs over MRs

(Sitruk-Ware & Spitz, 2003). Mifepristone binds to GRs with higher affinity than CORT, but leaves GRs biologically inert (Moguilewsky & Philibert, 1984). Thus, we expected that mifepristone treatment would counteract the effects of GR–CORT binding on a trait.

Mifepristone also binds to progesterone receptors (Sitruk-Ware & Spitz, 2003). To avoid potential confounds due to mifepristone's effects on progesterone receptors, we tested squirrels late in the active season, after females were more than 1 month past gestation and males were more than 2 months past mating season. In *U. beldingi* females, progesterone levels are drastically lower outside of gestation, and there is no relationship between postpartum levels of progesterone and vigilance or feeding behaviour (Nunes, Muecke, Ross, Bartholomew, & Holekamp, 2000). In male rodents, effects of progesterone primarily consist of interactions with testosterone (Wagner, 2006). Testosterone levels peak during mating season in adult male *U. beldingi*, and testosterone levels in juveniles, yearling and postmating-season adults are very low, often below detection levels of the assay (0.025 ng/ml plasma; Nunes, Duniec, Schweppe, & Holekamp, 1999). Our experiment took place roughly 2 months after the mating season, which should avoid these potential effects of mifepristone treatment. So although we must qualify our interpretations in light of the actions of mifepristone, the known seasonal hormone profiles and effects of CORT, progesterone and testosterone on behavioural and immune traits in *U. beldingi* (Brooks & Mateo, 2013; Mateo, 2007, 2008; Nunes et al., 1999; Nunes et al., 2000) support attributing treatment effects to the actions of GRs.

We delivered mifepristone for 3 days orally via a mixture of peanut butter and wheat germ, and selected a dose (50 mg per kg per day) corresponding to oral doses that effectively block GRs in humans (600 mg/person; Belanoff, Flores, Kalezhan, Sund, & Schatzberg, 2001) and rats (54 mg per kg per day; Wu et al., 2007). Every morning at 0700 hours, we gave squirrels the peanut butter and wheat germ mixture along with their daily chow. When in the treatment condition, squirrels received mifepristone in their peanut butter and wheat germ mixture, and only plain mixture in the control condition. Since peanut butter is a favourite food of *U. beldingi* (Mateo, 2008), the mixture was consumed soon after delivery in most cases.

Experimental Design

To test the effects of mifepristone on behavioural and immune variation of *U. beldingi* we used a repeated measures experimental design where each individual was its own control. Each squirrel was measured for all traits in both control and experimental treatments. We randomized and balanced the order of treatment so that half of the squirrels received mifepristone treatment on their first set of trait measurements and the other half received control treatment on their first set of trait measurements. We waited 3 days between measurements to ensure that mifepristone had exited the system of individuals who received mifepristone treatment first. We chose the 3-day treatment duration and inter-test interval based on data from rats showing that this ensures drug efficacy in the experimental condition and allows sufficient time for the treatment to be cleared from the body before testing the control condition (Sitruk-Ware & Spitz, 2003). We began the battery of tests by removing the squirrel from its home cage and placing it in a Tomahawk trap, from which it entered the behavioural test arena through a door in the wall. Tests were videotaped and scored later by an observer blind to the study's hypotheses and the squirrels' identities and treatment status. Unless otherwise indicated, all types of behavioural tests were recorded and scored in this way. After the 5 min trial testing exploration and activity, we shepherded the squirrel out the door and back into the trap so we could place an artificial burrow system in the arena, which took less than 1 min. We then released the squirrel back into the arena for the antipredator test. After the 10 min antipredator test, we shepherded the squirrel back into the trap after which we pulled it from the trap by hand and placed it in the restraint test. Finally, we handled the squirrel and obtained a small blood sample in a capillary tube via clipping a toenail for measurement of innate immune function. We collected a faecal sample from each squirrel during the battery of tests for measurement of faecal glucocorticoid metabolite levels. The full battery of behavioural tests and sample collections took 20–25 min to complete. This design gave us two measures of each variable for each squirrel in a balanced, randomized treatment order.

Ethical Note

Squirrels suffered no apparent negative effects from mifepristone treatment. Institutional Animal Care and Use Committees (IACUC) at University of Chicago (protocol no. 71255) and University of California Santa Barbara (protocol no. 5-03-532) approved this study, which adhered to standards set forth by the ASAB/ABS Guidelines for the Use of Animals in Research.

Statistical Tests

Control for multiple comparisons

This study required testing multiple hypotheses. Despite these hypotheses being planned comparisons, we must consider the risk of inflated type I error, especially with a relatively small sample size ($N = 18$). Two primary methods are used to control for this problem. First, Bonferroni correction adjusts α by dividing by the number of hypotheses tested. However, some authors argue that the Bonferroni correction is overly strict (Moran, 2003), especially for behavioural studies where small sample sizes inflate the possibility of type II errors (Nakagawa, 2004). These authors advocate calculating the probability of a given number of tests being significant by chance alone using a Bernoulli process, where a success is $P < 0.05$ (Moran, 2003). In this case, the number of significant results is given more weight than is the extremeness of P values. We made a function in R 3.0.2 (R Development Core Team, 2012) to perform this test, and we present the probability that the number of significant results in our study is due to chance as a control for our multiple tests.

Hypothesis 1: GR–CORT binding is a mechanism of phenotypic plasticity

To test GR–CORT binding as a mechanism of trait plasticity, we looked at the effect of CORT, mifepristone treatment, and their interaction on each trait in a generalized linear mixed-effects model. Because GR–CORT binding can be a mechanism of negative feedback in the CORT response (Sapolsky et al., 2000), we also investigated the effect of mifepristone treatment on CORT. Each squirrel was tested twice for each variable, so we included individual identity as a random intercept to account for the repeated measures. For exploration, we used a Poisson distribution. Activity and CORT approximated continuous normal distributions. Refuge use, time spent immobile and BKA are proportional variables, and we used a binomial distribution as recommended by Warton and Hui (2011). To prevent overdispersion in binomial models, we fitted an additional observation-level random intercept. For BKA, we also fitted a random intercept for sample to account for the duplicate plates used for each BKA sample. In every model, we retained sex and age as fixed effects. Treatment was included as a two-level categorical factor, and log-transformed CORT levels were included as a continuous covariate. We log transformed CORT to linearize its relationship with the response variables. For all models, we inspected residual plots and found that model

assumptions were met. In addition, for the Poisson and binomial models, we calculated the dispersion parameter (φ) and found that the mean–variance relationships of those distributions were not violated; therefore, standard errors of those models were accurate. We obtained *P* values from Markov chain Monte Carlo (MCMC) post hoc analyses for normally distributed variables using the LMERConvenienceFunctions package (Tremblay & Ransijn, 2012) in R 3.0.2 and nonparametric bootstrap *P* values for non-normally distributed variables using the R code provided in the supplement of Warton and Hui (2011). We ran these models using the 'lmer' function in the 'lme4' package (Bates, Maechler, & Dai, 2008).

In all models, we first evaluated the interaction effect between CORT and treatment to determine whether blocking GRs would influence the relationship between CORT and the trait. A significant interaction term would indicate that GRs affect the slope of the relationship between CORT and a trait, and therefore that GRs are a mechanism of plasticity. If that interaction term was insignificant, we evaluated the main effects of CORT and treatment. A significant main effect of mifepristone treatment in the opposite direction of the relationship between CORT and a trait would also indicate that GR–CORT binding is a mechanism of plasticity. In addition, we tested whether treatment affected body mass, as well as whether test order or two-way interactions between order, sex, age and treatment affected behavioural traits or BKA.

Hypothesis 2: GR–CORT binding is a mechanism of consistent individual differences

To test whether the variance of traits differed between experimental and control treatments, we used Pitman's test (Pitman, 1938). We used this rather than the more common *F* test for equality of two variances because the Pitman's test accounts for the dependence between experimental and control treatments introduced by our repeated measures experimental design. We used code from Medical Statistics in SPSS, SAS and R to run the test in R 3.0.2 (see Supplementary Material).

Hypothesis 3: GR–CORT binding is a mechanism of phenotypic integration

To test whether blocking GRs modulates correlations between traits, we used the Z_2^* statistic, which tests the hypothesis that two dependent correlations are equal (Equation 15 in Steiger, 1980). This test is appropriate for small sample sizes (Steiger, 1980), whereas other methods, such as multiresponse mixed-effects models, require substantially large sample sizes (Dingemanse & Dochetermann, 2013). Since most of our variables were non-normally distributed, we used Spearman's rank correlation coefficients, which are robust for use in the Z_2^* statistic (Myers & Sirois, 2006). For each pair of behavioural variables, we calculated the correlation in the control treatment and compared it to the correlation in the experimental treatment. A significant Z_2^* would indicate that mifepristone treatment affects the relationship between two variables and that GRs function as a mechanism underlying any correlation there may be between the two variables. We used R 3.0.2 (R Development Core Team, 2012) to obtain correlation values, then we obtained the Z_2^* statistic, drawing the accompanying *P* value from an online calculator (GraphPad Software, 2014).

RESULTS

Control for Multiple Comparisons

We obtained four statistically significant results ($P < 0.05$) from our 20 planned tests (plasticity of five traits, variance differences of five traits, and correlation comparisons between 10 pairs of traits). The probability of obtaining that number of significant tests due to chance is 0.01, indicating a low probability of study-wide type I error (Moran, 2003; Nakagawa, 2004).

Hypothesis 1: GR–CORT Binding is a Mechanism of Phenotypic Plasticity

Mifepristone caused significant differences between experimental and control treatments in *U. beldingi*. Because of missing data due to unsuccessful laboratory assays and a camera malfunction, our models have 31 observations on 17 squirrels. Treatment did not affect CORT levels of squirrels (estimate ± SE = −0.04 ± 0.16, $Z = -0.30$, $P_{MCMC} = 0.77$) or weight (estimate ± SE = 3.39 ± 2.29, $Z = 1.48$, $P_{MCMC} = 0.14$). The interaction between CORT level and treatment significantly affected exploration and resulted in a decrease in the positive relationship between exploration and CORT (estimate ± SE = −1.06 ± 0.39, $Z = -2.71$, $P_{bootstrap} = 0.03$; Fig. 1). Activity showed a similar pattern to exploration, but neither the interaction effect between CORT and treatment nor the main effects of CORT and treatment were significant (CORT: estimate ± SE = 10.83 ± 12.08, $Z = 0.90$, $P_{MCMC} = 0.38$; treatment: estimate ± SE = −4.93 ± 8.52, $Z = -0.58$, $P_{MCMC} = 0.57$). Experimental treatment did not affect refuge use (CORT: estimate ± SE = 1.80 ± 2.27, $Z = 0.80$, $P_{bootstrap} = 0.75$), but we found a tendency for mifepristone treatment to decrease time spent immobile in the response to restraint test (estimate ± SE = −0.99 ± 0.48, $Z = -2.05$, $P_{bootstrap} = 0.07$). BKA was significantly affected by treatment with mifepristone. Due to unsuccessful sample collection or laboratory assays, our sample size for this model was 27 samples on 16 squirrels. BKA significantly decreased with CORT in the control treatment (estimate ± SE = −0.93 ± 0.30, $Z = -3.08$, $P_{bootstrap} = 0.03$; Fig. 2), but significantly increased when animals were treated with mifepristone (estimate ± SE = 0.49 ± 0.22, $Z = 2.21$, $P_{bootstrap} = 0.04$; Fig. 2). Age, sex, and their interactions with mifepristone treatment were not significant in any model. Test order as a main effect was significant only for exploratory behaviour (estimate ± SE = −0.65 ± 0.26, $Z = -2.48$, $P_{bootstrap} = 0.02$), but its interaction with treatment was not significant for any of the variables

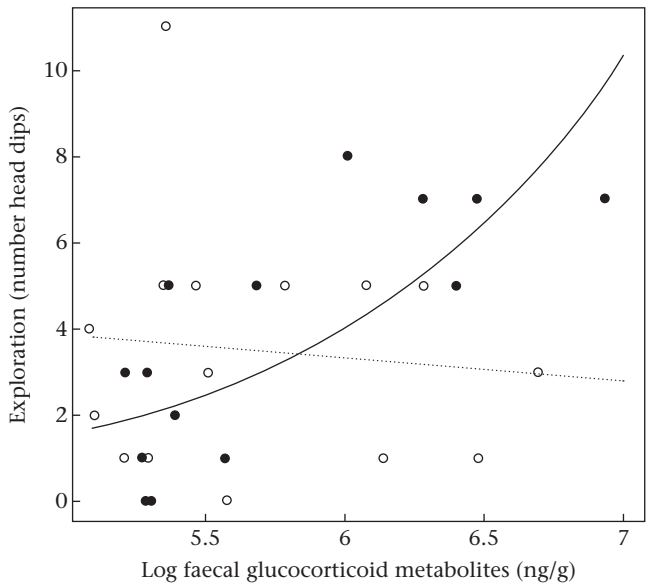

Figure 1. Relationship between faecal glucocorticoid metabolites and exploratory behaviour in squirrels tested both in the control treatment (filled circles, solid line) and the experimental treatment with mifepristone (open circles, dotted line). Lines represent separate Poisson regressions in each treatment, whereas statistical tests account for the repeated measures. We had 31 observations on 17 squirrels.

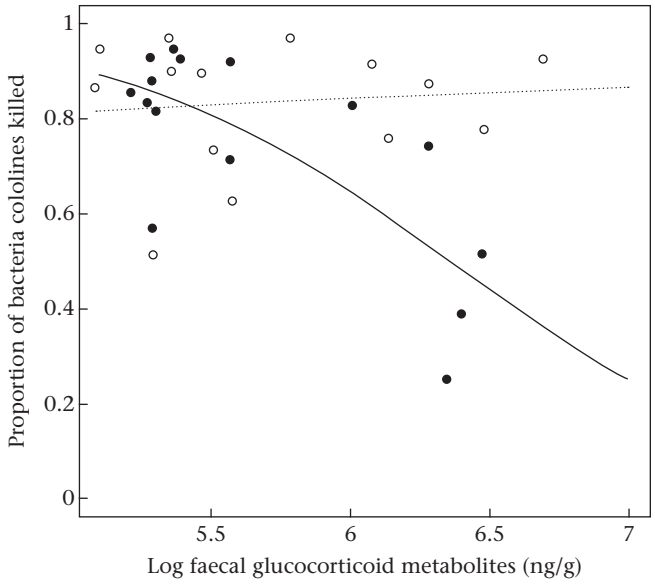

Figure 2. Relationship between faecal glucocorticoid metabolites and bacteria-killing ability in squirrels tested both in the control treatment (filled circles, solid line) and the experimental treatment with mifepristone (open circles, dotted line). Lines represent separate binomial regressions in each treatment, whereas statistical tests account for the repeated measures. We had 52 plate counts from 27 samples from 16 squirrels.

and the treatment effects were qualitatively unchanged with its inclusion in the model.

Hypothesis 2: GR–CORT Binding is a Mechanism of Consistent Individual Differences

Mifepristone treatment did not significantly affect the variance of any behavioural trait tested, but there was a strong tendency suggesting the variance of BKA was lower when squirrels were treated with mifepristone compared to control ($t_{16} = -2.07$, $P = 0.055$; Table 1).

Hypothesis 3: GR–CORT Binding is a Mechanism of Phenotypic Integration

Mifepristone treatment did not significantly affect the relationships among any behavioural traits (Table 2). However, BKA–behaviour relationships were modulated in two of four cases (Table 3). The BKA–activity and BKA–exploration correlations were significantly affected by mifepristone (BKA–activity: $Z_2^* = 3.13$, $P = 0.002$; BKA–exploration: $Z_2^* = 2.39$, $P = 0.017$; Table 3, Fig. 3). In both cases, a negative relationship in the control treatment contrasted with a positive relationship in the experimental treatment. For response to restraint, we found the opposite pattern, with a positive relationship in the control treatment and a negative relationship in the experimental treatment (BKA–time spent immobile: $Z_2^* = -1.75$, $P = 0.081$; Table 3).

Table 1
Pitman's test for homogeneity of variances between control and experimental treatment with mifepristone ($N = 17$)

Trait	$\sigma^2_{Experimental}$	$\sigma^2_{Control}$	t	P
Activity	1313.99	1333.68	−0.38	0.71
Exploration	7.94	7.38	0.53	0.60
Response to restraint	0.12	0.13	−0.49	0.63
Refuge use	0.20	0.17	0.60	0.56
Bacteria-killing ability	0.02	0.05	−2.07	0.055†

†$P < 0.10$.

Table 2
Z_2^* statistic testing differences in behavioural correlations between control and experimental treatments ($N = 16$ for bacteria-killing ability, $N = 17$ for behaviours)

Traits	$\rho_{Experimental}$	$\rho_{Control}$	Z_2^*	P
Activity–Exploration	0.59	0.43	0.55	0.59
Activity–Refuge use	−0.31	0.06	−0.99	0.32
Activity–Response to restraint	−0.02	−0.34	1.10	0.27
Exploration–Refuge use	−0.47	−0.67	0.78	0.44
Exploration–Response to restraint	−0.20	−0.03	−0.47	0.64
Response to restraint–Refuge use	0.11	−0.21	0.92	0.36

Table 3
Z_2^* statistic testing differences in behaviour–bacteria-killing ability correlations between control and experimental treatments ($N = 15$)

Traits	$\rho_{Experimental}$	$\rho_{Control}$	Z_2^*	P
Activity–BKA	0.52	−0.44	3.12	**0.002**
Exploration–BKA	0.45	−0.38	2.39	**0.017**
Response to restraint–BKA	−0.28	0.12	−1.75	0.081†
Refuge use–BKA	0.02	−0.24	0.68	0.50

BKA: bacteria-killing ability. Significant P values (<0.05) are shown in bold; †$P < 0.10$.

DISCUSSION

Many authors hypothesize that hormonal mechanisms control functional integration of traits or act as constraints to produce (co) variation of behavioural and immune traits (Ardia et al., 2011; Aubin-Horth, Deschênes, & Cloutier, 2012; Duckworth, 2010; Duckworth & Sockman, 2012; Garamszegi et al., 2012; Ketterson & Nolan, 1999; Koolhaas et al., 1999; Sih et al., 2004). In squirrels, activity, exploration and response to restraint constitute a behavioural syndrome (Dosmann et al., 2015). CORT positively relates to activity when squirrels are in good condition (Dosmann et al., n.d.) and treatment with exogenous CORT decreases BKA (Brooks & Mateo, 2013). Here we focused on binding of GRs with CORT as a potential mechanism of plasticity, consistent individual differences and phenotypic integration of behaviour and immunity in *U. beldingi*. Using wild-caught squirrels, we delivered mifepristone to block the potential effects of GR–CORT binding on a suite of behavioural and immune traits and test whether that mechanism affects phenotypic plasticity, individual variation, and correlations between behaviours and innate immunity.

Our results indicate a role for GR–CORT binding in the trait plasticity of *U. beldingi*. Indeed, mifepristone treatment abolished the positive relationship between CORT and exploration observed in the control treatment (Fig. 1). Although this result supports the hypothesis that GR–CORT binding is a mechanism of plasticity for exploration, it is not matched by a significant relationship between CORT and exploration in free-ranging squirrels (Dosmann et al., 2015). Because we controlled for multiple comparisons, it is unlikely that this discrepancy is due to a type I error (Nakagawa, 2004). Instead, we suggest that acclimation to captivity explains the difference in our study. The experience of the home cage prior to testing may have altered the perception of the false burrows in the experimental squirrels compared to free-ranging squirrels, which were measured in the same test but without experience of the unnatural home cage environment. This calls for an experiment manipulating GR–CORT binding in free-ranging squirrels to confirm the results. Nevertheless, when viewed alongside relationships between CORT and other behaviours in *U. beldingi* (Dosmann et al., n.d.; Mateo, 2007, 2008), the result points towards

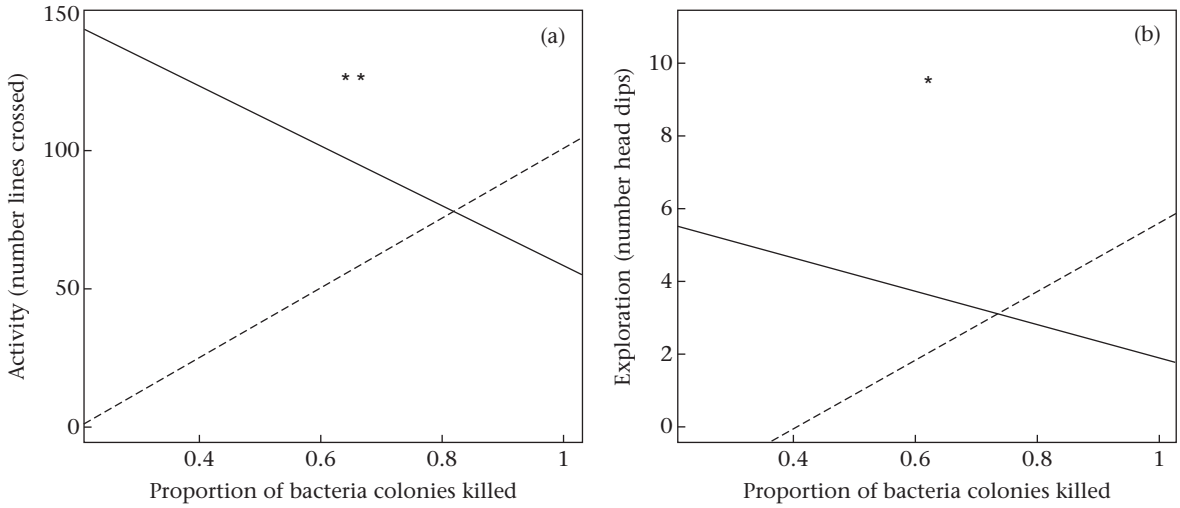

Figure 3. Regression lines showing the relationships between (a) bacteria-killing ability (BKA) and activity and (b) BKA and exploration in squirrels tested in both the control treatment (solid line) and the experimental treatment with mifepristone (dotted line). Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$) in the correlations of each behaviour and BKA between control and experimental treatments. Correlations were calculated from trait observations on 15 squirrels. See text and Table 3 for statistical details.

GR–CORT binding as a mechanism of plasticity for exploration. Mifepristone counteracted the suppressive effect of increased CORT levels on innate immunity (Fig. 2). The results align with a previous experiment on CORT and BKA (Brooks & Mateo, 2013), and suggest that GRs mediate the suppressive effect of CORT on innate immunity. Together, these results point towards a role for GRs in the plasticity of both behaviour and immunity of *U. beldingi*, and in turn a potential impact on whether squirrels succumb to predation, disease or starvation during winter hibernation.

Our results complement evidence from many vertebrate taxa establishing links between behavioural variation and glucocorticoids (Cockrem, 2007), as well as links between immunity and glucocorticoids (Demas et al., 2011). Many studies have found that CORT affects behaviours in wild animals (e.g. Breuner, Greenberg, & Wingfield, 1998; Costantini et al., 2012; Ferrari et al., 2013; Montiglio et al., 2012). However, there are few data from wild-caught individuals on the relationships between immunity and glucocorticoids (but see Bourgeon & Raclot, 2006; Brooks & Mateo, 2013; L. B. Martin et al., 2012; Sild, Meitern, Männiste, Karu, & Hõrak, 2014). For example, exogenous glucocorticoid treatment decreases humoral immunity in common eiders, *Somateria mollissima*, over the course of egg incubation (Bourgeon & Raclot, 2006). In contrast, Sild et al. (2014) found evidence that high feather corticosterone levels, indicative of chronic levels, are associated with greater resistance to experimental infection in greenfinches, *Carduelis chloris*, exemplifying how actual infection susceptibility may differ from various immune responses and that glucocorticoids can sometimes be immunoenhancing rather than immunosuppressive. In Belding's ground squirrels, experimental treatment with exogenous CORT decreases BKA while also muting the squirrels' ability to mount an immune response to a lipopolysaccharide challenge (Brooks & Mateo, 2013). Our results supplement these studies by additionally implicating GR–CORT binding and by assessing behaviour and immunity together. These are meaningful additions because pleiotropic effects of a single receptor on these traits may be more likely to act as an impediment to plasticity and/or response to selection than a single hormone whose level may change more rapidly and whose actions may operate via multiple pathways (Duckworth, 2010; Ketterson & Nolan, 1999; Stavreva et al., 2009).

Despite suppressing the plasticity of exploratory behaviour and immunity, treatment with mifepristone did not suppress among-individual variance in those traits or the other behavioural traits (Table 2). Such an effect would indicate that individual differences in density or sensitivity of GRs produce variation in behavioural or immune traits that mifepristone mutes. In spotted antbirds, *Hylophylax n. naevioides*, Canoine, Fusani, Schlinger, and Hau (2007) found that differing densities of testosterone receptors in the brain accounted for behavioural differences between individuals. We found a tendency for mifepristone to reduce variance of BKA, which is particularly interesting since it aligns with the direction of the predicted decrease in variance, pointing towards a need for more research. We found no evidence that blocking GRs modulated behavioural variance or correlations among behaviours, indicating no direct effect of GRs on personality or behavioural syndromes in *U. beldingi*. However, for both results, lack of statistical power may explain our inability to reject the null hypothesis.

Data regarding relationships between behaviour and immunity in wild animals remain rare and are mostly from studies of birds (e.g. Kluen et al., 2014; Krams et al., 2013; Loiseau, Sorci, Dano, & Chastel, 2008; L. B. Martin et al., 2012; Sild et al., 2011). Our results indicate that GR–CORT binding significantly modulates relationships among behaviours and innate immunity. Specifically, we found that the relationships between BKA and exploration and between BKA and activity were significantly affected by mifepristone treatment (Fig. 3, Table 3). In the control condition, both behaviours had a negative relationship with BKA, suggesting that squirrels trade off activity and exploration with immunity. This relationship was reversed when mifepristone was delivered to block GRs. Also, we found a strong tendency for mifepristone to affect the relationship between BKA and response to restraint (Table 3). Although this relationship was not significant, these results are interesting when considered together because the three behavioural traits constitute a behavioural syndrome in free-ranging squirrels. These behavioural traits covary between and within individuals (Dosmann et al., 2015). Although our sample size in this experiment was too small to statistically partition variation into within- and between-individual components, our experimental design indicates that the treatment effect was at the within-individual level. The directions of the treatment effects align with the correlations among the behavioural syndrome. The effect of mifepristone treatment on behaviour–BKA relationships was the same for activity and exploration, but opposite for response to restraint, which negatively correlates with both activity and exploration (Dosmann et al., 2015). This suggests that within-individual trade-offs among behaviour and innate immunity

underlie the between-individual correlations that constitute the behavioural syndrome in *U. beldingi*. Assessing both within- and between-individual correlations of immune and behavioural traits will be important in further understanding whether physiological mechanisms account for patterns of behavioural and immune (co) variation (Dingemanse & Dochetermann, 2013; Downs & Dochtermann, 2014). In addition, the prevalence of trade-offs within the immune system itself (Ardia et al., 2011) calls for cautious interpretation of the trade-offs between behaviour and the single immune trait we present here. Future research should include multiple immune traits to extend upon our evidence suggesting that GR–CORT binding mediates trade-offs among behaviour and immunity to produce observed patterns of (co)variation among those traits.

Although the results discussed above aligned with expectations of treatment effects, mifepristone treatment did not produce the expected increase of faecal CORT levels. GRs are involved in negative feedback of CORT production so that blocking GRs is expected to raise CORT levels (Sapolsky et al., 2000). This casts doubt on the efficacy of mifepristone treatment in blocking GRs, but there are potential explanations. In rats, MRs can enact negative feedback at baseline levels of CORT (Ratka, Sutanto, Bloemers, & de Kloet, 1989), and Mattson, Reynolds, Simonyte, Olsson, and Walker (2009) found both MRs and GRs affect negative feedback in humans. This raises the possibility that negative feedback in *U. beldingi* is partially independent of GRs. A second possibility is that impaired negative feedback was reflected in acute CORT responses to handling stress during testing, but because of the lag between plasma CORT and measurement in faecal glucocorticoid metabolites in *U. beldingi* (~6–30 h; Mateo & Cavigelli, 2005), we found no differences in faecal CORT on the test occasion. Since our data cannot evaluate these possibilities, we must qualify our interpretations. However, the interaction effects of CORT and mifepristone on traits, and parallels between this experiment and previously known relationships between CORT and innate immunity in *U. beldingi* (Brooks & Mateo, 2013), support interpretations that treatment effects demonstrate the actions of GR–CORT binding.

Overall, our study provides evidence that GRs contribute to observed patterns of behavioural and immune (co)variation seen in nature. These results echo similar relationships proposed to explain sexually selected traits (Ezenwa et al., 2012; Folstad & Karter, 1992; Rubenstein & Hauber, 2008; Safran, Adelman, McGraw, & Hau, 2008), wherein a balance of trade-offs among hormones, immunity and the trait produces correlations among them. In turn, this explains the phenotypic variance of the sexually selected trait, as some individuals pay a higher physiological cost to sustain the beneficial ornament whereas others have a less exaggerated ornament but pay a lower physiological cost. Our results raise the possibility that a similar process explains correlations among behaviours and immunity and their consistent individual differences over time, while suggesting that GR–CORT binding is a mechanism dictating these phenotypic patterns in Belding's ground squirrels. The greater flexibility of CORT compared to changes in GR levels in other rodents (Paskitti et al., 2000; Stavreva et al., 2009) also provides a potential biological explanation for the complex patterns of between- and within-individual correlations found in free-ranging squirrels (Dosmann et al., 2015) and other species (Downs et al., 2012; Ferrari et al., 2013). Given the ubiquity of the stress response in vertebrates, the GR–CORT mechanism is likely of broad importance in explaining patterns of trait variation at different timescales described by animal personality and ecological immunology, and our study provides evidence that the normally functioning GR (i.e. nonglucocorticoid resistant) plays a role in coordinating nonacute levels of CORT, behaviour and immunity.

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2014.12.026.

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